THE EVOLUTION THROUGH NATURAL HYBRIDIZATIONS OF THE UMATILLA DACE (Pisces: *Rhinichthys umatilla*), AND THEIR ASSOCIATED ECOLOGY AND SYSTEMATICS

by

GORDON ROBERT HAAS

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M.Sc., The University of British Columbia, 1988

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Abstract

Umatilla dace (*Rhinichthys umatilla* Gilbert and Evermann 1894) are determined to be a distinct species that has sympatrically speciated and evolved through ancient hybridizations between leopard and speckled dace. Principal components, univariate, and allometric analyses reveal three distinct morphotypes, with Umatilla dace intermediate and convergent. A field identification key is developed. Their body morphologies remain largely consistent across broad mutual ranges, and Umatilla dace are found in abundance and predominance in allopatry, parapatry, and strict sympatry with either or both leopard and speckled dace. All show reduction in their overall morphometric variability when in strict sympatric combinations. Mitochondrial and two nuclear DNA (ITS and D3B) sequences provide consistent evidence of the overall distinct identity, increased variability and past hybridization, and multiple hybrid origins of Umatilla dace. Umatilla dace are composed of three main types each more closely related to the leopard or speckled dace through their comparative nearby numerical dominance. Laboratory crosses demonstrate morphologies remain distinctive when reared under identical conditions. The two combinations of artificial hybrids of leopard and speckled dace strongly resemble Umatilla dace, with Umatilla dace still remaining more derived. Second generation artificial crosses demonstrate that Umatilla dace and both laboratory hybrids are viable with no differential mortality. Each species is specialized to particular water flow regimes as determined in the field and at spawning times in a laboratory flow tube using mature artificial crosses. Umatilla dace are specialized to intermediate water flows, as are both laboratory hybrids. The laboratory hybrids show a maternal relationship in their water flow tolerances that matches the molecular genetic data. Umatilla dace are only found in the interior Columbia River drainage in range overlap between leopard and speckled dace, and have an intermediate postglacial northern recolonization pattern and distance. Their main distribution strongly coincides with large Wisconsinan glacial lakes that existed in this area. The associated breakdown of water flow regimes is hypothesized to have caused the past interbreeding of leopard and speckled dace, with the difficult glacial / postglacial environment favouring their hybrids' increased genetic and ecological variability. The dace genus has disproportionately higher hybridization, as does its minnow Family Cyprinidae, particularly in western North America.

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PART I –

GENERAL INTRODUCTION

Chapter ONE

General Introduction

Biology and Background

Hybridization tends to be regarded as a minor force in the evolution of new bisexual animal species (Arnold 1992, 1997, Bullini 1985, DeMarais et al. 1992, Stone 2000). It generates much interest in speciation research (Coyne 1992, Endler 1989), but the emphasis is largely on why hybrids suffer differential mortality and why they have restricted distributions in hybrid zones (Barton and Hewitt 1985, Endler 1977, Hewitt 1989). The research into these issues is abundant, but the notion that hybridization itself can generate new animal species is rarely discussed (Arnold 1997,Hewitt 1990, Scudder 1974, Stone 2000, White 1959).

Hybridization has been to shown to produce new species. Many plants (Abbott 1992, Stebbins 1959), most angiosperms (Barrett 1989) and some insects (Bullini 1985, Bullini and Nascetti 1990, Spence 1990), snails (Goodfriend and Gould 1996), fish (refs. herein), amphibians (Maslin 1968, Uzell and Berger 1975), and lizards (Uzell and Darevsky 1975) are believed to have evolved through some hybridization event. However, these examples largely deal with polyploidy (Lewis 1980, Orr 1990) and related strong shifts in reproductive systems (Bullini 1985). In fact, the so-called 'hybrid theory' (Peacock and Harrison 1926, Bullini and Nascetti 1990) suggests that speciation through hybridization causes a tendency towards parthenogenetic reproduction.

There are few cases of natural animal hybrids forming a new bisexual species that maintains its integrity, evolves its own ecology, and coexists with both its parental forms (Arnold 1997, DeMarais et al. 1992, Dowling et al. 1989, Rao and DeBach 1969, Stone 2000). The best ones that are known from fishes still tend to be on a smaller geographic scale or restricted to an overall zone of overlap (Smith 1992). These fish examples are indeed species of hybrid origin, but not with the distributions and some other characteristics usually attributed to a distinct species. For instance, many of these few cases continue to hybridize with their original parental forms. A common difficulty with species of potential hybrid origin is that there is some evidence supporting their case, but these species and their evolution have not been convincingly demonstrated (Berger 1973, Ross 1958, Uzell and Darevsky 1973). Hybridization has nonetheless continued to be suggested as a viable and possible sympatric speciation mechanism (Arnold 1997, Dowling and Brown 1989, Hubbs 1955, Scudder 1974, Stauffer et al. 1979, Stone 2000).

Hybridization immediately produces heterozygotes and polymorphisms (Bigelow 1965, Lewontin and Birch 1966, Turner 1971) which can provide the genetic variation for speciation,

especially in an extreme or unpredictable environment (Bullini and Nascetti 1990, Carson et al. 1989, Cook and Johnson 1968, Kaneshiro 1990, Smith et al. 1983, Smith and Todd 1984, Svärdson 1970, Tegelström and Gelter 1990). Higher genetic variability is obtained through hybridization (Carson 1985, DeMarais et al. 1992, Lewontin and Birch 1966, Potts and Reid 1988) even at low levels of hybridization (Endler 1977,1989, Grant 1986,). Hybrids and polymorphic species are better buffered against environmental perturbations (Huxel 1999, Kornfield et al. 1982, Liem and Kaufman 1984).

Sympatric speciation or the sympatric establishment of heterozygous polymorphisms through ecological habitat specialization has been emphasized (Gillespie 1976, Hoekstra et al. 1985, Levene 1953, Maynard Smith 1966, Tauber and Tauber 1977a, Taylor 1976). This can occur through occupation of new habitats or through spatial partitioning of underexploited ones (Rosenzweig 1978, Schilthuizen 2000, Tauber and Tauber 1989). This was not usually considered by early speciation models, but those more recent ones demonstrate that reproductive isolation can evolve as a correlated character via disruptive or frequency dependent selection for habitat preference (Diehl and Bush 1989, Garcia-Dorado 1986, Rausher 1984; Rice 1984, 1987, Rice and Salt 1990, Udovic 1980). Sympatric divergence can proceed even with weak habitat preferences and moderate fitness differences (Diehl and Bush 1989, Felsenstein 1981, Futuyma and Mayer 1980, Futuyma and Moreno 1988, Grant and Grant 1989, Seger 1985,). As well, there appears to be additional data to support these models (Hewitt 1989, Jaenike and Holt 1991, Jones 1980, Jones and Probert 1980, Jordan 1991, Parsons 1983, Plante et al. 1989, Rice and Salt 1990, Rosenzweig 1991, Tauber and Tauber 1977b, Turner and Grosse 1980,). Habitat specialization by learning or conditioning (Diehl and Bush 1989, Futuyma 1989, Grant 1986, Immelman 1975, Maynard Smith 1970,), social selection (Larson et al. 1984, Lens et al. 2000, West-Eberhard 1983) and sexual selection (Blouws 1998, Kaneshiro 1990)

Plant and laboratory hybrids are known to be better adapted to a "middle" environment (Stebbins 1959, 1971, Straw 1955). Laboratory hybrid fish even have intermediate brain patterns and behaviours (Masai and Sato 1965). Hybrids are often more fit in hybrid zones (Butlin 1989, Harrison and Rand 1989, Littlejohn and Watson 1985, Moore and Buchanan 1985,) and can even geographically shift them when environmental or human interactions change their optima (Hewitt 1989, Hillis and Simmons 1986, Lens et al. 2000, Rising 1983, Van der Meer et al. 1985). Such intermediate environments for hybrids could also become 'density troughs' for appropriately adapted species as is argued for hybrid zones (Hewitt 1989). There is even evidence that mating within habitat may not be necessary for some hybrids to evolve into separate species (Arnold 1997). Laboratory examples exist where parents and first generation hybrids showed resistance to backcrossing (Rao and Debach 1969, Sailer 1954, Sears 1947).

There is additional inferential support for the possibility of speciation and evolution through hybridization in fishes (DeMarais et al. 1992, Smith 1992). Fish not only readily hybridize, but seem to be particularly able to evolve through polyploidy. For instance, the suckers (Family Catostomidae) are of tetraploid origin (Uyeno and Smith 1972) and are closely related to cyprinids and endemic to North America (Nelson 1984). Suckers are also known for their high level of intrafamilial hybridization (Hubbs 1955, Hubbs and Hubbs 1947, Nelson 1968, Smith et al. 1983). They reproduce bisexually, but at a higher ploidy, as do some plants (Carson 1985, Grant 1981, Rieseberg et al. 1990), insects (Bullini and Nascetti 1990) and other fish (Allendorf and Thorgaard 1984).

Unisexual fish are not uncommon in North America and are generally triploid females of hybrid origin from two bisexual species (Echelle et al. 1989, Vrijenhoek 1989). These also occur in minnows (*Phoxinus* spp.) in the family Cyprinidae (Dawley and Goddard 1988, Joswiack et al. 1985) of which the parentals and possibly the unisexual hybrids even exist in northeastern British Columbia (Cannings and Ptolemy 1998, Das and Nelson 1989, Haas 1998, McPhail and Carveth 1993). The 'frozen-niche variation model' (Vrijenhoek 1979, 1984) proposed for unisexual poecilid fish states that hybrid unisexuals best survive with their parent species when they utilize different habitats (Vrijenhoek 1978). Poecilid unisexual fish usually do this on a small geographic scale pertaining to water flow characteristics (Schenck and Vrijenhoek 1986).

Unisexual fish can even displace their sexual counterparts if their origin was multiclonal and thus more ecologically diverse (Bell 1982, Vrijenhoek 1979,). Genetic diversity favours exploitation of heterogeneous environments by unisexual fish (Vrijenhoek 1985, Vrijenhoek and Lerman 1982). There may be ecological parallels here to the possible hybrid origins of the bisexual fishes and animals. The increased diversity resulting from hybridization may permit their foothold to be established, particularly in a changed or diverse environment. Such ecological segregation is also known for other polyploid and clonal animal assemblages (Bell 1982).

Many other cyprinids have been described as distinct nominal or generic forms but then later identified as hybrids (Bailey and Gilbert 1960, , Gilbert 1961, 1978, Hubbs 1951, Hubbs and Bailey 1952, Hubbs and Miller 1943, Hubbs and Moore 1940, Legendre 1970, Menzell 1977, Miller 1945, Schultz and Schaefer 1936, Weisel 1954). In one situation involving an eastern North American dace, the species (*Rhinichthys bowersi* (Goldsborough and Clark)) was originally described from specimens collected in 1899 (Goldsborough and Clark 1908). It was later dismissed as being an intergeneric hybrid (Raney 1940b), but more recently its species status and origins have been re-assessed since four year-classes of the bisexual hybrid form have successfully persisted throughout the river system with both hypothesized parental

forms (Matthews et al. 1982, Stauffer et al. 1979). A similar situation appears to exist in a different cyprinid genus (Meagher and Dowling 1991, Menzel 1977, Miller 1968). As well, several bisexual species of fish of possible hybrid origin (Balon 1992, DeMarais et al. 1992, Krupka and Holcik 1976, Manwell et al. 1963, Meagher and Dowling 1991, Schultz and Smith 1936, Sola et al. 1989) and other bisexual fish species of possible introgressive origin (Kosswig 1963, Smith 1992, Smith et al. 1983, Svärdson 1970) have been tentatively described.

New World cyprinids are believed to share a common ancestry when a relatively few migrated to North America over the Bering Land Bridge in the Miocene (Miller 1959). The approximate 40 genera and 250 species (Nelson 1984) all share the same basic diploid chromosome number of n = 50 and are not highly genetically differentiated (Avise et al. 1975). The dace species of specific interest to this study are particularly homogeneous in karyotype (Howell and Villa 1976). Old World cyprinids are significantly different in karyotype and it ranges from a diploid number of n = 44 - 104 (Avise and Gold 1977). Other species-rich groups tend to be chromosomally diverse (Barton 1989, Bush et al. 1987, Wilson et al. 1974) and closely related species usually differ karyotypically (Lande 1979, White 1973). Hybrids are deleterious under such conditions, but not in the genetically similar North American cyprinids. This lack of genetic differentiation also emphasizes the importance of ecological separation in maintaining cyprinid species (DeMarais et al. 1992, Howell and Villa 1976, Otte 1989, Patton and Smith 1989) and that many likely are of recent origin (McPhail 1992, Ricklefs 1989,). Similar circumstances have been discussed for salamanders (Good 1989, Larson 1984, 1989).

Hybridization in freshwater fish is more prevalent than in any other vertebrate group (Avise and Smith 1974, Hubbs 1955, Smith 1992, Smith and Todd 1984). The family Cyprinidae has been recognized for its widespread interspecific and intergeneric hybridization (Chapter 10; DeMarais et al. 1992, Hubbs 1955). Western North America has always been more depauperate in freshwater fish species (Smith 1981) than eastern North America and it was strongly affected by Pleistocene glaciation (McPhail and Lindsey 1986, Miller 1961). Natural hybridization occurs more in depauperate regions (DeMarais et al. 1992, Hubbs 1955, Smith 1992) and in areas affected by glaciation (DeMarais et al. 1992, Dowling and Brown 1989, Dowling and Hoeh 1991, Kat 1985, Remington 1968, Smith 1992, Spence 1990). Hybrids may also be well suited to survive glaciation and to better disperse (Potts and Reid 1988, 1990, Schemske and Morgan 1990) into the new empty habitats present after glacial retreat (Hewitt 1989, 1996, Stebbins 1971). This disproportionate level of hybridization in a region historically presenting environmental conditions favouring it suggests some of its cyprinid species may have sympatrically evolved through hybridization events.

Specific Hypothesis – Umatilla Dace

This study examines three minnow species endemic to western North America that are members of the Family Cyprinidae. These are speckled dace (*Rhinichthys osculus* (Girard)), leopard dace (*R. falcatus* (Eigenmann and Eigenmann)), and the putative species Umatilla dace (Gilbert and Evermann 1894, Hughes and Peden 1989, Peden and Hughes 1988). Umatilla dace are presently recognized only as a subspecies of speckled dace (*R. osculus umatilla*; Robins et al. 1991), with Canadian jurisdictions somewhat arbitrarily giving it species status (*e.g.* Cannings and Ptolemy 1998, Peden and Hughes 1989). These three dace will be referred to as species for ease of communication and because this thesis demonstrates the validity of Umatilla dace as a distinct species (*R. umatilla* (Gilbert and Evermann)).

There was some original morphometric and meristic data (Bond 1973, Carl et al. 1977, Haas unpubl. data, Hughes and Peden 1989) that could be, but had not been, interpreted as indicating that Umatilla dace was a distinct species that had formed and evolved as a stable sympatric hybrid derivative of speckled and leopard dace (Haas unpubl. data and this thesis; for drawings and photographs see Figs. 14-15 in Chapter 2). Furthermore, this possibility of it being a hybrid form was something noted even in first description of Umatilla dace (Gilbert and Evermann 1894). Based on these data, an original hypothesis for this possible evolution of Umatilla dace through past hybridizations with leopard and speckled dace was constructed for testing in this study.

During the last Pleistocene glaciation, North America was largely covered with ice to approximately just south of the present border of Canada and the USA (Fig. 18 in Chapter 3). Any of the three dace species found north of this point would have been wiped out or survived the Wisconsinan glacial epoch in the Pacific refugium. For fish, this glacial refuge corresponded to the Columbia River region, which is a old drainage at least predating the Miocene/Pliocene formation of the western coastal mountain ranges (McKee 1972, McPhail and Lindsey 1986). Along the southeastern edge of the ice sheet at the Pacific refugium, ice tongues and river channel constrictions blocked off major Columbia River tributaries and large, ephemeral glacial lakes were ponded upstream (Allen et al. 1986, Bretz 1919, Bunker 1982, Fig. 18 in Chapter 3, Malde 1965, 1968). These huge lakes were accentuated by increased rainfall and runoff.

During deglaciation, complex patterns of ice retreat, stagnation, and local readvances produced more large glacial lakes and a shifting pattern of drainage connections (Armstrong 1981, Bretz 1919, Tipper 1971). The glacial lakes also drained suddenly with catastrophic flooding when ice blockages were floated or temporarily removed (Jarrett and Malde 1987, Waitt and Atwater 1989). Furthermore, the early drainage pattern of the Fraser River was into the Columbia River since the Fraser River was blocked with ice in the Fraser Canyon. The

Fraser River then also flowed through a succession of glacial lakes to enter the Columbia River (Fulton 1967, 1969, Mathews 1944, McPhail and Lindsey 1986).

These fluvial glacial lakes had no or little water flow which presently appears to be the natural isolating mechanism between some other dace species (Bartnik 1970a,1972, Gee 1968, Gee and Northcote 1963, Gibbons and Gee 1972). Under these glacial conditions, dace could have interbred, both hybridizing and perhaps partly introgressing (DeMarais et al. 1992, Dowling and Hoeh 1991, Hewitt 1996). Dace hybridize (Butcher 1980, Nelson 1966, 1973) and other cyprinid hybrids even dominate (Greenfield and Deckert 1973) in modern reservoirs behind dams under no or low flow conditions. Similar habitat intergradation also results in hybridization of different fish (Avise and Felley 1979, Hubbs 1955) and other cyprinids (Aspinwall et al. 1993a/b, Cross and Minckley 1960, Hubbs and Miller 1943, Izyumov et al. 1998, Weisel 1955). The same environments that produced the hybridization might also favour hybrids and may offer unexploited ecological niches for newly formed hybrid species (Bullini and Nascetti 1990, Goodfriend and Gould 1996, Larson 1989, Smith 1992, Wright and Lowe 1968). Other north temperate fish that survived glaciation in glacial lakes are believed to have inherited enough variability through hybridization and introgression to evolve into many ecotypes (Hubbs et al. 1974, Kosswig 1963, Mayden 1987, Miller 1961, Svärdson 1970, Taylor 1999, Wiley and Mayden 1985, Zardoya and Doadrio 1999).

Study Objectives and Organization

This study combines several large and small scale approaches (e.g. Brooks 1985, Gorman 1992, Larson 1989, Ricklefs 1989) to attempt to convincingly demonstrate and reproduce the viability of this speciation event and mode. A hypothesis, particularly one involving geological time, can never be absolutely proven. This study has tried to obtain sufficient support for the plausibility of the hypothesis and render it as the most parsimonious explanation. There are three basic criteria for the hypothesis addressed here:

(1) Umatilla dace are a distinct biological and evolutionary species from leopard and speckled dace. Umatilla dace exist distinctly in allopatry and sympatry with these other two dace species.

(2) Umatilla dace originally evolved from hybridizations of leopard and speckled dace. Mechanisms for the original hybridization event and the subsequent differentiation and maintenance of Umatilla dace are required.

(3) This original evolution was made possible by, and occurred during and immediately after, the last Pleistocene glaciation. The hybridizations of leopard and speckled leading to the formation of Umatilla dace were in particular intimately connected to the large glacial lakes formed during that period.

The thesis is organized into three sections to address these three criteria. Each section does not just correspond to one of the criteria, but rather all the criteria are addressed. The four chapters in the first two sections each essentially deal with data from (i) morphometrics / meristics, (ii) biogeography / distribution, (iii) molecular genetics, and (iv) ecology / habitat. The first section is composed of data that was collected from specimens in the field, and the second section involves tests of the results of that data largely based on the creation and laboratory rearing of the three dace species and artificial hybrid crosses of speckled and leopard dace. Each of the first two sections thus provides the data to assess the three criteria for the hypothesis that Umatilla dace evolved through past hybridizations of leopard and speckled dace. The third section generalizes the conclusions from the first two sections through an overall analysis of hybridization related to fish and to these dace in particular.

PART II – SPECIFIC DATA

Chapter TWO

Morphometric and Meristic Data

Materials and Methods

1. Sample Categories (3)

All morphometric and meristic data was analyzed in three sequential but inter-related sample categories (appendix A). These were established for the distinct purposes of (1) species discrimination, (2) detailed species examination, and (3) overall species evaluation. The distribution data for each species is necessarily introduced here, but will actually be fully discussed in Chapter 3.

1 - 1. 'Original 150 Fish' (Canada) - *Species Discrimination*

The 'original 150 fish' sample category was represented by nine allopatric sample sites in Canada only (appendix A). It was composed of 50 speckled dace (*R. osculus*) from two overall sites, 50 Umatilla dace from four sites, and 50 leopard dace (*R. falcatus*) from three overall sites. These allopatric sites were the Kettle and Granby rivers for speckled dace, the Fraser River and its tributaries for leopard dace, and the lower Columbia River and its tributaries for Umatilla dace.

These fish were used to develop and establish a discrimination protocol for the three dace species. They came from sites in Canada that were presumed or recognized as allopatric, and which were later verified as such by morphometric, meristic, and genetic (see Chapter 4) data. Originally, these sites of presumed or recognized allopatry were identified from the literature (Carl et al. 1977, Hughes and Peden 1989, Lee et al. 1980, McPhail and Lindsey 1986, Peden 1991, Peden and Hughes 1988) and from unpublished information (Haas and McPhail, pers. comm.).

1 - 2. 'Columbia and Fraser rivers' (Canada and USA) - *Species Examination*

The 'Columbia and Fraser rivers' sample category was represented by 77 sample sites (appendix A). It consisted of 407 fish composed of 110 speckled dace from 22 sample sites, 181 Umatilla dace from 35 sample sites, and 116 leopard dace from 20 sites. Twelve sites were defined as strictly sympatric, meaning that combinations of two or more of the dace species were found in the same museum sample, in museum samples from the same small-scale location, or collected at the same location at the same time. The strict sympatric combinations of dace were of all possible species' pairings and of one sample site for all three species together. The most prevalent strict sympatric species pair was speckled and Umatilla

dace, with Umatilla dace dominant in terms of abundance. More sympatric sites would have been recognized under a looser definition.

The only species examined that exists outside the Columbia and Fraser river watersheds is speckled dace. Twenty-three sample sites for speckled dace were outside these two overall drainage basins. Leopard dace are found only in the Columbia and Fraser river watersheds, and Umatilla dace are presently known only from the Columbia River drainage. The subsequent examination of the systematics and tests of the evolution of these three dace species logically involved just those population samples from within their mutual ranges.

It would have been preferable to examine only Columbia River dace since Umatilla dace seem to only occur there, but four reasons necessitated the inclusion of the Fraser River drainage: (1) there were assumed to be no definitive populations of leopard dace in the Columbia River in Canada when the 'original 150 fish' sample category was created to develop the species discrimination. This assumption has been corroborated. My original samples were only from Canada due to many unknowns at that stage and because of collection ease and permissions since I was working there. The leopard dace samples from the Fraser River are included as they needed to be used and carried through; (2) leopard dace appear rare in most of the Columbia River and insufficient numbers would have been obtained to undertake analyses with confidence; (3) leopard dace found in the Columbia River were often not in allopatry and it was desirable that the samples for development of the dace discrimination came from allopatric sites; (4) the Fraser River was also more intimately connected to the Columbia River after the last Pleistocene glaciation than any other surrounding watersheds (Fulton 1967, 1969, Kershaw 1978, Mathews 1944, McPhail and Lindsey 1986). This is the time of my hypothesized hybrid evolution for Umatilla dace and the Fraser River watershed will be seen as a legitimate inclusion since it provides pertinent information to this scenario (Chapters 3 and 4).

1 - 3. 'Total 500 Fish' (Canada and USA) - *Species Evaluation*

The 'total 500 fish' sample category was represented by 100 sample sites (appendix A). It was composed of the complete sample of 203 speckled dace from 45 sample sites, 181 Umatilla dace from 35 sample sites, and 116 leopard dace from 20 sample sites.

These dace were mainly examined to definitively establish the incompletely known range of Umatilla dace, and to some extent that of leopard dace. The proper development of a best species discrimination protocol, and a realistic examination of the systematics and evolution of these dace, required this information. In the end, leopard and Umatilla dace were not found outside their suspected ranges, and it was known at the outset that speckled dace were much more broadly distributed (Bond 1973, Lee et al. 1980, McPhail and Lindsey 1986, Wydoski and Whitney 1979).

2. Study Approach and Outline

Principal component analyses (PCAs) were used to objectively verify and define the typological species statuses of the three dace. After establishing that the three dace types in the 'original 150 fish' sample from respective regions of species' allopatry were morphometrically distinct, a protocol for their discrimination was developed. Similar PCA results were consistently obtained for and within the three sample categories and species.

This identification protocol was mainly based on one variable that correctly and easily classified all the 'original 150 specimens', but on another measure that almost did too. This main variable worked completely and effectively in Canada. Canada is where the entire 'original 150 fish' sample came from. Further sampling in Canada later confirmed the utility of these two characters for species discrimination there.

The main variable was known to sometimes not be consistent for speckled dace outside Canada (Bisson and Bond 1971, Bond 1973, Peden and Hughes 1988, Wydoski and Whitney 1979). Unfortunately, the second characteristic turned out to be insufficient to bolster the main one for all identifications. A third discriminating variable was necessary, but only in some areas of the USA when a dace specimen could otherwise not be identified to species using the main or first two characters. The sample category of the 'total 500 fish' from throughout the Pacific Northwest in North America was evaluated and identified using this protocol based on the three characters and on locality information. The third variable correctly classified all speckled dace in the US that could not be with the first two characters. Leopard and Umatilla dace remained identifiable, even in the USA, using the first two variables. Eleven other univariate / bivariate variables are provided to assist and further verify the species' identifications. All these discriminations of the various sample categories based on univariate / bivariate characters were later objected defined and verified by PCA on the 42 morphometric variable set.

The three discriminating variables, ultimately along with collection locality data, were used to build a diagnostic key for these species. The distributions of the species were then established, particularly areas of strict sympatry (Chapter 3). The resultant 'Columbia and Fraser rivers' sample category represented their mutual ranges. This 'Columbia and Fraser rivers' sample category was the basis of the analyses of the evolution of Umatilla dace, and also for the further development and refinement of the systematic identifications of all three dace species.

The species statuses of the three dace are primarily interpreted using the criteria of coexistence in the biological species concept (Mayr 1963, 1969), but also that of distinct evolutionary lineages usually first attributed to the evolutionary species concept (Wiley 1978, Wiley and Mayden 1985). Although the biological species concept has operational difficulties

under certain circumstances and for particular taxa (Ehrlich 1961, Hull 1970, Scudder 1974, Sokal and Crovello 1970), it is generally applicable in sexually reproducing species occurring in sympatry (Paterson and McNamara 1984, Cracraft 1989). Under these conditions, the biological species concept is operational and does not necessarily preclude other species concepts or engender particular mechanistic viewpoints (Chandler and Gromko 1989, Coyne et al. 1988, Masters and Spencer 1989, Templeton 1989). Since these dace are sexually reproducing and evidence of strict sympatry was found, the biological species concept is mainly employed. Strict sympatric distributions are more fully discussed in Chapter 3, while the typology and distinct evolutionary pathways of species based on regular discrete morphometric and meristic data are evaluated here in Chapter 2.

The evolutionary hypothesis for hybrid origin of Umatilla dace in this Chapter 2 is evaluated using allometry coefficients and through PCAs of the species comparing them separately in allopatric and strict sympatric sites. The allometry coefficients permitted a direct analysis of the growth patterns for Umatilla dace in comparison to speckled and leopard dace. These growth patterns detailed which body shape regions and proportions were more similar or intermediate to either speckled or leopard dace, and also which were unique to Umatilla dace. The allopatry versus strict sympatry analyses provided information of how each character in cumulative PCA loadings for all the species under each distribution might have been altered.

3. Fish Collections and Sampling

The sampled dace came from new field collections listed as to be deposited in the University of British Columbia Fish Museum, or from four museums (appendix A). The samples cover all times of the year it was possible to collect, and in many cases multiple samples were made or were available for the same sites from different periods and years.

The new field collections were made by electrofishing, seining, and trapping. The dace were humanely euthanized with an overdose of various fish anaesthetics prior to preservation in 10% formalin. Once fixed, they were then flushed of formalin using fresh water, and then placed in approximately 50 % isopropyl alcohol for storage. Tissue samples consisting of a small fin clip were removed from new specimens at 23 sample sites (appendix A) and preserved in 95% ethanol and ultimately placed in a refrigerator.

4. Data

4 - 1. Morphometrics and Meristics

Fifty-eight morphometric measurements and four meristic characters were taken on all 500 dace. This was reduced to a set of 42 morphometric measurements used in all PCAs on all sample categories in this Chapter. One meristic variable was also not used (appendix B). This was counts of lateral line scales, which were found to not be consistent for discrimination of the dace species on this large data set (also see McPhail and Carveth 1993; but see Peden and

Hughes 1988). The lateral line scale counts also gave an overall impression of user subjectivity, and indeed were difficult to take consistently and accurately. The measurements and counts are explained in Figure 1 and Appendix B. Logarithmic transformation of morphometric data is almost always recommended (Bookstein et al. 1985, Burnaby 1966, Shea 1985, Bryant 1986) and was undertaken here for morphometric variables in all three sample categories.

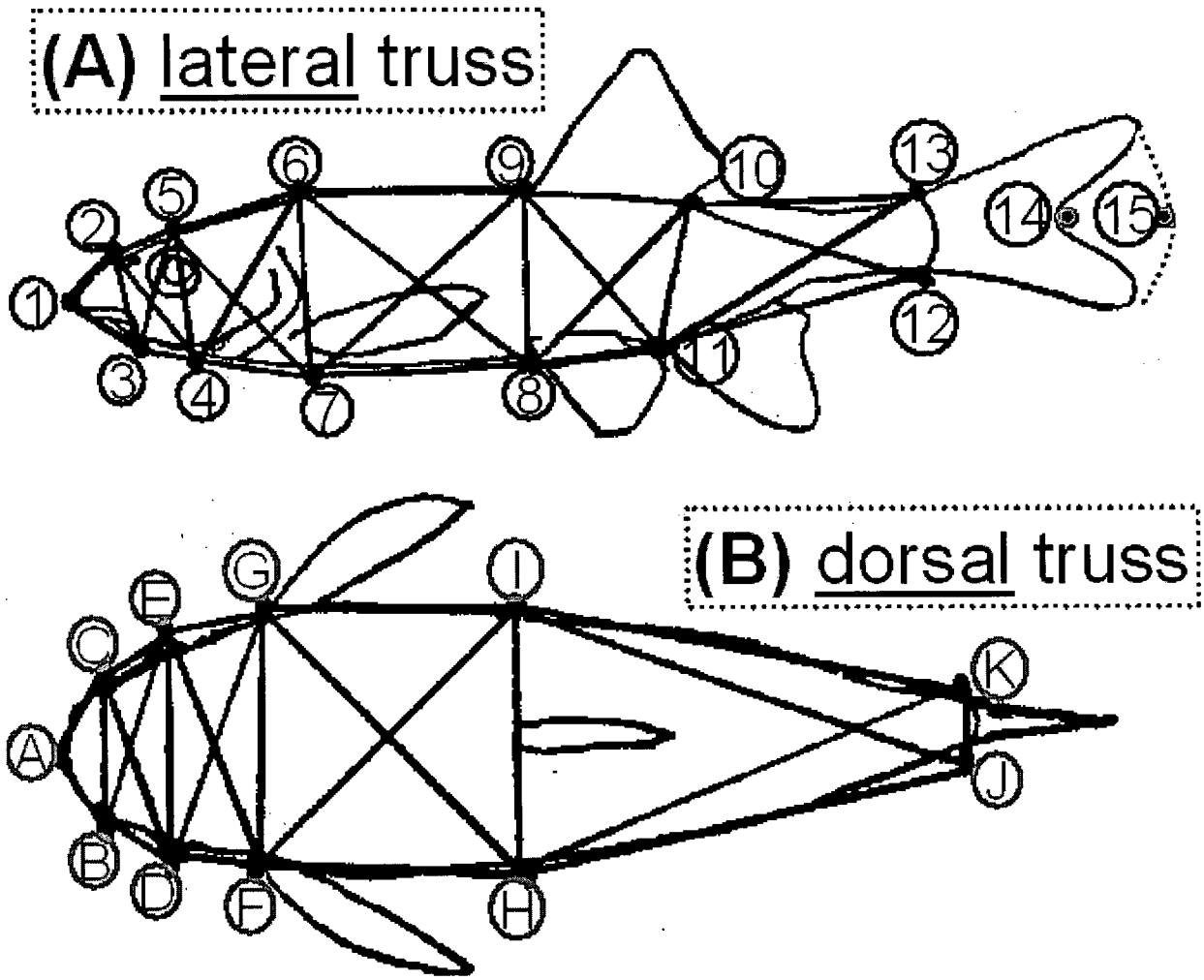
Two truss measurement networks taken from a lateral and a dorsal perspective were respectively computed from 15 and 11 landmark points (Fig. 1 and appendix B). The landmark points were marked with a straight probe into thick paper sheets directly from the dace specimens. These marked points were then digitized and their coordinates used to generate 26 lateral and 14 dorsal measurements (appendix B).

Non-truss data were originally selected from literature (Cornelius 1969, Hubbs et al. 1974, Hughes and Peden 1989, McPhail and Carveth 1993, Peden 1991, Peden and Hughes 1988, Schultz 1936) and from unpublished information (Haas pers. comm.). Non-truss measurements were made to 0.01 cm. Its morphometric data component was measured with Mitutoyo electronic digital calipers linked to a computer running a BASIC program written to automatically translate and tabulate its incoming electronic data signals. Where necessary, all non-truss data was collected under a binocular dissecting microscope. Accuracy was verified for all non-truss measurements and all counts by repeating them until the same or a reasonably similar number was obtained. By convention in ichthyology (Hubbs and Lagler 1964), the measurements were made on the left hand side of the body. The truss measurements were also verified on 10 fish of each species in the sample category of the 'original 150 fish' to verify the truss programs.

4 - 2. Characteristics and Statistical Assumptions

Measurement error was assessed on the sample category of the 'original 150 fish'. This was done by re-measuring, re-counting, re-marking, and re-digitizing 10 specimens of each species randomly chosen from their 50 dace each. An assessment of the measurement error was undertaken by a comparison of means (Winans 1984), and by examining the ratio of individual to total (among plus within) variation resulting from a one-way ANOVA (Baumgartner et al. 1988, Falconer 1981).

The data set is complete with no missing values. All data collection was done by me and is consistent throughout the study (Lee 1990). Meristic data was not used in the multivariate morphometric analyses since its variables here are not related to size and would have contributed no realistic information to shape. The meristic variables also discriminate the dace species groups and thus would have strongly affected any multivariate analyses. The individual morphometric characters did not have completely definitive or consistent species characteristics and their analyses provide an independent corroboration of the meristic discriminations.



(A) lateral truss landmark points:

1 – tip of snout
2 – posterior edge of nostril
3 – posterior edge of maxillary
4 – posterior edge of orbit
5 – posterior edge of orbit
6 – posterior edge of operculum
7 – origin of pectoral fin
8 – origin of pelvic fin
9 – origin of dorsal fin
10 – insertion of dorsal fin
11 – origin of anal fin
12 – posterior edge of hypural plate (ventral)
13 – posterior edge of hypural plate (dorsal)
14 – posterior edge of fork in caudal fin
15 – posterior tip of outstretched caudal fin lobes

(B) dorsal truss landmark points

A – tip of snout
B – posterior edge of nostril
C – posterior edge of nostril
D – posterior edge of orbit
E – posterior edge of orbit
F – origin of pectoral fin
G – origin of pectoral fin
H – origin of dorsal fin
I – origin of dorsal fin
J – posterior edge of hypural plate
K – posterior edge of hypural plate

Figure 1. Two truss measurement series taken on each dace specimen (total = 500 fish). The lateral truss as presented results in 30 measurements, and the dorsal truss as shown gives 14 measurements. The measurements for the dorsal truss characters that are bilateral are used as the mean from each of those two sides. Definitions for all measurement data are in appendix B.

Final character selection was made to adequately describe the dace and still meet the statistical requirements of the tests to be employed. Measurements were done in all three body dimensions to try to be more representative of its overall shape (Bookstein et al. 1985, Reyment et al. 1984). The morphometric characters were also selected to be highly correlated with size which is desirable as otherwise the separation of size from shape in multivariate analyses can result in false shape variables (Reist 1985, Somers 1986). The size ranges and means of the dace species are similar in all three sample categories, and individuals were chosen to encompass this full size range for each species. Any differences between the groups are thus most probably not based on size alone (Claytor and MacCrimmon 1987). The species are also closely related and roughly equivalently represented in their three sample categories and overall. Morphometric statistical procedures work best when groups have all such features (Corruccini 1975, Pimentel 1979, Siegel and Benson 1982, Thorpe 1983).

Ipsative measures are those which sum to a constant or to another measure. Their presence in data was possible here since a large number of variables were deliberately collected to provide a full representation of the dace phenotypes. Large character numbers provide balance to multivariate analyses that are highly affected by the data used. A sufficient number of variables help stabilize their analyses and strengthen comparisons based on the resulting information. However, the large number of characters is also then often high relative to numbers of individuals in the species in some sample categories. Ipsative measures were evaluated for the 'original 150 fish' sample category through logical screening and by an examination of the eigenvalues from the PCAs. The number of negative eigenvalues from a PCA tends to reveal the number of ipsative measures (Pimentel 1979).

Such data sets can also lead to matrix singularity (determinant $\neq$ zero) through linear dependence and redundancy. High character intercorrelation can result in a problem for multivariate statistics known as Rao's paradox (Corruccini 1987, Healy 1969, Rao 1966, Willig and Owen 1987). In this situation, size differences between groups are over-corrected if their shapes are very similar. This could have been the case with the three dace species. This effect brings the group centroids closer together than they would be in a univariate analyses and can obscure or incorrectly define relationships. This was checked using the 'original 150 fish' sample category.

The specimens from the 'original 150 fish' sample category were sexed when large and mature enough to do so accurately. This was done to evaluate possible effects of sexual dimorphism on other analyses. Two-way analysis of variance (ANOVA) (Thorpe 1976, 1980, Corruccini 1987) and two-way multivariate analysis of variance (MANOVA) (Lande and Arnold 1983, Thorpe 1976, Neff and Marcus 1980, Willig and Owen 1987) were used to separately examine the morphometric and meristic characters.

A sample of 50 for each species was used in the 'original 150 fish' sample category because it is twice the general 'rule-of-thumb' recommendation of a minimal sample size of 25 individuals per group for morphometric analyses (Mardia 1971, Neff and Marcus 1980, Reist 1985). The 50 dace for each species also gave an acceptable range for character selection and use since most multivariate statistical programs require that sample size exceed character number. This requirement is actually based on total sample size (at least ≥ 150 fish in the 'original 150 fish' sample category) exceeding character number, but it is often thought to be better used as the conservative number of specimens in each group in any multivariate analysis.

A univariate / bivariate test and a multivariate test were used to examine the sufficiency of the sample size for the 'original 150 fish' category. This was done separately on morphometric and meristic variables in the multivariate test. Both tests involve random selection of individuals and plotting against sample size. In the univariate case, the plotting is of cumulative means and associated standard deviations and standard errors, and for the multivariate instance it is the correlation matrices determinants. An adequate sample number is indicated by where the resultant curve stabilizes and asymptotes.

5. Analyses - Univariate / Bivariate

5 - 1. Ratios - Size Correction

For the univariate / bivariate data representation, the various untransformed morphometric measurements are individually adjusted for size by simple division by fork length. The meristic characters are not size related, compensated, or transformed. This ratio for size correction of the morphometric characters was tested by comparison to division with the two other body length measurements collected (appendix B).

5 - 2. Meristics and Morphometrics

One major and one other main univariate / bivariate discriminating meristics were developed using the 'original 150 fish' sample for identification of the three dace species throughout their mutual range (Figs. 2 and 3; appendix A). The main discriminator is a barbel score index ranging from one to three, which is based on its size, visibility and directionality (also see Peden and Hughes 1988). Barbel length is actually the key diagnostic and is what the three score index categories are based on. However, its measurement is almost certainly highly individualized and probably has low inter-user repeatability. Avoidance of these potential problems was the main rationale for a barbel score index, but this also permitted the incorporation of other barbel information that seemed to assist in identifications.

Pelvic fin stays are the second main discriminating meristic (also see Peden and Hughes 1988) and are based on a standard geometry equation for the are of a triangle. The pelvic fin is not a strict triangle but its area was most easily approximated this way. The formula for the

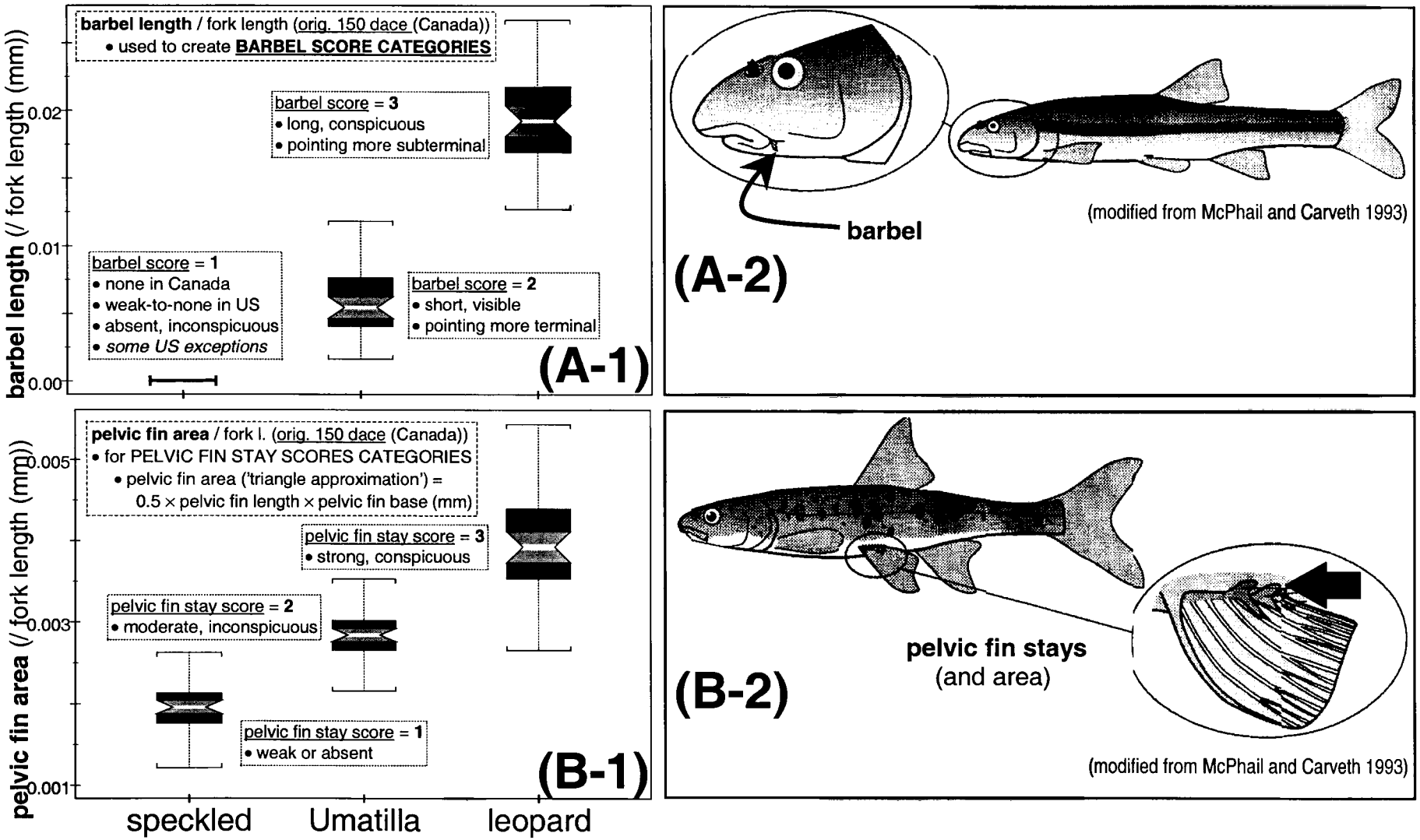


Figure 2. Boxplots showing how the three barbel and pelvic fin stay scores (Fig. 3) were generated. Barbel length (A-1) totally separates each species, and pelvic fin area (B-1) discriminates them on their main nonoverlapping components. The white horizontal line in each species' box is their data median, the boxes are their data quartiles, and the vertical dotted lines extending from them encompass their data ranges. A single horizontal line with vertical bar ends (instead of a box) represent a single species value. Nonoverlapping grey notches between species' boxes indicate statistically significant differences at about 5% (Chambers et al. 1983). Figure 2(A-2) shows the location and appearance of barbel for a dace, and Figure 2(B-2) that for pelvic fin stays.

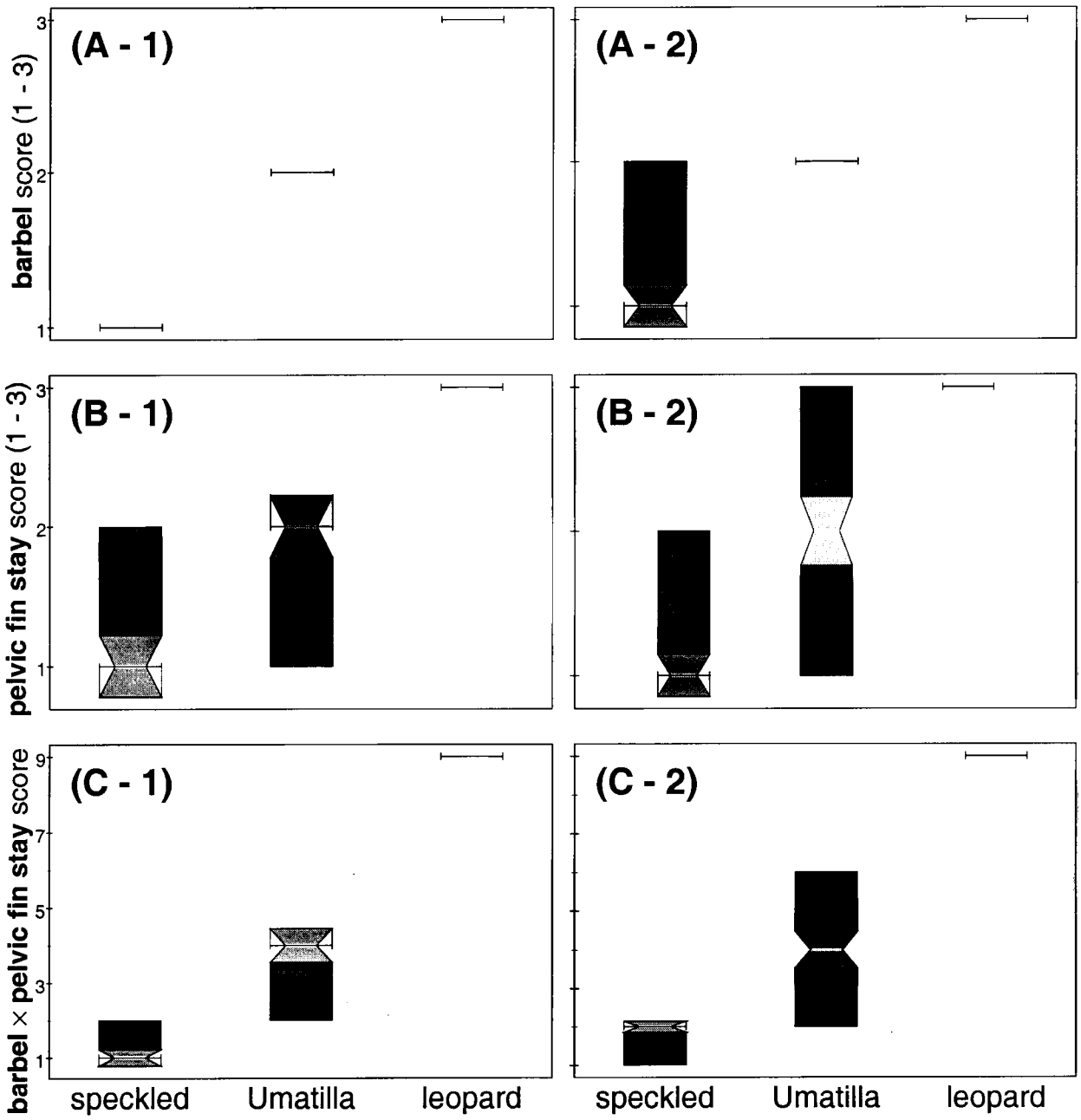


Figure 3. Boxplots of barbel scores, pelvic fin stay scores, and their product (see Fig. 2 for score definitions and categorization). Barbel and pelvic fin stay scores always discriminate leopard dace, but not always speckled and Umatilla dace in the USA. Their multiplicative product can further help to distinguish species in some instances. Plots A-1, B-1 and C-1 are for the 'original 150 fish' sample category from Canada used to establish the species discrimination. Plot A–2 is for the 407 dace sampled from the 'Columbia and Fraser river' watersheds sample category, in Canada and the USA. Plots B-2 and C-2 are for 330 dace sampled from the Columbia and Fraser River watersheds in Canada and the USA (appendix A). These latter two plots for the 330 dace (B-2 and C-2) are for *speckled dace with barbels only (USA)*, but for all specimens of Umatilla and leopard dace present in the USA. The white solid horizontal line in each box is each species' data median, the boxes are their respective data quartiles and the vertical dotted lines extending from each box encompass their respective full data ranges. A single line with vertical bars at each end instead of a box indicates a single score only for that species. Nonoverlapping grey notched areas between species' boxes in each plot indicate statistically significant differences at a rough 5% (Chambers et al. 1983).

area of the pelvic fin is *0.5 × pelvic fin length × pelvic fin base* in mm. This area measure was initially rationalized as increasing with stronger pelvic fin stays. The pelvic fin stay scores also incorporate a subjective assessment of their presence and conspicuousness.The third discriminating variable is dorsal fin ray number. These three characters for discrimination were further selected to be able to be used in the field and without necessarily sacrificing fish. Three other characters specific to identifying speckled dace, four for leopard dace, and four that worked for all three dace species were also developed.

6. Analyses - Multivariate

6 - 1. Statistical Assumptions

Univariate normality tests were the probability (quantile) plot correlation coefficient procedure (Ryan and Joiner 1974, Filliben 1975) and the Shapiro-Wilk statistic (Shapiro and Wilk 1965). Multivariate normality tests are uncommon so this was visually assessed through probability plots. If the probability plot approximates a straight line that is taken to indicate multivariate normality (Andrews et al. 1973, Everitt 1978, Healey 1968, Wilk and Gnanadesikan 1968, Wilk and Shapiro 1968). Multivariate normality was also further evaluated by looking for an ellipsoidal point pattern that should result from plotting the first two PCs from a PCA against each other if such normality is present.

Homoscedasticity, or homogeneity of group variances and dispersions, was conservatively tested (Phillips et al. 1973, Somers 1986) using Box's modification of Bartlett's test (Pimentel 1979). Homoscedastic data definitively enable the analysis of total data matrices for subsequent multivariate analyses such as PCA, rather than of the pooled within-group matrices sometimes suggested (Phillips et al. 1973, Pimentel 1979, Shea 1985, Somers 1986). Furthermore, my specimens are from compound localities (different combined populations) such rationale for pooling is additionally weakened (Sokal 1965, Thorpe 1976, 1980).

Nonetheless, PCAs were run as checks using pooled within-group data (Reist 1986) from the 'original 150 fish' sample category and using two related multivariate procedures which necessarily account for groups: sheared PCA (Bookstein et al. 1985, Humphries et al. 1981), and linear discriminant function analysis (Fisher 1936) which explicitly requires *a priori* group designation. Jackknife tests on the PCAs were also used to examine the effects on their output of different subsets of data (Gibson et al. 1984).

6 - 2. Principal Components Analyses (PCAs)

Q-mode PCAs were undertaken on total covariance matrices of the 42 $\log_{10}$ - transformed morphometric characters for all three sample categories (appendix B). Significance was conservatively tested using Bartlett's chi-square test of sphericity (Phillips et al. 1973, Pimentel 1979) and the graphical Scree test (Cattell 1966, Somers 1986). This significance and the overall resultant patterns from the PCA were further substantiated by the jackknife analyses

(Gibson et al. 1984). Another general 'rule-of-thumb' (Kaiser 1960) is that no eigenvectors (EVs) or principal components (PCs) with eigenvalues less then one should be interpreted. An alternate PCA based approach for size correction (Burnaby 1966, Rohlf and Bookstein 1987) was also tried in order to verify the results.

6 - 3. Allometry and Growth

The allometry coefficients are calculated using intraspecific PCA on a covariance matrix of only $\log_{10}$ transformed morphometric data for the 'Columbia and Fraser river' sample group. The PCAs are done separately on each of the three dace species, as otherwise the character loadings would only be for them as a single composite entity. The morphometric data consisted of 42 characters from the two truss networks (appendix B) because it was desirable to just represent overall body shape. Since size is not part of the meristic data, those characters could not provide realistic allometric information and were not used (Reist 1985, Somers 1986).

The characteristics, statistical assumptions, and procedural justifications as described and done for the other 42 character matrix (appendix B) used in the previous PCAs undertaken largely for discrimination of the three sample categories (Fig. 2) were carried out here with similar results and outcomes. These are not presented and discussed again as that lengthy information is already readily available here and thus also for the simple sake of brevity.

The multivariate allometry coefficients are calculated by making the first morphometric EV isometric for all characters (number = p) by transforming each of their loadings to values of $p^{-0.5}$ (Jolicoeur 1963, Mosimann 1970, Somers 1986). The actual first EV is then divided by this first isometric one. These calculations require the morphometric data to be log transformed.

The patterns from the multivariate allometry coefficients were corroborated using those obtained from two linear least square regressions on the morphometric data set. One is based on the mean of the $\log_{10}$- transformed morphometric data (Claytor and MacCrimmon 1987, Reist 1985, Shea 1985, Thorpe 1975) for each fish. The mean individuals represent shape for that particular specimen, and the allometry coefficients are the slope of the regression lines. The second is an examination of the residuals about the same regression line.

The least squares regression technique was itself also verified with reduced major axis regression (Gould 1966). The latter procedure is often deemed preferential on the theoretical grounds that the size measure (x-variable) in least-squares regression is not truly independent because of measurement error (Claytor and MacCrimmon 1987, Kuhry and Marcus 1977, Sacher 1970). This dependence could result in its regression estimates being unpredictably biased downward (Cock 1966, Leamy and Bradley 1982, Manaster and Manaster 1975, Zar 1968).

The multivariate allometry coefficients are preferentially used because they have several advantages. The main one here is that my data are cross-sectional (composed of different

individuals at different sizes and ages) from throughout the ranges in the Pacific Northwest of North America for these three dace species. It has been established that this type of data gives good and real ontogenetic information (Atchley and Rutledge 1980, Cheverud 1982, Gould 1971, Lande 1979, Shea 1985), but also that it is best represented by PCA. This is because the allometry or growth being assessed is not based on longitudinal data from individual organisms (Jolicoeur 1963, Pimentel 1979, Reyment et al. 1984). The assumption here then is that size is a realistic surrogate of chronological age (Bookstein et al. 1985, Cheverud et al. 1983, Creighton and Strauss 1986, Shea 1985). This assumption is very likely not unreasonable since these samples of cross-sectional data are quite large (Alberch 1980, Strauss 1987, Strauss and Fuiman 1985). It is further advantageous that this data set is for fish since they have theoretical indeterminate growth.

The multivariate allometry coefficients are also based on a composite multivariate size factor and not just single variable. There is no problem selecting a single representative size variable as must occur in regression, and the intercorrelations of all the characters are used (Lande and Arnold 1983, Reyment et al. 1984). The composite multivariate size measure is assessed as an age indicator and for its interspecific consistency and equivalency using correlations between the intraspecific PC1 size vectors and between the three intraspecific univariate body length measurements collected here (Cheverud 1982, Creighton and Strauss 1986).

As well, the multivariate allometry coefficients are not just empirical descriptions of growth patterns, but have been shown to be the solution to the differential equation relating growth rates of a character and body size to time (Lande 1985, Shea 1985, Strauss 1987). The allometry coefficients are thus equivalent to the growth rates of morphometric characters relative to body size (Alberch et al. 1979, Creighton and Strauss 1986).

Regression techniques are not as robust but they do still provide a good check as they are accepted as the best univariate / bivariate approximations of allometry (Corruccini 1978, Gould 1966, Reist 1985). The regression procedures are related to the power function $y=ax^b$ (Huxley 1932, Snell 1891) which describes exponential growth of each part of an organism and has a basis both cellular (Gerhart et al. 1982, Katz 1980, Laird et al. 1965, 1968) and morphometric (Blackstone 1987, Creighton and Strauss 1986, Strauss and Fuiman 1985).

6 - 4. Allopatry versus Strict Sympatry

PCAs were done separately on (i) total allopatric and (ii) total strict sympatric samples of the three dace species combined in each of these distribution categories. These were used to assess possible overall and character specific body shape changes under each of these distribution patterns. The PCAs were done separately on the allopatric and sympatric samples, as otherwise their character loadings would only be for them as a single composite entity. A

single total PCA would also not really be valid for this descriptive examination since its results are obviously data specific. Such a PCA could be biased by combining the data from two discrete entities and this combined PCA could then also compromise the variability from each distribution category and their subsequent accurate and realistic description.

PCAs could also have been undertaken on each individual species in allopatry and strict sympatry. This was not done due to strict sympatric sample sizes being considerably smaller in sympatry than allopatry. The important associated concern was that the character number in the data set would easily exceed that of the fish number of any strictly sympatric sample for one species, and would then have to be greatly reduced and be very much less informative. The combination of the three species into a single strict sympatric sample and analysis helped circumvent these sample size issues. Nonetheless, their combined analysis does not permit as detailed an assessment as analyses of individual species would because the combined data output cannot be assigned to any specific single dace species. Instead, the combined results are interpreted as overall changes that might occur to the dace species in sympatry or allopatry.

Since information was *a priori* available on group designation, a linear discriminant function analysis (DFA) of the total data in this sample category could also have been argued for. This was similarly not undertaken because biologically informative character loadings on a shape vector were the desired analytical outcome. The loadings from a DFA are not necessarily indicative of the scaled importance of characters to a description of shape and are not necessarily based on shape alone. DFA loadings usually are the numbers by which their individual characters would be multiplied to obtain that DFA discrimination. PCA was thus the preferred analysis for obtaining realistic character information on a shape axis.

The scatterplots resulting from the two PCAs are not presented because the main interest was in the PC2 shape loadings and their particular presentation in Figure 9 was desirable for interpretation purposes. The PCA scatterplots were also still very similar to those done for the 42 morphometric characters (Fig. 7) and such a traditional representation would not have been anywhere as informative for these analyses.

The boxplot that is presented instead has the PC2 shape vector scores 'scaled' on its y-axis so that sympatric and allopatric groups from two PCAs could be equivalently compared (Fig. 8). This 'scaling' was done simply by taking the largest and smallest PC2 scores from both PCAs and scaling the largest to the smallest by dividing it by the largest difference in PC2 scores from the two PCA data sets. This brought both PC2 ranges in line with each other through the use of a single constant scalar in order to make their comparisons legitimate and equivalent. The scaling of the scores for the fish did not affect the loadings for their measured characters presented in Figures 9 and 12.

Two morphometric variables, snout overhang and barbel length, used in the previous PCAs were discarded here because their discriminatory power (Figs. 2, 3, and 4A) could have biased the PC scores here which were best absolutely related only to shape for this analytical purpose. The univariate body size measure of standard (or fork or total) length (appendix B) was removed from the analyses since it would have been inappropriate, and because it was already being used to evaluate the validity and utility of the multivariate size vector. Accurate and realistic description of shape was much more important than just discrimination based on it in this case. The meristic characters were not used for the same reason (Figs. 2 and 3; appendix B).

The characteristics, statistical assumptions, and procedural justifications as described and done for the regular 42 character matrix (appendix B) used in the previous PCAs undertaken largely for discrimination of the three sample categories (Fig. 7) were carried out here with similar results and outcomes. The necessary interspecific consistency and equivalency of the intraspecific PC1 size vectors were also evaluated and confirmed via the same correlations as for the allometry coefficients. All these are not presented and discussed again as that lengthy information is already readily available elsewhere here and thus also for the simple sake of brevity.

The data used here are for the 'Columbia and Fraser river' sample group and consist of 39 $\log_{10}$ transformed morphometric characters (appendix B) multivariately analyzed in a covariance matrix. There were 78 allopatric and 32 strict sympatric speckled dace in 22 samples, 154 allopatric and 27 strict sympatric Umatilla dace from 35 sample sites, and 104 allopatric and 12 strict sympatric leopard dace in 20 samples. Twelve sample sites were strictly sympatric, and represented all possible species' pairings and one sample site for all three species together (appendix A). The most prevalent strict sympatric species pair was speckled and Umatilla dace, with Umatilla dace dominant in terms of abundance at five sites and speckled dace dominating at two sites. All other species pairings consisted of one site each.

Results

The numbering of the headings in the 'Results' section is deliberate and coincides with that for the preceding 'Materials and Methods' section. When no 'Results' exist for a particular heading from the 'Materials and Methods' section then its heading number is absent here in the 'Results' section. There is a lot of detail and information in these two sections, and this numbering is meant to help keep track of and coordinate them.

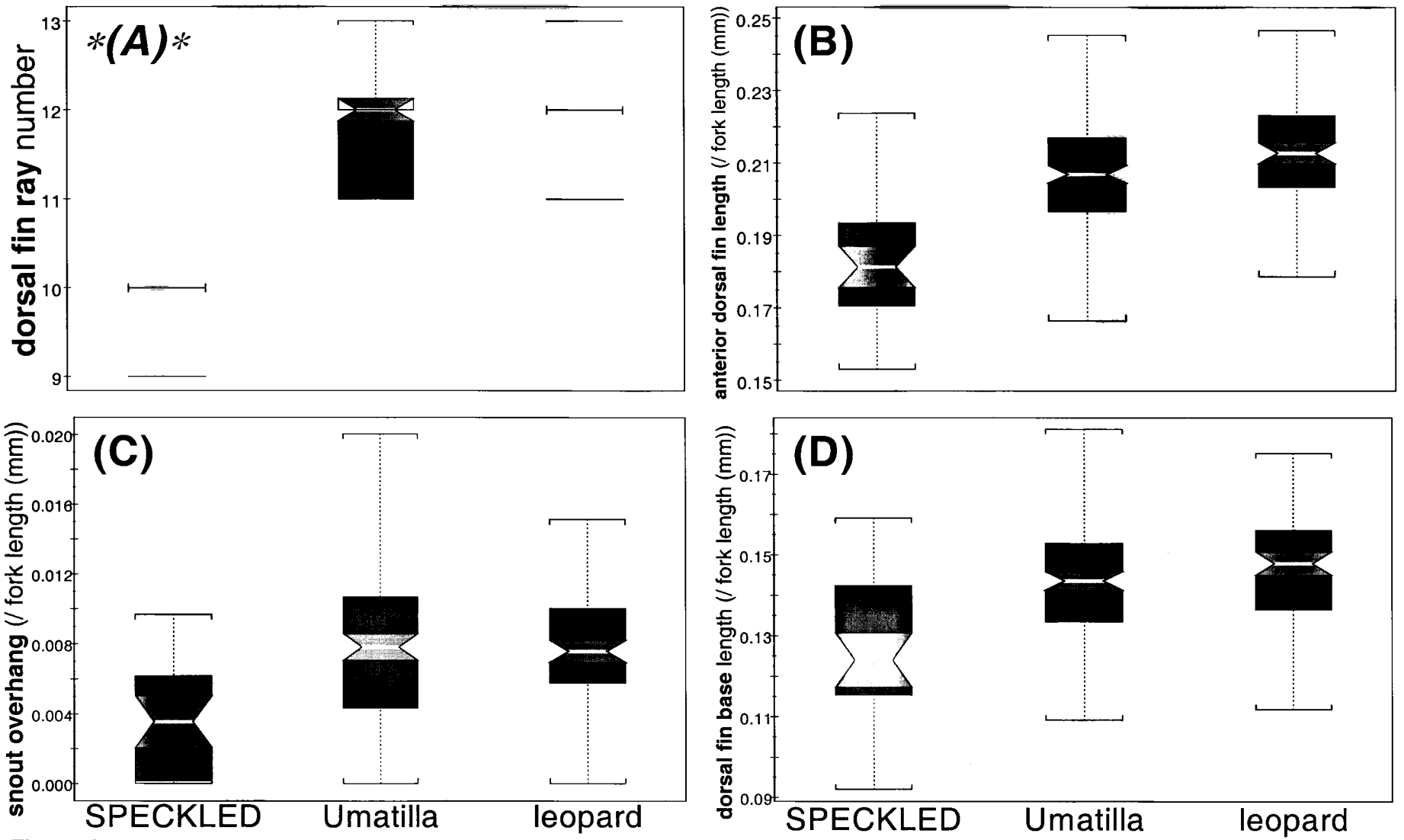


Figure 4. Boxplots of the four best other measurements for distinguishing speckled dace (note plot (A) in particular). The data are for 330 dace sampled from the Columbia and Fraser river watersheds in Canada and the USA, and are for *speckled dace with barbels only (USA)*, but for all Umatilla and leopard dace in the USA. Speckled dace with barbels can otherwise not be fully discriminated (Fig. 3). The white solid horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical dotted lines extending from each box encompass their full data ranges. Nonoverlapping grey notched areas between boxes in A-D indicate statistically significant differences at a rough 5% (Chambers et al. 1983).

4. Data

4 - 2. Characteristics and Statistical Assumptions

The mean amount of measurement error for morphometric and meristic data is negligible. The one-way ANOVA revealed that the ratio of individual to total (among plus within) variation are large (close to one) indicating that measurement repeatability is high and measurement error is insignificant. Each of the species also had similar patterns of insignificant error.

The morphometric characters are all highly correlated with size, but no evidence of potential related problems such as Rao's paradox were evident for the multivariate analyses. In support of this, the dace data have more statistically significant group differences based on MANOVA than on ANOVA. Better group separation was thus obtained in the multivariate than univariate / bivariate analyses.

No ipsative measures were detected in the reduced 42 morphometric measurement data set. There were no negative eigenvalues. The data are not singular because the matrix is at least positive semi-definite. Its analysis also ran under available statistical programs that generally will by default not analyze singular data matrices.

No statistically significant or strongly consistent univariate or multivariate sexual dimorphism for morphometric or meristic data was detected in any of the species (p at least > 0.1). The PCAs done on the morphometric variables also reveal no differences between these males and females. Pectoral fin length did have some sexually dimorphic patterns (Hubbs et al. 1974), but not consistently for all samples or throughout their ranges, and particularly not at levels of statistical significance. Pectoral fin length might be used in some situations for the identification of males and females, but this would probably have to be developed on a population or smaller regional level. Where such sexual dimorphism was noticeable, males had larger pectoral fins. In some of these instances, the larger male pectoral fins also seemed to have a downward arch in comparison to the females (Hubbs et al. 1974).

A sample size of 25 – 30 dace would have been sufficient as determined by both univariate tests of both the morphometric and meristic data, and by multivariate tests of the morphometric data. The fish numbers for the other sample categories were much higher and they were assumed to be sufficient based on these results. In all cases, the number of fish representing any of the dace species was higher than the number of characters analyzed.

5. Analyses - Univariate / Bivariate

5 - 1. Ratios - Size Correction

Size correction of all morphometric characters by division with the two other body length measurements collected (appendix B) produced identical results. Fork length was used in these ratios because it is a more recognizable standard by fish biologists in the Pacific Northwest in North America, most of who much more regularly work on salmonid fishes. The

use of standard length has caused measurement error problems for previous species discriminators (Haas and McPhail 1991, 2001a/b).

Ratios are the oldest morphometric method for size correction, but also have inherent statistical problems (Burnaby 1966, Pimentel 1979, Reyment et al. 1984). They are nonetheless used here, and often elsewhere, because of their ease and simplicity. Ratios were used here because individual characters can be examined and size-adjusted in the field without need or ready access to calculators or computers. For this purpose, ratios here crudely but effectively accounted for body size and still provide sufficient discrimination of the three dace groups.

It is nonetheless important to be aware of the potential problems with ratios. Ratios assume a correlative and linear relationship between the variables involved (Alberch 1978, Atchley et al. 1976, Reist 1985). Many variable-to-size relationships still meet these criteria, but it is also assumed that the axis describing their linear relationship passes through the origin, which is rare (Thorpe 1983). Other issues are that ratios do not remove scaling effects (Atchley et al. 1976) and compound error terms (Reist 1985).

5 - 2. Meristics and Morphometrics

Barbel length completely separates the three dace species in the 'original 150 fish' sample (Fig. 2A; also see Peden and Hughes 1988). The development of the barbel index with three score categories related to these barbel length species' divisions seemed justified (Fig. 3(A1)).

Pelvic fin area appears to be reasonably approximated by its calculation based on a triangle (Fig. 2B). Pelvic fin area separated most, but not all, of the specimens of the three species in this sample category (Figs. 2B). Pelvic fin area was still deemed sufficient as a basis for the desired development of a similarly based and related score index to that for barbels. The pelvic fin stay index also had three score categories similarly ranging from absent or weak to strong (Fig. 3(B1)). The pelvic fin stay scores came from the non-overlapping pelvic fin areas that were based on the data quartiles and non-overlapping boxplot 'whisker' that existed for each species (Fig. 2B). The combination of barbel and pelvic fin stay scores as a multiplicative product even further improved the discrimination of the three species (Fig. 3(C1)), and could be used to assist in identifications that might seem inconclusive using either score index by itself.

The absence of barbels was known to sometimes not be consistent for speckled dace outside Canada and within the USA (Bisson and Bond 1971, Bond 1973, Peden and Hughes 1988, Wydoski and Whitney 1979). The development of these two score indexes and their multiplicative product here was hoped to be sufficient to still identify those speckled dace known to have barbels. The 'original 150 fish' category confirmed that speckled dace in Canada did in particular not have barbels (Fig. 3(A1)) so the discriminators worked very well in that country (Figs. 3(A1-C1)). This did unfortunately not end up being the case (Figs. 3(A2-C2)). The

identification issue that then remained was for the identification of speckled dace that had barbels, with such speckled dace only being found in the USA (Hughes and Peden 1989, McPhail and Carveth 1993, Peden and Hughes 1988). Figures 3(B2-C2) show only those speckled dace with barbels because those without them would not have needed these other two discriminators for their identification.

Dorsal fin ray number completely discriminates these speckled dace with barbels (Fig. 4A). It could not separate Umatilla and leopard dace but this was unnecessary since the barbel and pelvic fin stay score indices could. The combination of these three univariate / bivariate discriminating characters and indices separated all three dace species in all situations examined here. Some of these identifications of speckled dace could nonetheless still be difficult so three more univariate / bivariate morphometric measurements were developed to act as additional discriminators and as backups for any such more tenuous situations (Figs. 4(B-D)). These three characters, aside from dorsal fin ray number (Fig. 4A) are anterior dorsal fin length (Fig. 4B), snout overhang (Fig. 4C; also see Peden and Hughes 1988), and dorsal fin base length (Fig. 4D).

Four additional univariate / bivariate morphometric measurements were developed to assist in the other remaining species discrimination of leopard and Umatilla dace (Fig. 5). With speckled dace having barbels already being discriminated, leopard and Umatilla dace should always have been identifiable using the barbel and barbel $\times$ pelvic fin stay score indices (Figs. 3(A2) and 3(C2)). However, it was recognized that these two score indices can still be somewhat subjective and individualized (also see McPhail and Carveth 1993, Peden and Hughes 1988). Therefore, these four other characters can further assist in the discrimination of leopard and Umatilla dace in situations where speckled dace have barbels. There should be no problem in distinguishing these species in regions where speckled dace have no barbels (Figs. 3(A1-C1)). None of these four characters could completely categorize leopard or Umatilla dace, but rather worked in the majority of cases. In descending order of probable utility, these four size-corrected morphometric characters are pelvic fin base (Fig. 5A), mouth width (Fig. 5B), tail falcation which is total length – fork length (Fig. 5C), and lateral 'thinness' which is fork length / body depth taken at the dorsal fin origin (Fig. 5D).

As a final assistance to species discrimination, four more morphometric measurements were developed that further help identify all three dace species in the 'Columbia and Fraser rivers' sample category (Fig. 6). This discrimination included all speckled dace, with and without barbels, in this sample category. In descending order of probable utility, these four morphometric measurements are pelvic fin length (Fig. 6A), anterior anal fin length (Fig. 6B), caudal peduncle depth (Fig. 6C), and eye size (Fig. 6D).

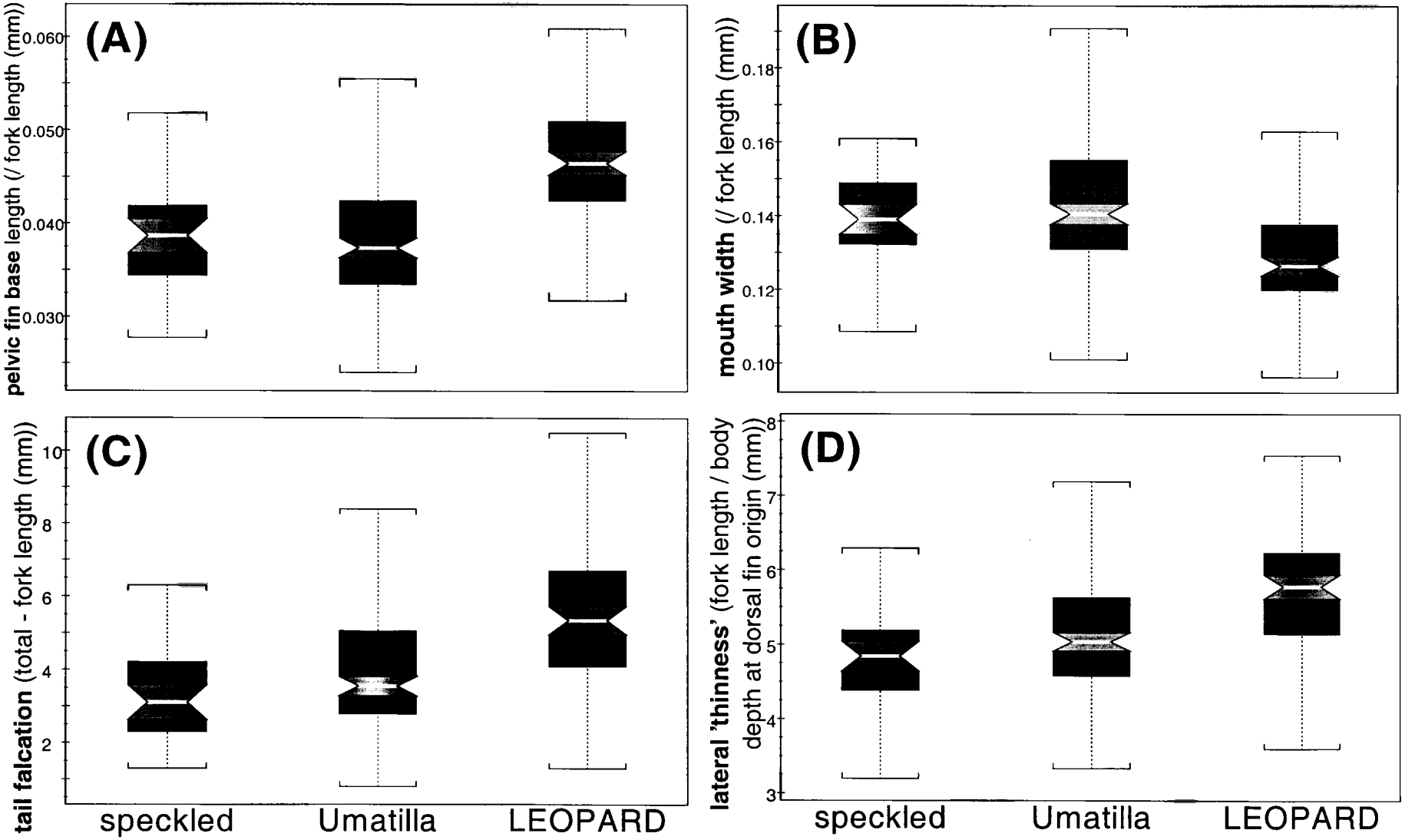


Figure 5. Boxplots of the four best other measurements for distinguishing leopard dace. The data are for 330 dace sampled from the Columbia and Fraser river watersheds in Canada and the USA, and are for speckled dace with barbels only (USA), but for all Umatilla and leopard dace in the USA. Leopard dace can be fully discriminated by barbel and pelvic fin stay scores, but these can be subjective (Figs. 2 and 3). The white solid horizontal line in each box is each species' data median, the boxes are their data quartiles, and the vertical dotted lines extending from each box encompass their data ranges. Nonoverlapping grey notched areas between boxes indicate statistically significant differences at a rough 5% (Chambers et al. 1983).

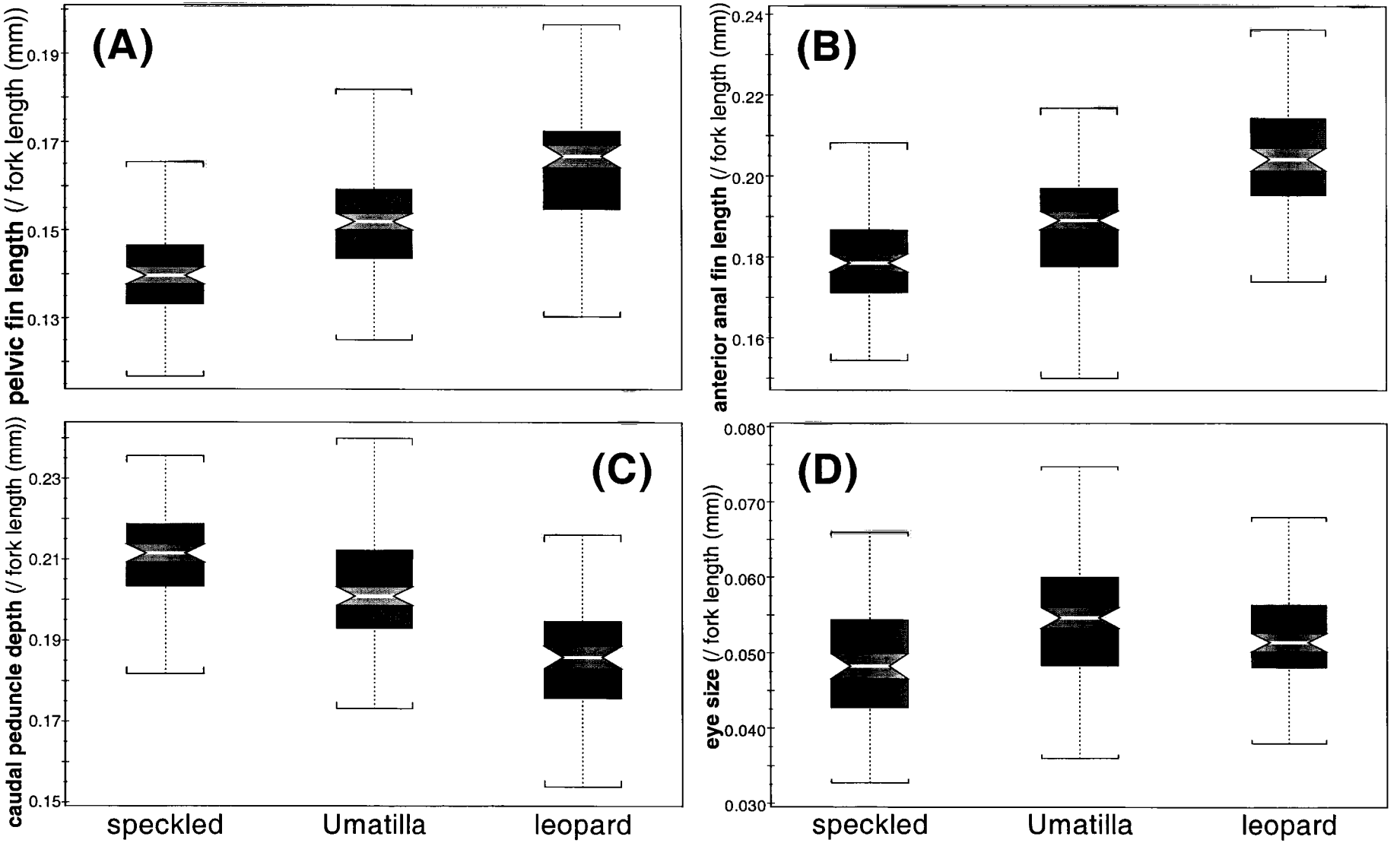


Figure 6. Boxplots of the four best other measurements that could further help identify all three dace species. The data are for 407 dace sampled from the Columbia and Fraser river watersheds, in Canada and the USA (appendix B). The white solid horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical dotted lines extending from each box encompasses their respective data ranges. Grey nonoverlapping notched areas between species' boxes in each set (A-D) indicate statistically significant differences at a rough 5% (Chambers et al. 1983).

This large number of characters provided for additional discrimination that should make correct identification of the three dace species very probable. These additional characters can be used for backup identification in ambiguous, uncertain, or preferable instances.

Ten of the 11 additional discriminating variables work in an identical manner to the four key discriminators of barbel and pelvic fin stay score indices, their multiplicative product, and dorsal fin ray number. All these characters range from low to high, with speckled dace always being in the lowest category and leopard dace in the highest (Figs. 2-6(A-C)). Eye size is a minor exception. Speckled dace are still in the smallest category with the smallest eyes, but Umatilla dace have a trend toward largest eyes with leopard dace being more intermediate (but see speculations in Hughes and Peden 1989). The differences in eye size between the boxes in the plots appear slight, but they are nonetheless statistically significant for all three dace species.

6. Analyses - Multivariate

6 - 1. Statistical Assumptions

The morphometric data are normally distributed under univariate and multivariate conditions when $\log_{10}$ transformed (Pimentel 1979, Smith 1980). Such transformation also helped standardize the individual character variances and scales (Jolicoeur 1963, Smith 1980). Multivariate normality was further confirmed by later PCAs that had ellipsoidal point distributions in the plots of their first two PCs (Thorpe 1976, Reyment et al. 1984).

Linearity of the morphometric data sets are also assumed based on all these results since linearity is very strongly related to normality (Neff and Marcus 1980, Pimentel 1979). The transformed morphometric data are homoscedastic ($p > 0.5$) for all species and all sample categories.

The PCAs on pooled within-group data, the jackknife tests of the PCA itself, and the pooled within-group procedures of sheared PCA and linear discriminant function analysis all produced very similar results to the PCA. A direct analysis of the total data matrix is justified. Such decisions on analyses of total versus pooled within-groups could have been important because PCA was specifically selected since it does not require *a priori* grouping. Prior group designation can be subjective, is better used for discrimination than description, and assumes there is only one taxon per group that can be completely distinguished (Humphries et al. 1981, Pimentel 1979, Thorpe 1976, 1980). The basis of statistics involving *a priori* group designation also is group ordination rather than individuals, which was not necessarily desirable here.

The PCAs undertaken here on morphometric data naturally demonstrated and discriminated any groups and were not subjectively predisposed to doing so. A covariance matrix analysis was used since it is generally believed to be more realistic and representative of the associated character and individual data (Pimentel 1979, Corruccini 1983). Re-doing these multivariate analyses using a correlation matrix of the same transformed variables also produced essentially

identical ordination results. The same results were also obtained from an alternate size correction protocol (Burnaby 1966). These consistent similarities in results from multiple approaches strongly suggest that the PCAs are realistic.

6 - 2. Principal Components Analyses (PCAs)

In all the PCAs, only the first two EVs and PCs were significant under all tests and guidelines. The second EVs and PCs accounted for all the statistically significant variation (Bartlett's test, $p<0.05$; Scree test) remaining after the first. Only these first two were subsequently analyzed. In all multivariate procedures in this Chapter, the first EVs account for the majority of the variation and are general (loadings all have same sign). The second EVs are orthogonal (uncorrelated), account for the remainder of the statistically significant variation, and are bipolar (loadings have mixed signs). The first are characteristics of a composite size vector that has effectively removed non-contributing multivariate size and its rate of change from subsequent EVs (Campbell 1976, Reyment et al. 1984). If the second EV is also statistically significant, and particularly if it is the only other one that is, then it is an excellent estimate of multivariate shape (Lande 1985, Leamy and Bradley 1982, Somers 1986, Strauss 1987). These general interpretations have been justified mathematically (Rao 1964), and makes intuitive sense since large size-related variation should normally predominate in morphometric data (Sacher 1970).

Further support for these size and shape interpretations are that larger and smaller dace in my data respectively have larger and smaller scores on the first PC (Cheverud 1982, Creighton and Strauss 1986), whereas the second PC does not distinguish between different sized individuals but rather between their groups. This is the case for all three dace species.

The PCAs show the three dace species in all the sample categories to be clearly and overall similarly distinct (Fig. 7). The species' centroids (Figs. 7(A1-C1)) and their medians, quartiles and ranges (Figs. 7(A2-C2) show the same pattern. The group distinctions are solely based on the second PC that has been demonstrated here to represent multivariate shape. Umatilla dace, and any measures of central tendency for its data, are consistently intermediate to speckled and leopard dace.

Some difference can be seen in the slopes of the regression lines from robust local weighting (Cleveland 1979) for the 'original 150 fish' sample category in comparison to the other two sample categories (Figs. 7(A1-C1)). These differences are most likely not realistic and representative of body shape because only the speckled dace in the 'original 150 fish' from Canada sample category are composed solely of fish with no barbels (also see McPhail and Carveth 1993, Peden and Hughes 1988). The barbel length measurement (appendix B – D(14)) did affect this since removing it from the PCA did horizontally flatten out the regression

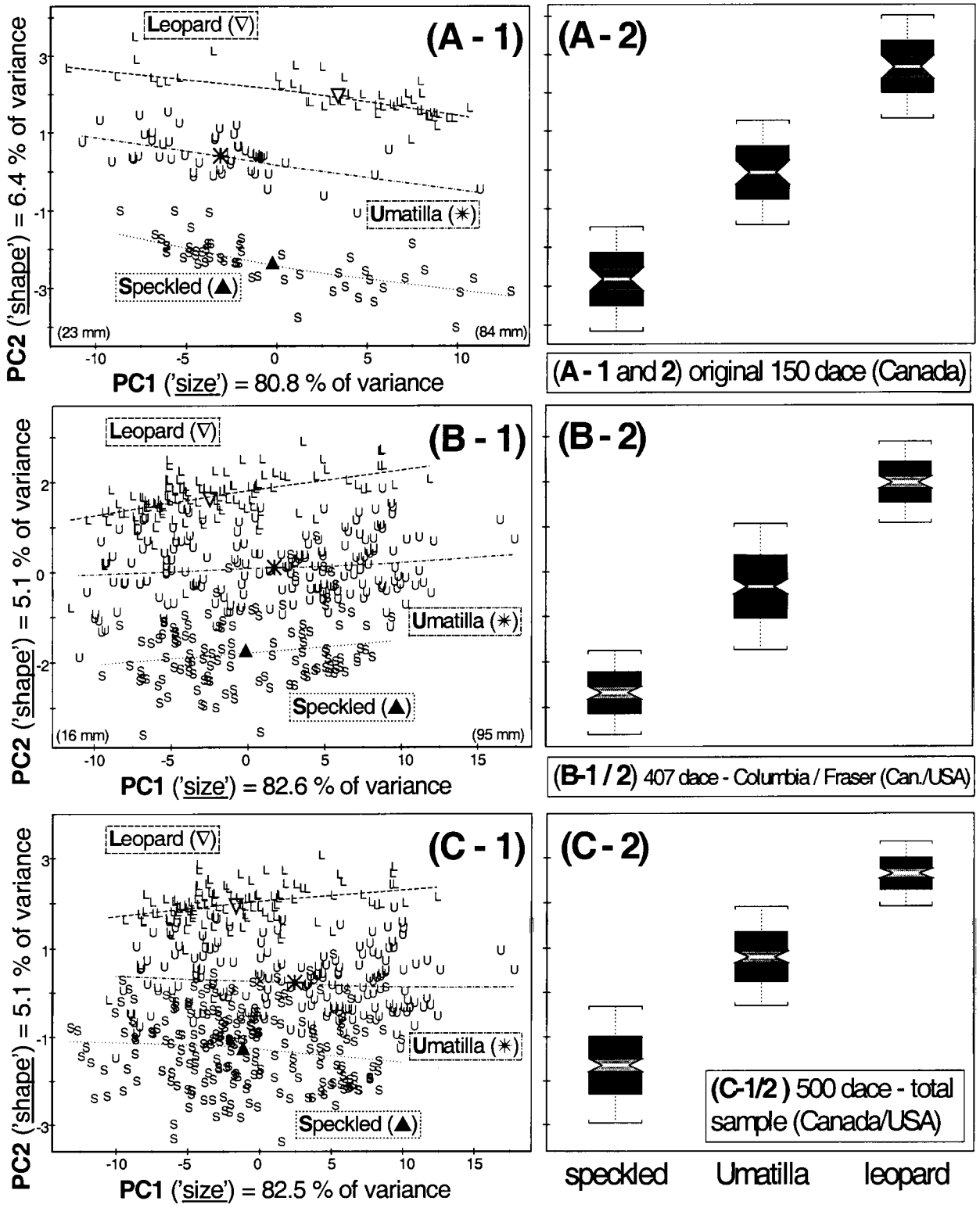


Figure 7. PCA scatterplots (A1-C1) and boxplots (A2-C2) of those PC 2 scores. In each scatterplot, the species are denoted by a symbol for their centroids and by a regression line from robust local weighting (Cleveland 1979). The boxplot PC2 scores are 'smoothed' by linear interpolation to demarcate them completely perpendicular to their axis. The white solid horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical dotted lines extending from each box encompass their respective full data ranges. Nonoverlapping grey notched areas between species' boxes in each plot indicate statistically significant differences at a rough 5% level (Chambers et al. 1983).

lines. Another reason for the different regression line slopes is probably the strongly reduced sample size in the 'original 150 fish' sample category.

The PC2 shape scores on the boxplots (Figs. 7(A2 - C2)) were 'smoothed' by linear interpolation to demarcate them completely perpendicular to their y-axes so that species discriminations could be equivalently compared between the specimen categories without being affected by these different overall grouping directions. The directionality of the groups and the actual scores for the individuals can still be seen on the scatterplots (Figs. 7(A1-C1)). The boxplots were also intended to assist in the interpretation of the data rich PCA scatterplots. Furthermore, these particular boxplots are for the discrimination rather than description of the three dace species. Since no other biological interpretations are being made here, the boxplot representations via an identical 'smoothing' technique for all sample categories are much less potentially problematic.

No differences were seen in the boxplots of the PC2 shape scores (Fig. 7). The varying vertical sizes of some boxplots are simply related to the number of specimens for each dace species in the three sample categories. This can be easily seen by the equal vertical boxplot sizes for the 'original 150 fish' category which was composed of 50 dace per species versus the other two sets of boxplots which had different numbers of specimens for each species.

6 - 3. Allometry and Growth

Similar results for allometry coefficients were obtained under multivariate and regression procedures. Least-squares and reduced major axis regression also produced virtually identical results (Gould 1966, Sacher 1970), with no bias detectable from the least-squares protocol. This similarity could have been expected since the data had minimal and statistically insignificant measurement error.

The validity of the intraspecific PC1 size vectors as age indicators and of their interspecific consistency and equivalency was confirmed by their high correlations between species and to the three body length measurements taken here. These similar size ranges and several central tendency measures for them can also be seen in the single overall PCA based on the total data sets for the various sample categories (Fig. 7), and specifically that for the 'Columbia and Fraser river' dace (Fig. 7B). This further validated the use of this large cross-sectional data set for measuring allometry and ontogenetic growth, and also confirmed that the species comparisons would not be overly skewed by size differences for the three dace. In summary, the differences being examined are realistic and relate to body shape and not overall body size.

Allometry coefficients provide a relative measure of allometry and its direction for each character. The numbers are equivalently centred about an isometric value of one (where body part growth would be constant with size) so that the allometric pattern for each variable can be

seen. If the allometry coefficient if greater than one then positive allometry is present, and if less than one negative allometry is. The size of the allometry coefficients, less than or greater than one, indicate how strong their positive or negative allometry is. This magnitude of the allometry coefficients gives the allometric growth rates of each morphometric character.

All this is how and why the allometry coefficients are given as they are in Figure 8, where allometry coefficients for Umatilla dace are mainly presented in comparison to those for speckled and leopard dace. Umatilla dace have overall growth more similar to leopard dace in the front region of their head (snout-to-eye region – Fig. 8A) and in the posterior region of their body (under and post dorsal fin – Fig. 8C). Umatilla dace have overall growth more similar to speckled dace in their anterior body region (behind snout-to-eye region and before dorsal fin – Fig. 8B). There in fact are no similarities in allometry coefficients between leopard and Umatilla dace in the anterior body region, and none between speckled and Umatilla dace in the snout-to-eye or posterior body regions.

Umatilla and leopard dace specifically have more similar growth patterns for truss measurements 2-3, 3-5, 4-5, B-D (C-E), and D-E in the snout-to-eye region and for truss characters 10-12, 10-13, H-J (I-J) and H-K (I-J) in the posterior body region (Figs. 8A, 8C, and 9; also see Fig. 1 and appendix B). This largely translates to several snout depth and width growth patterns, and the horizontal and transverse growth of the caudal peduncle and the area behind the dorsal fin, being more similar in Umatilla dace to leopard dace. Umatilla and speckled dace have more similar specific growth patterns for truss measurements 6-7, 7-8, 7-9, D-F (E-G), D-G (E-F), and F-I (G-H) (Figs. 8B and 9; also see Fig. 1 and appendix B). This mainly involves growth in all planes of the region behind the head and before the dorsal fin.

Umatilla dace themselves also have their most strongly unique growth patterns for the specific truss measurements 1-2 and 3-4 in the snout-to-eye region (Fig. 8A) and for 6-8 and 6-9 in the anterior body region (Fig. 8B). There were no unique growth patterns in the posterior body region (Fig. 8C). These translate into growth more specific to just Umatilla dace in the length of very tip of the snout, the ventral distance behind the mouth and before the branchiostegals, the horizontal and transverse length of the dorsal area behind the head, and before the dorsal fin (Fig. 9; also see Fig. 1 and appendix B). No strongly unique allometry coefficients for Umatilla dace were found in the posterior body region (Fig. 8C and 9; also see Fig. 1 and appendix B). These growth patterns strongly unique to Umatilla dace are somewhat loosely, but still easily, defined as those clearly different from both leopard and speckled dace. For instance, they often involve allometry coefficients that are positive or negative for leopard dace and the opposite sign for speckled dace. These instances provide clearer assignment of growth patterns for Umatilla dace as more similar to leopard or speckled dace.

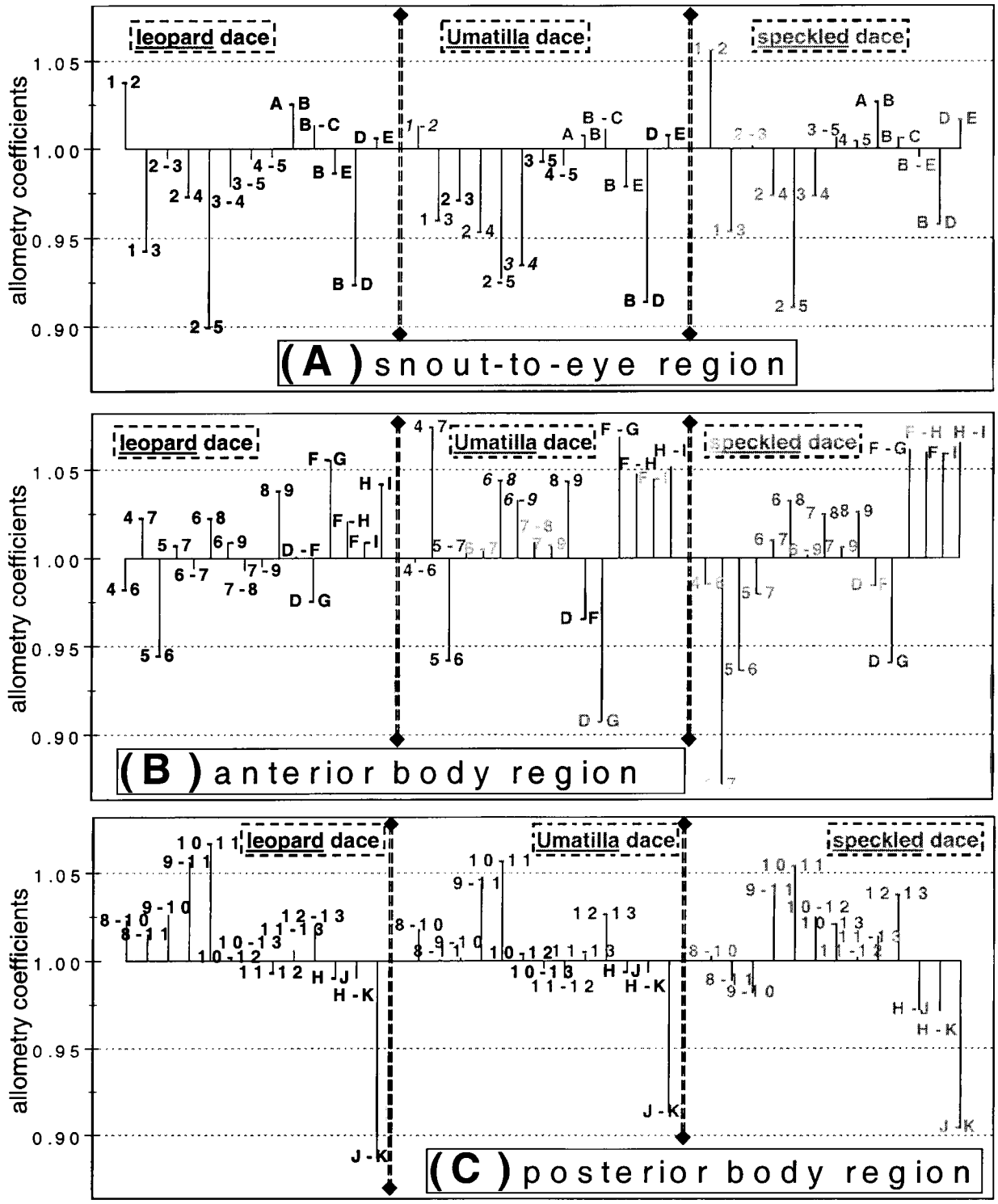


Figure 8. Allometry coefficients for each species' total shape in three body regions represented by truss measurements (Fig. 9; also see Fig. 1 and appendix B): (A) snout-to-eye; (B) anterior body (post-snout and pre-dorsal fin); (C) posterior body (under and post-dorsal fin). An allometry coefficient of one indicates isometry, meaning that growth of that measurement area is directly proportional to overall size. If it is greater than one then its growth is faster, and if less than one then slower. The size of the allometry coefficients indicate the magnitude of the growth difference from that for overall size (isometry). These growth patterns for Umatilla dace are compared to those for leopard and speckled dace (Fig. 9). Growth patterns for Umatilla dace more similar to leopard dace are in black, and those more similar to speckled dace are in light grey. Growth patterns strongly unique to Umatilla dace are in dark grey italics and its nondescript patterns are in dark grey. Patterns for Umatilla dace distinctly intermediate to the other two species can be seen on the plots.

A specific growth pattern for Umatilla dace that is distinctly intermediate to those for leopard and speckled dace snout-to-eye region is B-C (Fig. 8A). In the anterior body region, these distinctly intermediate and specific growth patterns are F-H (F-G) and H-I (Fig. 8B). In the posterior body region, they are 8-11, 9-10, 9-11, and 10-11 (Fig. 8C). The mainly involve growth in the width of the most anterior measurable point of the head, in body width at the origin of the dorsal fin, and in all planes beneath the dorsal fin (Fig. 9; also see Fig. 1 and appendix B).

The allometry coefficient data on Figure 8 could also be used to compare the growth patterns of leopard and speckled dace. These are not emphasized or fully discussed since this study mainly involves systematic discrimination and evolutionary hypotheses about Umatilla dace. In general, the similarities and differences in growth patterns between leopard and speckled dace are already summarized by comparative growth patterns to Umatilla dace. Their comparison to Umatilla dace only needs re-interpretation in terms of just leopard and speckled dace.

6 - 4. Allopatry versus Strict Sympatry

The separate PCAs of allopatric and strict sympatric samples of the combined three dace species do seem to provide possible and realistic overall and character specific body shape changes under each of these distribution patterns. The PC1 size vectors were consistent and equivalent between the two PCAs, as were their three body length measurements taken here (appendix B). These similar size ranges and several central tendency measures for them can also be seen in the single overall PCA based on the total data sets for the various sample categories (Fig. 7), and specifically that for the 'Columbia and Fraser river' dace (Fig. 7B). The combined species comparisons here would not be overly skewed by size differences, with body shape instead forming the realistic basis of the discussion of the results.

The only statistically significant difference in species between allopatry and sympatry was for speckled dace (Fig. 10). The ranges of the PC2 shape vector scores for speckled dace were similar, but were consistently reduced in allopatry. Umatilla dace have almost identical median and data quartile distributions in sympatry and allopatry, but the range of shape values is higher in allopatry. Leopard dace are very similar in allopatry and sympatry, but had slightly lower PC2 shape vector scores and a very slightly larger range of shape values in allopatry. Overall, there would appear to be a tendency towards the range of shape variability being reduced in sympatry. Leopard dace shape in sympatry are also slightly displaced further out from its shape in allopatry, while that for speckled dace in sympatry is displaced more towards the other two dace species in sympatry and again at a statistically significant level. The PCAs also verify the same discrimination and placement of the three dace species as was found in PCAs on other sample categories of the data set (Fig. 7).

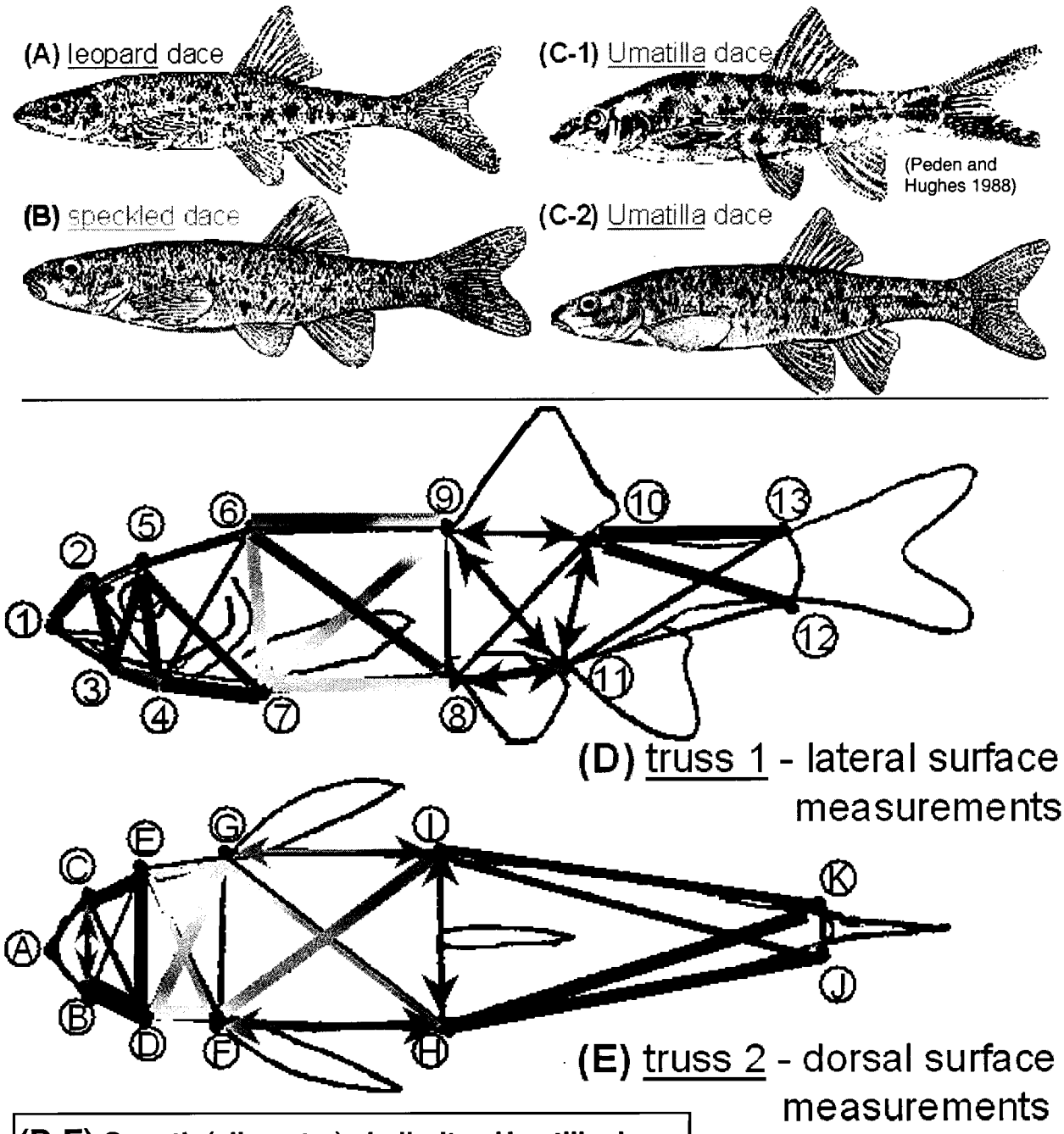


Figure 9. Allometry (growth) coefficient patterns (data - Fig. 8) for Umatilla dace compared to those for leopard and speckled dace on the actual truss measurement diagrams (drawings D - E; also see Fig. 1 and appendix B). Composite drawings of each species (A, B, and C-1/2) are shown to directly visualize these growth pattern results. Growth patterns for Umatilla dace more similar to leopard dace are in thick black lines, and those more similar to speckled dace are in light grey thick lines. Growth patterns for Umatilla dace distinctly intermediate to leopard and speckled dace are in dark grey thinner double-ended arrows, and those strongly unique to Umatilla dace are in dark grey thick lines. Nondescript patterns are in thin black lines.

When the morphometric characters for these individual and group PC2 shape vector scores are analyzed, their most notable overall changes are that their PCA loadings are often much stronger in sympatry than in allopatry (Figs. 11 and 12; also see Fig. 1 and appendix B). These stronger loadings in sympatry are particularly concentrated in the head area. There are more, but less strong, differences in the loadings in allopatry. The increased allopatric loadings are largely concentrated in the diagonal dorsal-to-ventral planes, and in the horizontal plane of the ventral surface of the anterior body region. Snout overhang also has increased loadings in allopatry.

There are several characters that are loaded in opposite directions (reversed signs), and these are again classified as strongly or less strongly loaded (Figs. 11 and 12; also see Fig. 1 and appendix B). The strongest changes in loading signs are in the ventral snout and dorsal head-to-dorsal fin areas. Somewhat less strong loading sign reversals are in the pelvic fin, and in the diagonal dorsal-to-ventral plane of the caudal peduncle. The direction of the reversals in the signs of these loadings are not consistent from allopatry to sympatry, but twice as many of them shift from negative loadings in allopatry to positive loadings in sympatry or vice-versa. Those loadings that are negative in allopatry and positive in sympatry are truss measurements 1-3, 5-6, 10-12, and 10-13 (for character definitions see Fig. 1 and appendix B). The loadings that are positive in allopatry and negative in sympatry are pectoral length and truss measurement 6-9. The largest shifts in sign from allopatry to sympatry or vice-versa are for truss measurement 1-3, 5-6, and 6-9.

The largest differences in patterns of character loadings between allopatry and sympatry are called 'strongly' or "much' more strongly' (Fig. 12). This definition is somewhat loose, but still easily defines those character loadings that are most clearly and numerically different. The pattern differences that are present, but not as clear or numerically different, are not described by the terms 'strongly' or "much' more strongly'. Patterns termed 'nondescript' have no strong differences between allopatry and sympatry.

Discussion

Systematics

The three dace species are consistently differentiated and this discrimination is similar between the three data subsets analyzed both univariately and multivariately (Neff and Smith 1979). This is also the case for the comparative assessments of allometry and growth in Umatilla dace to leopard and speckled dace. More importantly, the species distinctions remain similar and strong in strict sympatry (Dowling et al. 1989, Greenfield and Greenfield 1972, Hubbs 1955, Meagher and Dowling 1991, Stone 2000). The three dace species and their

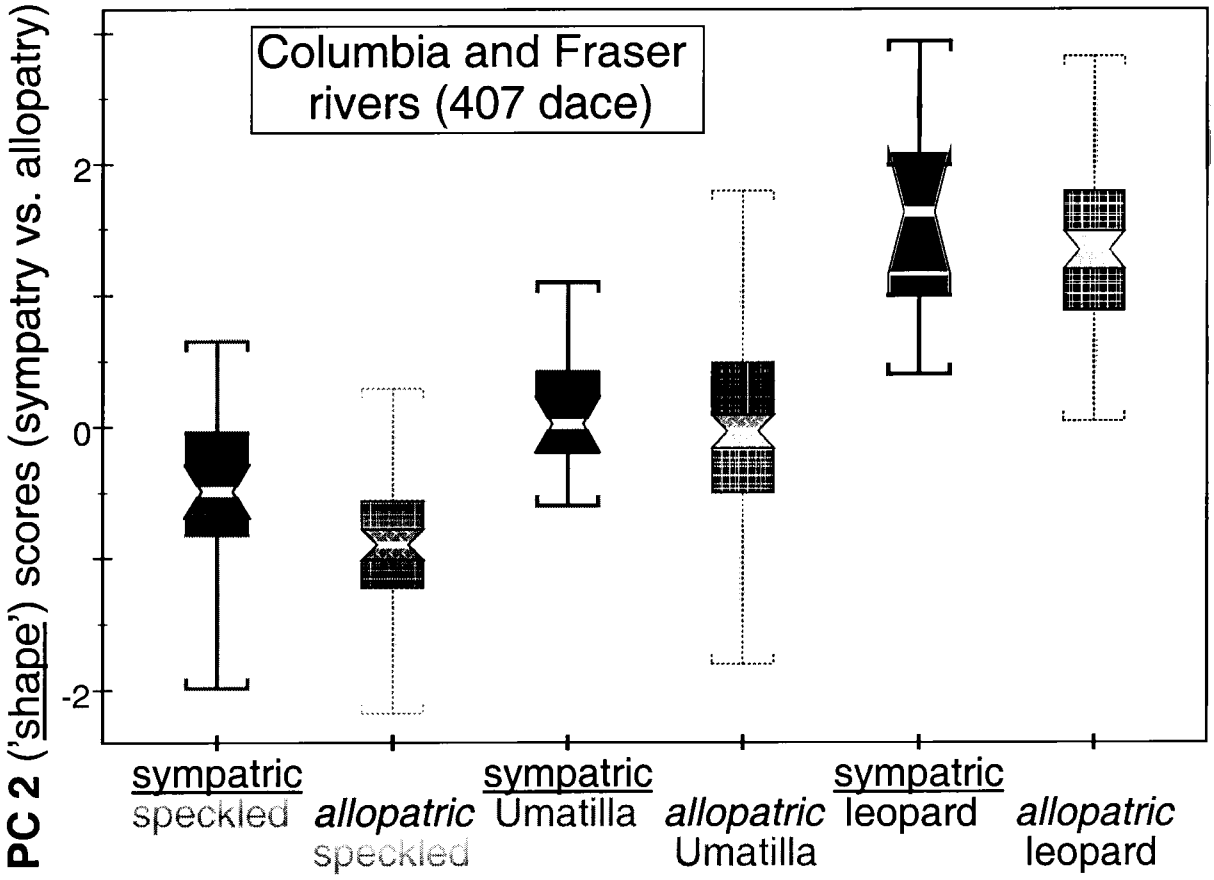


Figure 10. Boxplots of the principal component analyses of the three species analyzed separately as strict sympatric and allopatric site samples from the Columbia and Fraser river watersheds (total = 407 dace). The resultant scores for PC2 represent each dace species' overall 'shape' for 39 measurements (Figs. 1 and 12; appendix B). The y-axis is 'scaled' so the two groups and analyses can be equivalently compared. In the boxplots, speckled dace are plotted in light grey, Umatilla dace in dark grey, and leopard dace in black. Using these colours, strict sympatric site samples for each species are plotted in a solid pattern and allopatric site samples in a hatched template. The white or black solid horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical lines extending from each box encompass their full data ranges. Nonoverlapping notched areas between species' boxes within sympatry and allopatry indicate statistically significant differences at an approximate 5% level (Chambers et al. 1983). The sympatric PC2 accounts for 5.4 % of variance, and allopatric PC2 for 3.6 %.

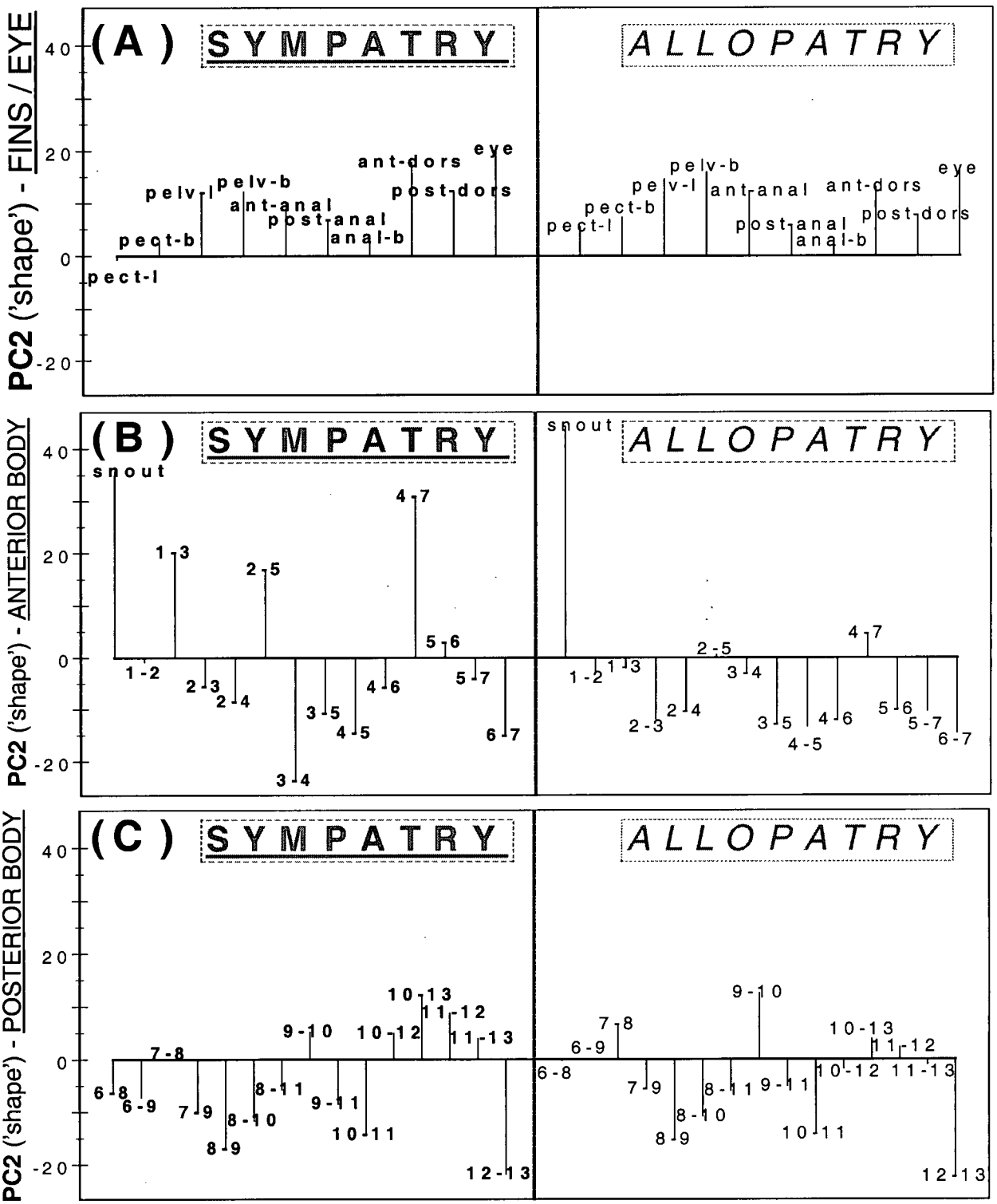
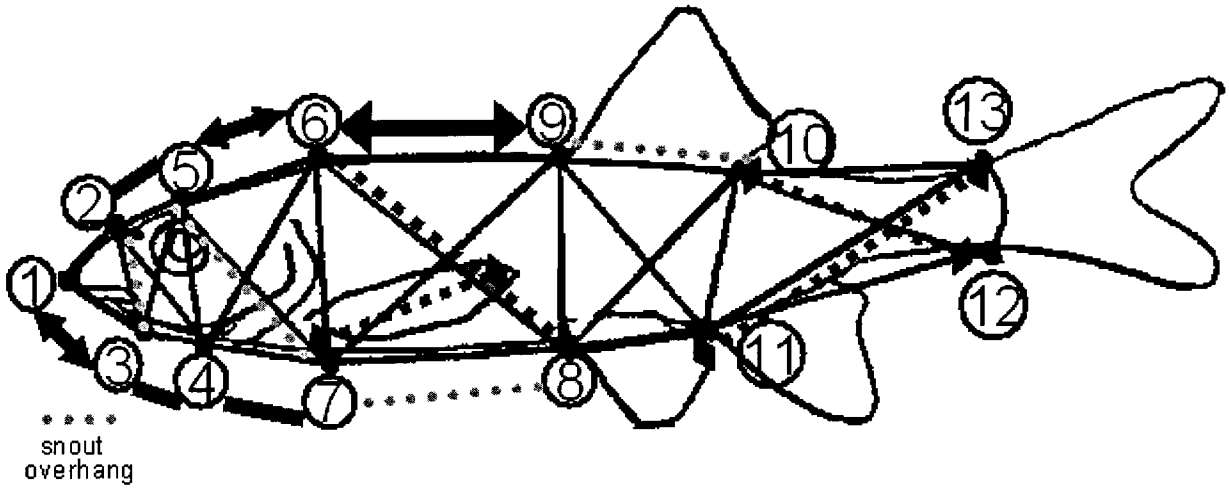


Figure 11. Principal component analyses of the three species analyzed separately as strict sympatric and allopatric site samples (Fig. 10 and appendix A) from the 'Columbia and Fraser river' watersheds (total = 407 dace). The resultant loadings for PC2 are plotted about zero and represent each species' overall shape in three body segments for 39 measurements (Figs. 1 and 12; appendix B): (A) fins / eye; (B) anterior lateral body truss (pre-dorsal fin); (C) posterior lateral body truss (under- and post-dorsal fin). The sympatric loadings are shown in dark bold font and those for allopatry are in lighter regular font. The magnitudes and signs of the PC2 ('shape') loadings indicate the individual significance and directionality of each measurement to the discrimination of the three dace species within the strict sympatric and allopatric site groupings. Their collective loadings indicate the overall character relationships for total body shape. The sympatric PC2 accounts for 5.4 % of the variance and the allopatric PC2 for 3.6 %.



PC2 ('shape') differences - **sympatry** vs. *allopatry*

- **strongly opposite loadings (reversed sign)**
- opposite loadings (reversed sign)
- **much more strongly loaded in sympatry (same sign)**
- more strongly loaded in sympatry (same sign)
- more strongly loaded in allopatry (same sign)
- nondescript loading

Figure 12. Principal component two (PC2) loading differences for 39 measurements (Fig. 1 and appendix B) representing each dace species' overall shape analyzed separately as strict sympatric and allopatric site samples (data - Fig. 11; for sites see appendix A) from the 'Columbia and Fraser river' watersheds (total = 407 dace). PC2 shape loadings that are strongly opposite (reversed sign) in sympatry versus allopatry are in thick solid black arrows, and those not as strongly opposite are in thin dashed lines ending in diamonds. PC2 shape loadings that are much stronger in sympatry are in thick solid dark grey lines, and those stronger in sympatry, but somewhat less so, are in thin dashed dark grey lines. PC2 shape loadings that are somewhat stronger in allopatry are in thin dotted light grey lines. Nondescript loadings that show no differences from sympatry to allopatry are in thin black lines.

1. **(A)** Barbels absent or hidden / inconspicuous (Figs. 2A and 3(A1); score = 1) **- speckled dace** (Canada and USA)
    - *note:* some speckled dace in USA have barbels (see step 3)
    - pelvic fin stays absent / weak [to moderate / inconspicuous] (Figs. 2B and 3(B1-2); score = 1 [to 2])
        - barbel score × pelvic fin stay score ≤ 2 (Figs. 3(C1-2))
            - for other characters to assist identification see Figs. 4B-D and 6 (also Figs. 14-15 and qualitative description in Chapter 2)

    (B) Barbels present; short / visible / more terminal to long / conspicuous / more subterminal (Figs. 2A and 3(A1-2); score = 2 or 3) - go to *step 2*
    - pelvic fin stays [absent or weak to] moderate / inconspicuous to strong / conspicuous (Figs. 2B and 3(B1-2); score = [1 to] 2 or 3)
        - barbel score × pelvic fin stay score ≥ 2 to 6 (Figs. 3(C1-2))
            - for other characters to assist identification see Figs. 4B-D, 5, and 6 (also Figs. 14-15 and qualitative description in Chapter 2)

2. **(A)** Barbels present; strong / conspicuous / more subterminal (Figs. 2A and 3(A1-2); score = 3) - leopard dace (Canada and USA)
    - pelvic fin stays strong / conspicuous (Figs. 2B and 3(B1-2); score = 3)
        - barbel score × pelvic fin stay score = 9 (Figs. 3(C1-2))
            - *note:* score product index indicates that barbel and pelvic fin stay scores both = 3
            - for other characters to assist identification see Figs. 5-6 (also Figs. 14-15 and qualitative description in Chapter 2)

    (B) *In Canada only* - barbels present; short / visible / more terminal (Figs. 2A and 3(A1); score = 2) **- Umatilla dace** (Canada)
        - *note:* some speckled dace in USA have barbels (see step 3) • for USA go to *step 3*
        - pelvic fin stays [absent / weak to] moderate / inconspicuous (Fig. 2B and 3(B1); score = [1 to] 2)
            - barbel score × pelvic fin stay score ≥ 2 to 6 (Figs. 3(C1-2))
        - for other characters to assist identification see Figs. 4B-D, 5, and 6 (also Figs. 14-15 and qualitative description in Chapter 2)

3. **(A)** *In USA only* - barbels present; hidden / inconspicuous [to short / visible / more terminal] (Fig. 3(A2); score = 1 [to 2]) **- speckled dace** (USA)
    - pelvic fin stays absent / weak [to moderate / inconspicuous] (Fig. 3(B2); score =1 [to 2])
        - barbel score × pelvic fin stay score ≤ 2 (Figs. 3(C1-2))
            - *note:* maximum of score product index indicates that barbel and pelvic fin stay scores both ≠ 2 for any one dace
        * dorsal fin ray number ≤ 10 (Fig. 4A)
    - for other characters to assist identification see Figs. 4B-D and 6 (also Figs. 14-15 and qualitative description in Chapter 2)

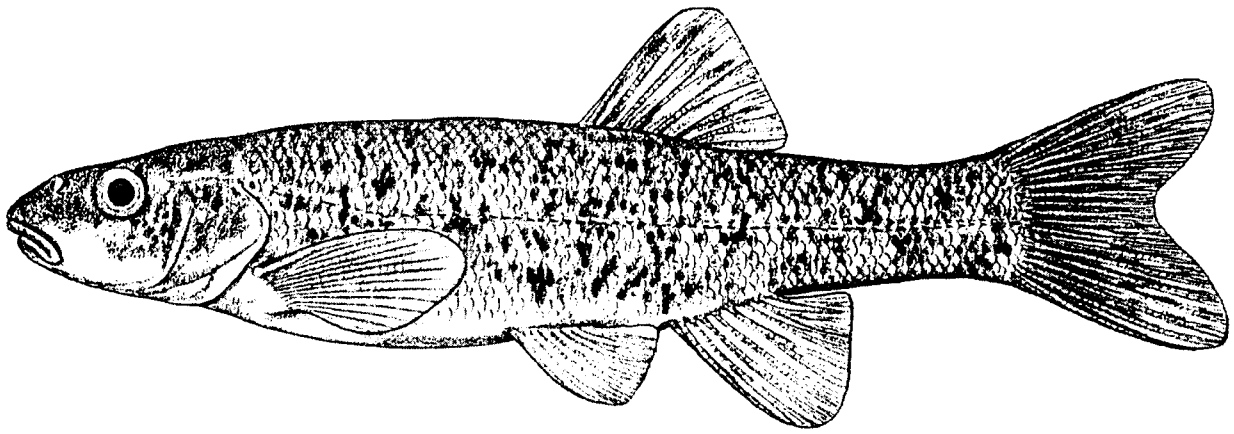
    (B) *In USA only* - barbels present; short / visible / more terminal (Fig. 3(A2); score = 2) **- Umatilla dace** (USA)
        - pelvic fin stays [absent / weak to] moderate / inconspicuous [to strong / conspicuous] (Fig. 3(B2); score = [1 to] 2 [to 3])
            - barbel score × pelvic fin stay score ≥ 2 to 6 (Figs. 3(C1-2))
        * dorsal fin ray number ≥ 11 (Fig. 4A)
        - for other characters to assist identification see Figs. 4B-D and 6 (also Figs. 14-15 and qualitative description in Chapter 2)

Figure 13. Key for identification of three dace species (*Rhinichthys* spp.) in Columbia and Fraser river drainages of Canada and USA: (i) speckled dace (*R. osculus*), (ii) Umatilla dace (*R. o. umatilla*), and (iii) leopard dace (*R. falcatus*). Longnose dace (*R. cataractae*) are not in this key, but are readily identified as the only species with a frenum, meaning the snout is attached to the upper lip (McPhail and Carveth 1993, Wydoski and Whitney 1979).

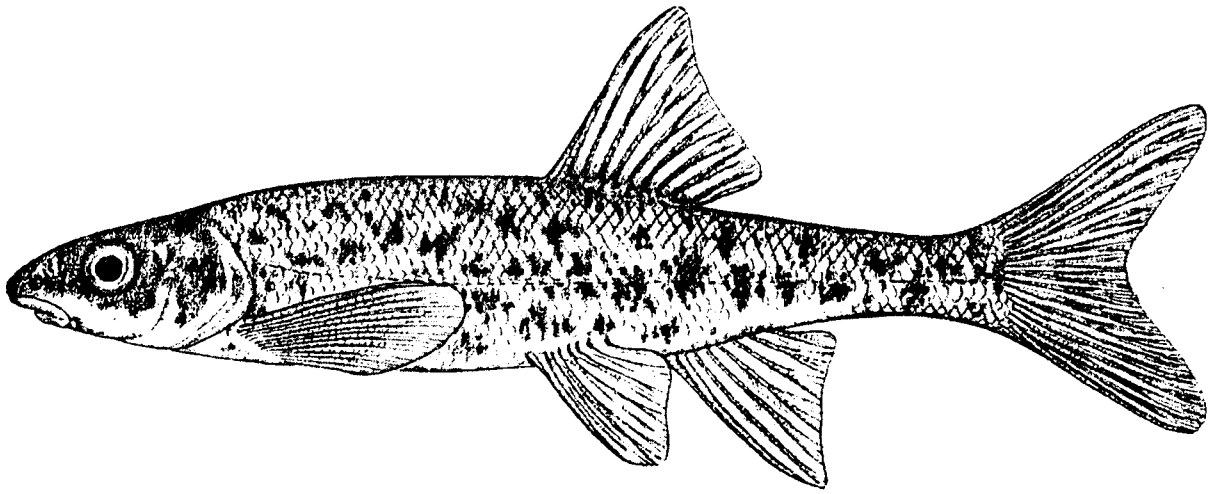
distinguishing characteristics remain distinctive (Figs. 14-15; also see drawings in Peden and Hughes 1988) and relatively consistent across their broad geographic ranges and in parapatry / sympatry as sampled here (also see Chapter 3). Umatilla dace are differentiated from both leopard and speckled dace, and their differences are as large as those designating these latter two accepted dace species (Howes 1991, Schaefer 1991). This is all very strong support and endorsement for the recognition of Umatilla dace as a distinct species from speckled and leopard dace (also see Hughes and Peden 1989, Peden 1991, Peden and Hughes 1988).

Umatilla dace further maintain and even enhance their species distinction in strict sympatry with either or both leopard and speckled dace (Arnold 1997, Larson 1989, Stone 2000). This is critical evidence of their species status under species concept like the biological one (Mayr 1963, 1969, Nelson and Hart 1999, Paterson and McNamara 1984) being used here as part of the theoretical basis for the evaluation of species' statuses (also see Persson 1991, Poly and Sabaj 1998). The data indicate a general trend of reduction in overall multivariate shape variation and a strengthening of specific character loadings in strict sympatry for all three dace species, but particularly for Umatilla dace, when compared to them in allopatry. Each dace species in coexistence with either or both of the other dace species is less variable and more uniform in its multivariate morphometric shape and has more defined specific morphometric characters that are both important to their distinction as species (Cherry et al. 1982). The specific changes that occur in strict sympatry in individual morphometric characters are also strongly correlated to the ecology and distribution / range of each of the three dace species that probably permits or at least enhances their species separation (Chapters 5 and 9). This has been a standard theoretical expectation, and is again in compliance and support of distinct species status for Umatilla dace under the biological and other species concepts (Nelson and Hart 1999).

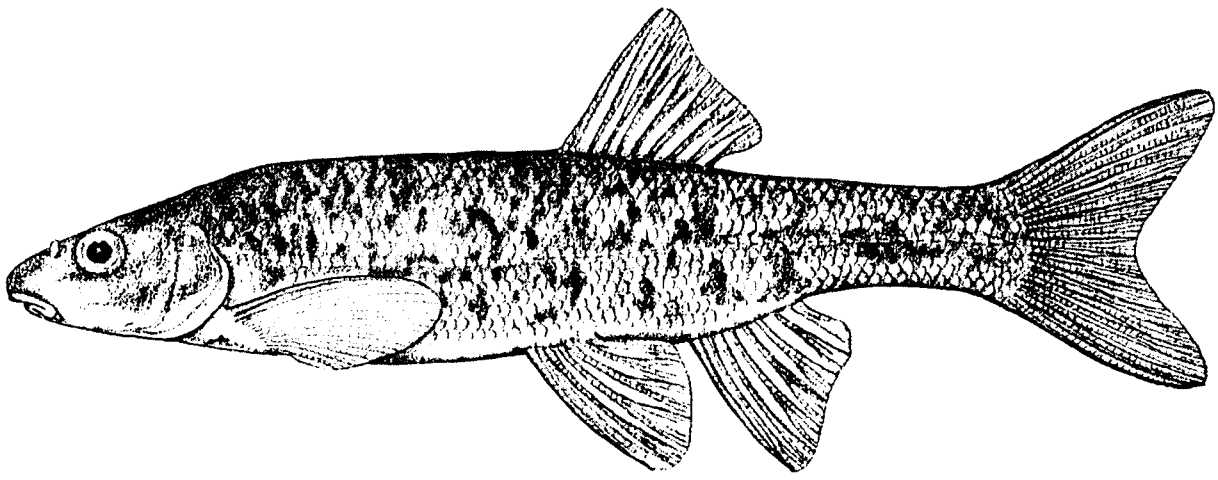
It was also possible to construct a diagnostic key for all three dace species that would work on the complete large data set examined here from throughout their ranges in the Columbia and Fraser river drainages in Canada and the USA (Fig. 13). This key is based on qualitative and quantitative characters that can be measured or taken on live specimens in the field. Each of the individual variables in the key can themselves also somewhat discriminate the three dace species, particularly in more localized and specific portions of their ranges. Each of the characters specifically noted in the key is also supported by seven more univariate / bivariate measurements for the separation of speckled dace from Umatilla and leopard dace, and for eight more univariate / bivariate variables for the separation of leopard dace from Umatilla and speckled dace. The combination and utility of this large set of varied characters should permit the correct identification of the three dace species. They also help confirm the uniqueness of



speckled dace (*Rhinichthys osculus*)



leopard dace (*Rhinichthys falcatus*)



Umatilla dace (*Rhinichthys o. umatilla*)

Figure 14. Composite drawings of the three dace (*Rhinichthys* spp.) species at maturity (drawn at ~ 70 mm). The drawings were done for me by Karen Klitz.

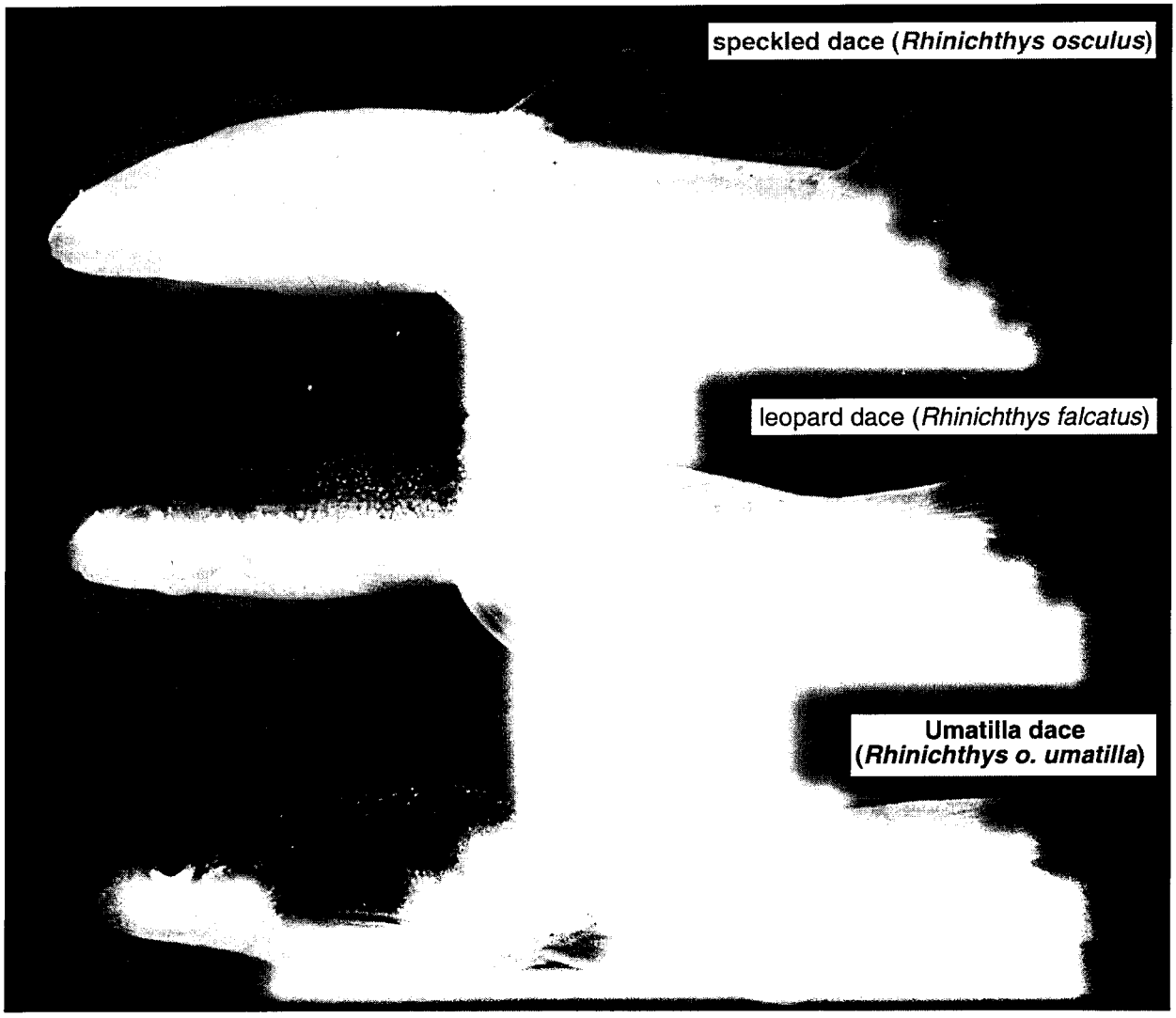


Figure 15. Photograph of laboratory reared mature specimens of the three dace (*Rhinichthys spp.*) species (~ 70 mm).

Umatilla dace, and support its designation as a distinct dace species (Funk 1985, Howes 1991, Schaefer 1991, Stone 2000).

Some level of caution in the application of this key is probably still warranted since it was developed from and for this data set which does not completely cover the ranges of at least speckled and Umatilla dace in the Columbia River drainage in the USA. This caution would particularly be warranted for speckled dace in unsampled regions of the USA that have barbels, and for co-occurring Umatilla dace.

Other keys specific only to the Canadian component of the range of the three dace species are in McPhail and Carveth (1993) and Peden and Hughes (1988). Umatilla dace were previously noted as species in older keys to the freshwater fishes in Washington State (Schultz 1936), and recognized as a distinct form of speckled dace in Oregon (Bond 1973). Umatilla dace are to be again recognized as a species in the forthcoming in the new edition of the Freshwater Fishes of Washington (Wydoski pers. comm.; old edition is Wydoski and Whitney 1979). These other keys would not be successful in discriminating the three dace species, at least not across their ranges as studied here. Some specific characters mentioned and used in them are also not supported by this data. Nonetheless, the distinction and recognition of Umatilla dace in these other identification protocols is once more supportive of their designation as a distinct species.

If pressed for a general qualitative comparative description of the three dace species, the following characteristics for each of them would generally be valid and could assist in their identifications (Figs. 14-15, and also see Peden and Hughes 1988). Reliance on these or other qualitative descriptions and / or avoidance of undertaking the quantitative measurements for their more accurate identifications is not recommended (Fig. 13). It is often possible and desirable to differentiate these dace and other such species on a smaller localized geographic scale using qualitative characteristics. However, this did not work here across the ranges of these three dace species, and also would first have to be properly developed from their correct quantitative identifications.

(1) Leopard dace are the most streamlined in body form and specific characteristics, with a thin, slender, ventrally flattened lateral appearance that has also been overall described as delicate. Their fins and fin bases are the largest and most falcate (pointy). Their tails have the largest fork and size, and are connected to the narrowest caudal peduncle. Their barbels and pelvic fin stays are conspicuous and visible, and their actual mouth size is smallest and most subterminal. Their eyes also *appear* particularly large, probably due to the comparative perspective of their streamlined narrow heads. Leopard dace have a set of larger and more conspicuous irregular spots in accordance with their name, and their body colouration often appears more white, silver, or just lighter in tone.

(2) Speckled dace are the least streamlined in body form and specific characteristics. They are more robust, ventrally and dorsally rounded, and 'chunky' in lateral appearance. Their fins and fin bases are the smallest and most rounded. Their tails are smallest in size and fork, are preceded by the thickest caudal peduncle. Their pelvic fins stays are generally weak or absent. Their barbels are absent in Canada and either absent or still usually smaller and hidden or inconspicuous in the USA. Their mouths are much more terminal, and they have the smallest eyes. Speckled dace have a spotting pattern again in accordance with their name that generally consists of more regular distributions of very small speckles or spots. Their bodies tend to be darker in colouration and appearance. Speckled dace in Canada often have greatly reduced to no such obvious speckling.

(3) Umatilla dace are intermediate in virtually all measurements to leopard and speckled dace, and in overall appearance as well. Their misidentifications are with both speckled and leopard dace, and specific populations or geographic areas of Umatilla dace usually appear more similar to one of these two other dace species. Overall, Umatilla dace could be said to have the somewhat more streamlined, ventrally flattened appearance of leopard dace with a similarly subterminal mouth, while retaining the darker colouration and more robust look of speckled dace especially for the latter in particular features like the larger caudal peduncle. Umatilla dace nonetheless have some characteristics that provide particular recognition, while still actually being intermediate. They particularly tend to have a notable dorsal 'hump' that rises sharply just posterior to their heads at about their opercula. They also have a distinctive spotting pattern which consists more of irregular mottled blotches often concentrated more towards the dorsal surface, and that look like overall blended spot amalgamations.

Eight populations of the three dace species are categorized as 'unusual' in appearance (appendix B). While these are still definitely classified as one dace species, their specimens had an unusual or distinctive appearance, characteristics, and / or morphometric(s) and meristic(s). This specification as 'unusual' was also often complicated by these same specimens also being 'poor' quality and / or 'small' size (appendix B). A further clarification of these six 'unusual' populations that are in the Columbia River drainage (Fig. 16 in Chapter 3) is warranted to highlight them and make other personnel aware. The speckled dace samples that had some 'tendency' toward Umatilla dace are the Cowlitz River and Lacamas Creek in Washington State. The Umatilla dace samples with some leopard dace 'tendencies' are from Otter Creek (also see Carl et al. 1977) and the Pend d'Oreille River in British Columbia (BC), and from the Owyhee River in Oregon.

A particularly noteworthy site with 'unusual' dace is Mission Creek in BC. The single dace from this location was classified as speckled dace, but was also a very poor specimen. It is

biogeographically conceivable that speckled dace have entered Mission Creek since its headwaters do meet those of the Kettle River, which is the only location where speckled dace do exist in BC. However, many and interannual collection attempts in Mission Creek did not only fail to produce more speckled dace but none of the three dace species studied here were found at all. The only dace species found in the entire Okanagan basin in BC into which Mission Creek drains were a very few Umatilla dace (appendix A; Fig. 16 in Chapter 3). More Umatilla dace were also found in the Similkameen River, but this does not drain into the Okanagan system in Canada and is also potentially isolated above a barrier at which Enloe Dam now sits. The supposed speckled dace was not directly collected as part of this study, and no other possible specimens of any of the three dace species were ever found or turned in again from the fisheries fence enumeration operation for kokanee (*Oncorhynchus nerka* (Walbaum)) that provided the single specimen. It seems more likely that this single speckled dace is in fact a hybrid of some sort and it is further possible that it is not even one of the three dace species in this study. The specimen was not of sufficient quality to permit a more definitive assessment, and its remote possibility of being a speckled dace was still worth noting but only with this qualified clarification.

Beaverdam Creek is also significant because its dace were clearly classified as, and looked like, speckled dace but also had frenums (Fig. 13). The presence of a frenum means the snout is attached to the upper lip. This unique characteristic is always attributed only to longnose dace (*R. cataractae* (Valenciennes)) (McPhail and Carveth 1993, Wydoski and Whitney 1979). The speckled dace in Beaverdam Creek may be hybrids of some sort with longnose dace because the frenum does appear to be a dominant characteristic in longnose dace hybrids which all appear to have one (Calhoun 1940, Child and Solomon 1977, Cooper 1980, Haas unpubl. data, Nelson 1966, 1973, Raney 1940b, Ross and Cavender 1981, Schultz and Schaefer 1936, Smith 1973, Suttkus and Cashner 1981). These speckled dace could also be a uniquely characterized population. This location is in Nevada and outside the Columbia and Fraser river drainages studied in detail here.

Evolution

Umatilla dace warrant species recognition as distinctive morphometric and meristic type, but more importantly here they are intermediate overall to leopard and speckled dace and also for almost every individual univariate / bivariate character. This intermediacy of Umatilla dace is consistent with and supportive of the hypothesis that they originally speciated through multiple hybridizations of leopard and speckled dace (Arnold 1997, Brust et al. 1998, Dowling et al. 1989, Funk 1985, Greenfield and Greenfield 1972, Neff and Smith 1979, Stauffer et al. 1979). Those few characters that Umatilla dace are not intermediate for could be further interpreted as supporting its independent evolution (Bloom 1976, Jordan 1991, Stone 2000). These non-

intermediate variables may not have existed or been as strong in the early generation hybrids (Chapter 6), but may have been selected for over the longer time period of the evolution of Umatilla dace (Goodfriend and Gould 1996, Turner 1971). Hybrids are almost always intermediate to their parental species (Hubbs 1955), and this has been the case for other dace hybrids and for dace hybrids with other species from different genera within its Family Cyprinidae (Calhoun 1940, Child and Solomon 1977, Cooper 1980, Cross and Minckley 1960, Haas unpubl. data, Ross and Cavender 1981, Schwartz 1972, 1981, Suttkus and Cashner 1981, Weisel 1955; Chapter 10). Long term evolutionarily distinct bisexual hybrid lineages are not generally thought to often exist in animals and thus have received little if any study (Arnold 1992, 1997, Bullini 1985, Dowling and DeMarais 1993).

The specific intermediate characters and overall intermediate shape of Umatilla dace also correlate strongly with an ecology and distribution / range that probably permits or at least enhances their species separation (Cherry et al. 1982, Lewontin and Birch 1966, Persson 1991, Stauffer et al. 1979, Stone 2000). This ecology is also intermediate to leopard and speckled dace, which is itself again supportive of the possible hybrid origins of Umatilla dace (Chapters 5 and 9). Such specialization and maintenance of a specific ecology has been argued as important or critical in the formation and ongoing evolution of a species like Umatilla dace (e.g. Arnold 1997, Diehl and Bush 1989, Rao and DeBach 1969, Stone 2000, Tuner and Grosse 1980).

Umatilla dace are also intermediate to leopard and speckled dace in comparative assessments of allometry and growth (Goodfriend and Gould 1996). Umatilla dace are most similar to leopard dace in the depth and width of the snout region, and most like speckled dace in the dorsal area posterior to the head and anterior to the dorsal fin. Umatilla dace also maintain some unique and species-specific allometric features, particularly in body depth and in head and body width. These allometric and growth characteristics of Umatilla dace are again strongly associated with a similar and distinctive ecology and distribution / range (Chapters 5 and 9). Furthermore, they are in complete congruence with the 'direct' morphometric assessments of the same characters and are in full agreement with the general appearance of Umatilla dace (Figs. 14-15). This congruence is tight enough to be able to further explain morphometrics details such as the appearance of the distinctive dorsal 'hump' in Umatilla dace through its differential growth of the aforementioned specific body parts and regions (Cherry et al. 1982).

Umatilla dace and their distinguishing characteristics remain distinctive and intermediate in several strictly sympatric locations across a broad range (also see Chapter 3). In strict sympatry, there is a general trend of reduction in overall multivariate shape variation and a strengthening of specific character loadings when compared to allopatry. These overall and

specific changes are strongest in Umatilla dace. Umatilla dace in coexistence with either or both of the other dace species are less variable and more uniform in their multivariate morphometric shape and overall appearance. They also have more defined specific morphometric characters in sympatry. The specific characters that are most impacted in sympatry are also those that have the strongest association with the specific ecology determined for Umatilla dace (Chapters 5 and 9). This specific ecology and associated morphometry permits or at least enhances the separation in sympatry of Umatilla dace (Blouws 1998, Gorman 1992, Wiley and Mayden 1985). This has been a standard theoretical expectation for speciation in general and for the speciation or at least maintenance of hybrids in particular (Arnold 1992, 1997, Futuyma and Mayer 1980, Kondrashev and Mina 1986, Larson 1989, Rao and DeBach 1969, Stone 2000).

Chapter THREE

Biogeography - Distribution and Range Data

Materials and Methods

The distributions of the speckled (*R. osculus*), Umatilla (*R. o. umatilla*), and leopard (*R. falcatus*) dace in the Pacific Northwest region of North America were established from the species' identifications of 500 fish specimens. These 500 dace are from 100 sample sites (appendix A), later determined to represent most of the total and / or mutual ranges of the three species. These species' identifications are based on a dichotomous key developed from three main meristic characters. This is supported and validated by an additional 11 morphometric measurements to assist species' identifications as desired or needed. Principal components analyses based on an independent data set composed of 42 morphometric measurements (appendix B) for the same and subsets of the 500 dace were used to objectively and independently verify the species identifications and identities. All the necessary and further information for the morphometric and meristic data and their analyses are fully presented and detailed in the 'Materials and Methods' and 'Results' sections of Chapter 2.

The distributions and ranges of the three dace species were examined for overlap, and particularly for strict sympatry of two or all three species. Strict sympatry is defined as combinations of two or more of the dace species found in the same museum sample, in museum samples from the same small-scale location, or collected at the same location at the same time.

The samples of strict sympatry and the regions of mutual range overlap are particularly important because the species statuses of the three dace are primarily interpreted using the criteria of coexistence in the biological species concept (Mayr 1963, 1969). Although the biological species concept has operational difficulties under certain circumstances and for particular taxa (Ehrlich 1961, Hull 1970, Scudder 1974, Sokal and Crovello 1970), it is generally applicable in sexually reproducing species occurring in sympatry (Cracraft 1989, Paterson and McNamara 1984). Under these conditions, the biological species concept is operational and does not necessarily preclude other species concepts or engender particular mechanistic viewpoints (Coyne et al. 1988, Masters and Spencer 1989, Templeton 1989). Since these dace are sexually reproducing and evidence of strict sympatry was found, the biological species concept is mainly employed.

Results

Study Samples - Confirmed

The 500 fish sampled are composed of 203 speckled dace from 45 sample sites, 181 Umatilla dace from 35 sample sites, and 116 leopard dace from 20 sample sites (Fig. 16 and appendix A). The mutual range of the three dace species is the Columbia River (Figs. 16 and 17A, and appendix A).

Umatilla dace were only found in the interior Columbia River watershed in Canada and the USA. Leopard dace were only found in the Columbia River basin in the USA, and are the only of these three dace species to be found in the Fraser River watershed (Canada). Speckled dace are the only one of these three species much more broadly distributed outside the Columbia and Fraser rivers (Bond 1973, McPhail and Lindsey 1986, Wydoski and Whitney 1979). Speckled dace are commonly found in all lower elevation drainages in and surrounding the Columbia River watershed throughout the western USA (Lee et al. 1980). Speckled dace are not found in the Fraser River basin. Speckled dace in the Columbia River basin in Canada are only in the Kettle and Granby rivers, where they are allopatrically isolated above the substantial barrier at Cascade BC. Speckled dace are in fact the only dace species (*Rhinichthys* spp.) in this isolated Kettle River area that also is one of the very few drainages where longnose dace do not also exist (Haas, unpubl. data). Longnose dace were found at all other sites in Canada for leopard and Umatilla dace and at nearly all other sites examined in this total study. All three dace species in Canada are now found in only in allopatry with respect to each other.

It would have been logical for subsequent detailed examinations of the systematics and evolution of these three dace species to only be from those population samples and their resultant species' distributions and ranges within the Columbia River drainage. The Fraser River is necessarily included for three reasons: (1) leopard dace appear rare in most of the Columbia River and insufficient numbers would have been obtained to undertake analyses with confidence; (2) leopard dace found in the Columbia River were often not in allopatry and it was desirable that such allopatric samples would also be analyzed; (3) the Fraser River was more still intimately connected to the Columbia River after the last Pleistocene glaciation and likely more so than any other surrounding watershed (Fulton 1967, 1969, Kershaw 1978, Mathews 1944, McPhail and Lindsey 1986). This is the time of my hypothesized hybrid evolution for Umatilla dace and the Fraser River watershed was seen as a legitimate inclusion providing pertinent information to this scenario. The 'Columbia and Fraser rivers' sample category thus forms the basis of the detailed analyses of the evolution of Umatilla dace, and also for the further development and refinement of the systematic identifications of all three dace species.

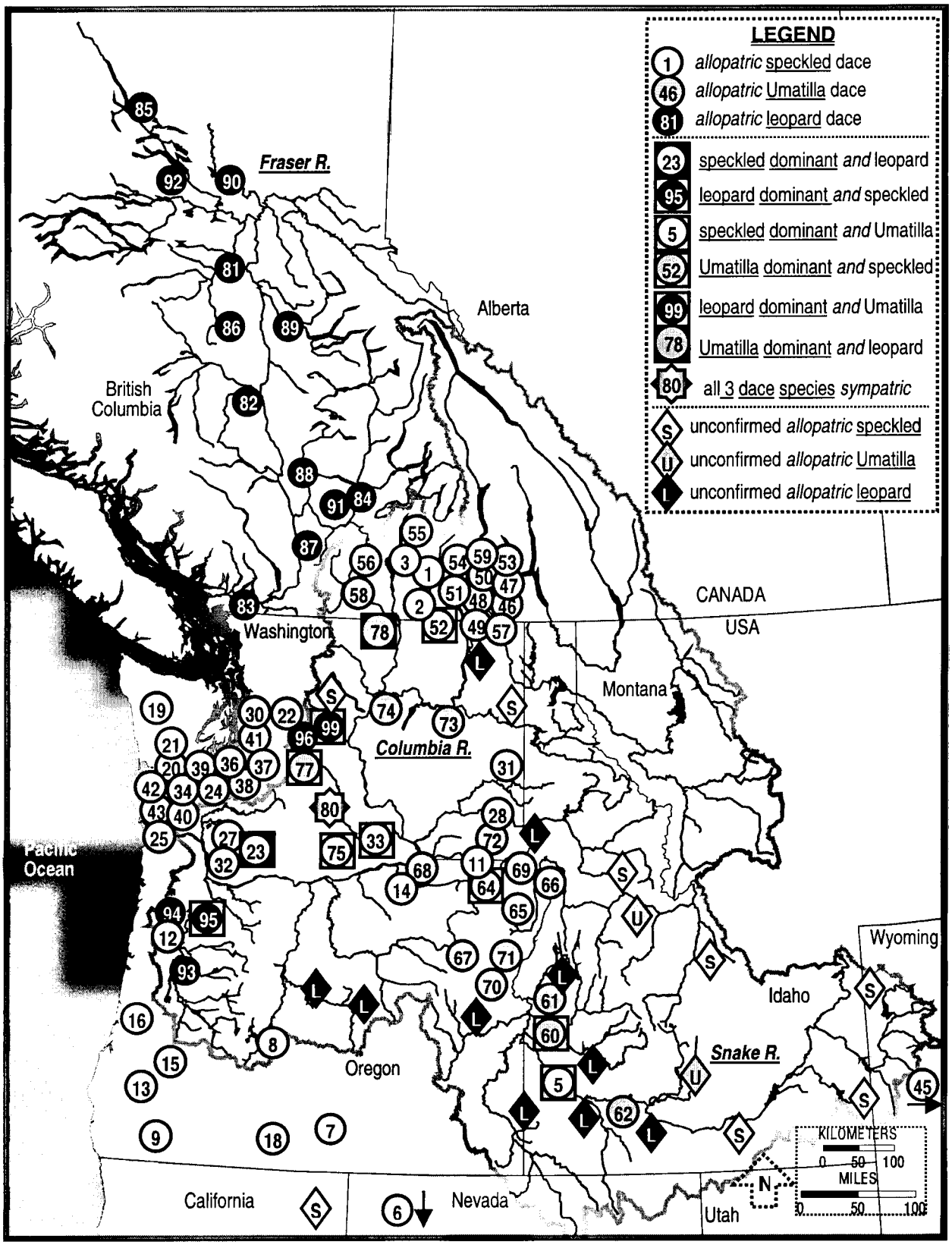


Figure 16. Map of allopatric and sympatric sample collection or museum specimen sites in the Pacific Northwest region of Canada and the USA (appendix A). Unconfirmed samples were not available (see Methods section) and are listed in their source literature as allopatric. These unconfirmed samples were only used to fill out species' distributions. Two unconfirmed Umatilla dace are all those in Peden and Hughes (1988, 1989), and ten unconfirmed leopard dace are all those in Peden (1991), not confirmed in this study. Speckled dace are ubiquitous throughout the US Columbia River basin and western US (Bond 1973, McPhail and Lindsey 1986, Wydoski and Whitney 1979). Their eight unconfirmed records are only a few from Lee et al. (1980).

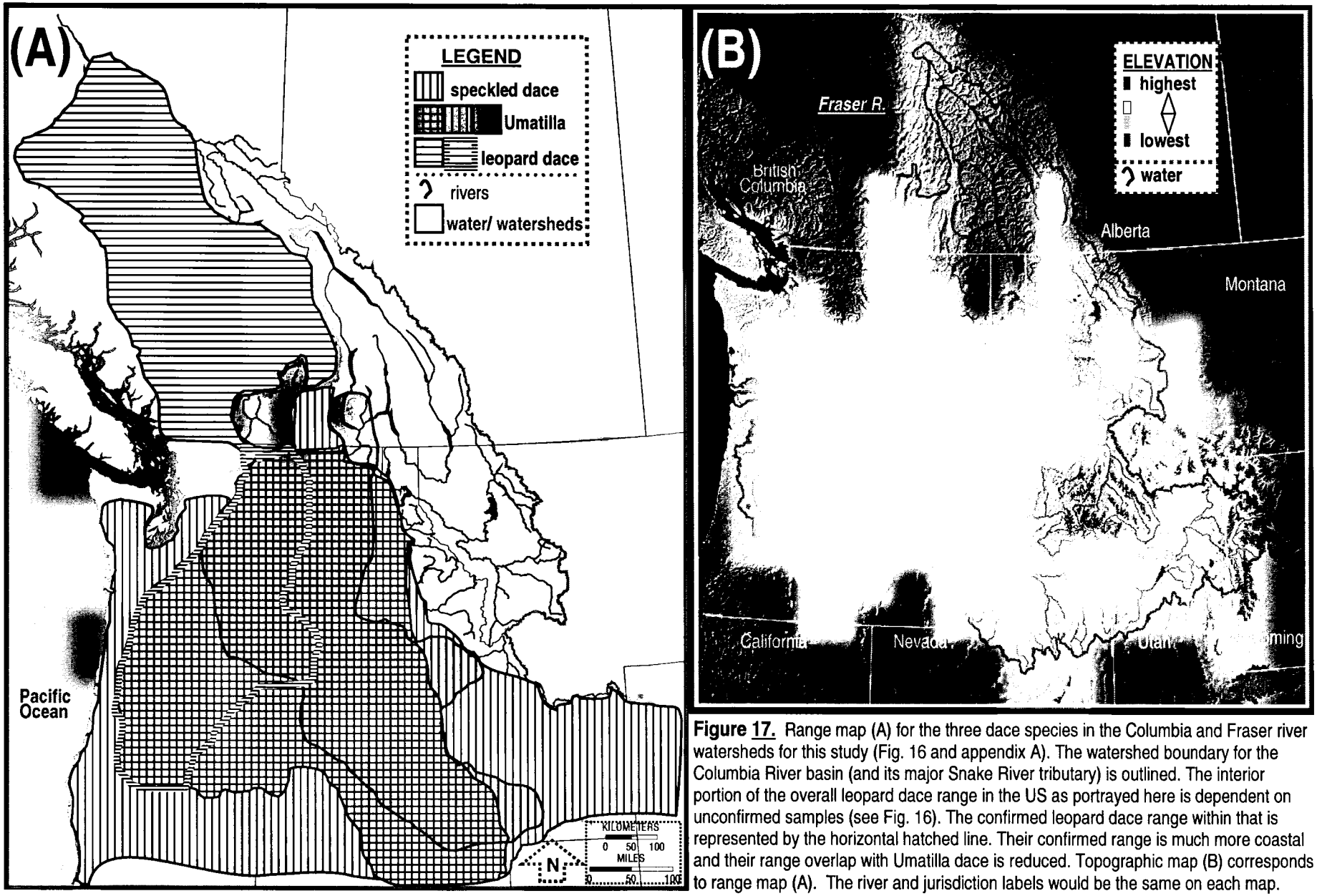


Figure 17. Range map (A) for the three dace species in the Columbia and Fraser river watersheds for this study (Fig. 16 and appendix A). The watershed boundary for the Columbia River basin (and its major Snake River tributary) is outlined. The interior portion of the overall leopard dace range in the US as portrayed here is dependent on unconfirmed samples (see Fig. 16). The confirmed leopard dace range within that is represented by the horizontal hatched line. Their confirmed range is much more coastal and their range overlap with Umatilla dace is reduced. Topographic map (B) corresponds to range map (A). The river and jurisdiction labels would be the same on each map.

This 'Columbia and Fraser rivers' sample category was represented by 77 sample sites (Fig. 16 and appendix A). It consists of 407 fish composed of 117 speckled dace from 22 sample sites, 176 Umatilla dace from 35 sample sites, and 116 leopard dace from 20 sites. Twelve sites were strictly sympatric (Fig. 16 and appendix A). The strict sympatric combinations of dace were of all possible species' pairings and of one sample site containing all three species. More sympatric sites would have been recognized under a looser definition, and parapatry was very common (Figs. 16 and 17A). Twenty-three sample sites for speckled dace were outside of the Columbia and Fraser river basins (Fig. 16 and appendix A).

The most prevalent strict sympatric species pair is seven sets of speckled and Umatilla dace (Fig.16 and appendix A). Umatilla dace are dominant in terms of numerical abundance in five of these seven samples, and speckled dace in two. These seven strict sympatric pairings of Umatilla and speckled dace are found most prevalently in tributaries draining into the Snake River and also in the north central portion of the Columbia River basin near and in the lower / middle portions of the Yakima River.

The northern boundary of this interior range for Umatilla dace closely matches that of ice cover at the maximum extent of Pleistocene glaciation (Fig. 18A). Umatilla dace have not postglacially advanced into Canada much beyond this former glacial boundary, barely getting into the lower portion of the Columbia River and the very lowest point of the Kettle River in Canada below the large barrier at Cascade BC. Umatilla dace have penetrated somewhat further into the Similkameen River in Canada, but there remain even more sporadically distributed, rare, and in low numbers. Postglacial recolonization of Umatilla dace in Canada has really been only into regions of allopatry in terms of speckled and leopard dace being absent. Their recolonization might have been hampered in most instances by higher elevations (Fig. 17B), although exceptions exist.

The westerly limit of the interior range of Umatilla dace corresponds to the coastal mountain range (Fig. 17B) and within the Columbia River to roughly a traditional strong downstream / upstream demarcation known as "The Dalles". The overall distribution of Umatilla dace below the maximum extent of Pleistocene glaciation largely matches the overlapping interior ranges of speckled and leopard dace (Fig. 17A). Umatilla dace are usually common and numerically dominant where they occur here. The interior overlap of leopard and speckled dace further coincides with the major downstream and lower elevation impact zone from the repetitive flooding of Glacial Lake Missoula (Figs. 17B and 18B; Bretz 1919, Waitt and Atwater 1989). The most heavily impacted region from this flooding is generally and appropriately referred to as the 'channelized scablands', with the evidence for and affects from it very clearly visible even today (Allen et al. 1986, Bunker 1982). The more southerly populations of Umatilla dace are

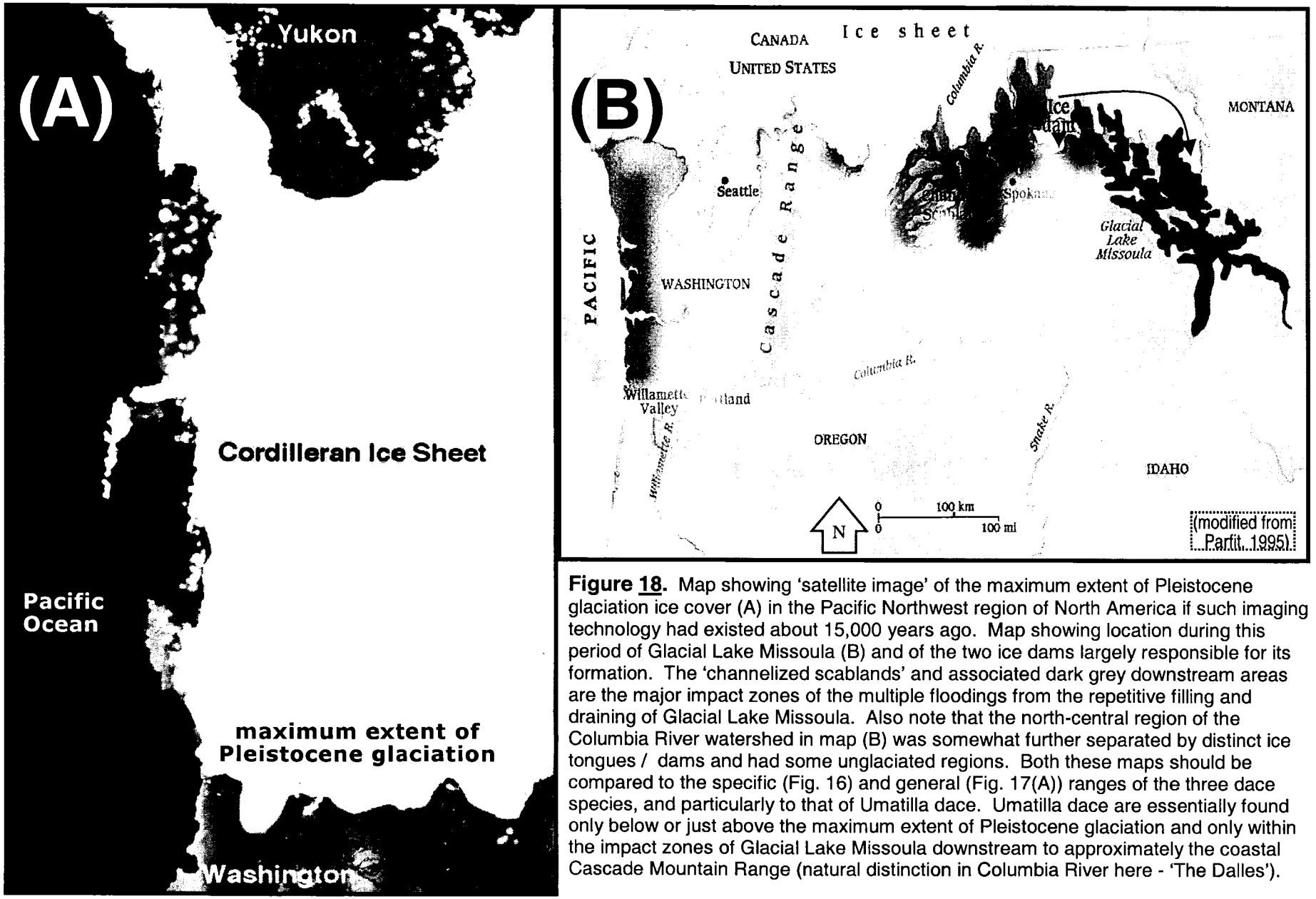


Figure 18. Map showing 'satellite image' of the maximum extent of Pleistocene glaciation ice cover (A) in the Pacific Northwest region of North America if such imaging technology had existed about 15,000 years ago. Map showing location during this period of Glacial Lake Missoula (B) and of the two ice dams largely responsible for its formation. The 'channelized scablands' and associated dark grey downstream areas are the major impact zones of the multiple floodings from the repetitive filling and draining of Glacial Lake Missoula. Also note that the north-central region of the Columbia River watershed in map (B) was somewhat further separated by distinct ice tongues / dams and had some unglaciated regions. Both these maps should be compared to the specific (Fig. 16) and general (Fig. 17(A)) ranges of the three dace species, and particularly to that of Umatilla dace. Umatilla dace are essentially found only below or just above the maximum extent of Pleistocene glaciation and only within the impact zones of Glacial Lake Missoula downstream to approximately the coastal Cascade Mountain Range (natural distinction in Columbia River here - 'The Dalles').

also within the similar downstream flooding and impact zone of Glacial Lake Bonneville (Malde 1965, 1968, Jarrett and Malde 1987)

Each of the other four possible strict sympatric species pairings are represented by one sample (Fig. 17 and appendix A) dominated by one or the other species. The two strict sympatric pairs of leopard and speckled dace are both found in the more westerly and south-westerly regions downstream of the Coastal Mountain range (Fig. 17B) and of "The Dalles". These two river basins are the Cowlitz and Willamette. These two coastal leopard and speckled dace sympatric samples do not coincide with any Umatilla dace. These two watersheds, particularly the Willamette River, were still affected by flooding from Glacial Lake Missoula (Fig. 18B), but there was less damaging impact. These regions were not as badly channelized, and the force of each flood from Lake Missoula had presumably been somewhat dissipated by distance and the Coastal Mountain range. The dace from these two watersheds also never very likely had any access to, and would not have come from, distant Glacial Lake Missoula.

The two pairs of leopard and Umatilla dace are both found in the uppermost higher portions of the north-central interior region of the Columbia River in Washington, specifically in the Similkameen and Wenatchee rivers. These drainages were distinctly isolated during Wisconsin glaciation by the differential extent and penetration of ice (Figs. 18A and B). They also were more on the northern, higher, and less heavily impacted limits of flooding from Glacial Lake Missoula (Fig. 18B). These regions also had their own temporary smaller glacial lakes resulting from the same ice damming as Glacial Lake Missoula, but on a much smaller scale and usually over a shorter timeframe (Mathews 1944, Fulton 1967, 1969).

Overall, leopard dace were only found in two areas of the Columbia River basin in the USA. The drainages west of the Coastal Mountain range and in the north central portion near the Yakima and Similkameen river basins. In Canada, leopard dace were not found in the Columbia River system (but see 'Study Samples – Unconfirmed' section of this Chapter). Leopard dace were only found allopatrically in sporadic locations and local abundance throughout the Fraser River system. Leopard dace are the only of these three dace species to have postglacially recolonized regions north of the maximum extent of the Wisconsinan ice sheets, and have done this to considerable distances in the Fraser River (Fig. 18A). This postglacial recolonization would have been through areas of higher elevation (Fig. 17B).

The one strict sympatric sample of all three dace species is from the Yakima River (Fig. 16 and appendix A). Umatilla dace are numerically dominant, but still with substantial numbers of speckled dace present. Its leopard dace sample consists of a single specimen, and no more were ever collected in spite of intensive efforts by myself and by very regular and thorough surveys of the Yakima River by US Fisheries Agencies (e.g. Pearsons et al. 1992/93/94).

These US personnel have expressed an admirable interest in non-game fish species and received training from me in identifying dace. This single leopard dace runs somewhat counter to data published in an earlier survey of the Yakima River (Patten et al. 1970) and some other data from nearby watersheds (Grayy and Dauble 1977, Reimers and Bond 1967). However, there are few museum specimens available from these earlier collections in the Yakima River and those dace have turned out to be Umatilla dace. There remains some possibility though that leopard dace were until recently more prevalent in the Yakima River system.

Study Samples - Unconfirmed

This study found few specimens readily available mainly from one particular region of the Columbia River in the USA, this being the more upper regions of the Snake River in Idaho in the USA (Fig. 16 and appendix A). This was due to distance, inaccessibility, collecting permissions, and simple cost of undertaking a field trip there. It was also the result of its few museum specimens being held in loan by another researcher and not made available upon multiple requests. The concern is that the only dace species determined for the upper Snake River basin in this study are Umatilla and speckled dace, and the status of leopard dace there is unknown.

This is partly based on an apparent conundrum that there are no leopard dace from the upper Snake River basin listed in the fish collection at the University of Idaho (Wallace, pers. comm.) and that the original type specimen for leopard dace nonetheless came from the Boise River in Idaho (Eigenmann and Eigenmann 1893). The Umatilla dace in the Boise River also demonstrate the only variable association with both leopard and speckled dace based on nuclear DNA sequence data (Chapter 4). While definitely remaining distinct as an overall species, all other Umatilla dace are more closely related to either speckled or leopard dace (Kat 1985). It is possible that rivers like the Boise and Yakima are undergoing species displacement or shifts (Huxel 1999) with respect to their leopard dace (also see Hughes and Peden 1989, Peden and Hughes 1988) and / or renewed hybridization although evidence of more hybridization was not definitively found in the single specimen examined with molecular genetic techniques.

For the sake then of completing the distributions of dace throughout the whole Columbia basin, a few species' allopatric samples and identifications were used from the literature (Fig. 16). This was largely done out of necessity for leopard dace and in spite of concerns. Ten unconfirmed sample locations for them were used (Lee et al. 1980, Peden 1991). Two unconfirmed samples were also added for Umatilla dace (Hughes and Peden 1989, Peden and Hughes 1988). To be clear, these 12 samples could not be confirmed as to their definitive species identifications based on the protocols developed in this study. Eight unconfirmed

samples for speckled dace (Lee et al. 1980) were also utilized, but much more so only for representativeness and completeness with less concern about their proper identification.

There is not much concern about the two unconfirmed Umatilla dace samples identified by Hughes and Peden (1989, Peden and Hughes 1988) because all other samples named by them as Umatilla dace were also confirmed as such in this study. However, there are particular concerns about the unconfirmed leopard dace samples since all their previously identified samples from the Columbia River in Canada (Hughes and Peden 1989, Peden and Hughes 1988, Peden 1991) that were available elsewhere for my confirmation were deemed Umatilla dace (Fig. 16 and appendix A). To be fair, most of these disputed identifications of leopard dace are based on small and / or poor specimens, and the samples are also often of dace with notably unusual morphometric and / or meristic characteristics (appendix A). Nonetheless, other more regular and intensive surveys of these same Columbia River drainages in Canada also found no leopard dace and only Umatilla dace (Johnson et al. 1994, R.L.&L. 1991, 1995, Rosenfeld 1996).

The issues of access to this region and to its unconfirmed museum samples could not be resolved within the time-frame of this thesis. The unconfirmed samples were put on the distribution maps (Fig. 16), but are highlighted and are interpreted with knowledge and caution. This is also the case for the range map developed here for leopard dace. The entire range of leopard dace based on confirmed and unconfirmed samples is presented (Fig. 17A). However, only the confirmed range for leopard dace is also given. The confirmed and unconfirmed samples have leopard dace spread throughout most of the Columbia River system in the USA.

Discussion

Systematics

The three dace species exist in allopatry, parapatry, and in all possible pairings of strict sympatry in several broadly scattered locations throughout the interior regions of the Columbia River drainage in the USA. The three dace species are also found in strict sympatry in the Yakima River. Umatilla dace are abundant in allopatry and sympatry across this large range. Umatilla dace are common and also usually numerically dominant in the majority of their strict sympatric samples with speckled and leopard dace.

This is very strong support for the distinctiveness of Umatilla dace as a species (Arnold 1997, Bullini 1985, Smith 1992, Stone 2000), and also for their designation as such within the biological and other related species concepts (Mayr 1963, 1969, Nelson and Hart 1999, Wiley and Mayden 1985). Umatilla dace display their own sustained evolutionary lineage and do not loose their species integrity in the presence of leopard and speckled dace (Wiley 1978).

Umatilla dace appear to be reproductively isolated from leopard and speckled dace, with no good indications of present hybridization (Chapters 2 and 4; Blouws 1998, Howes 1991, Smith 1992).

Evolution

The distribution of Umatilla dace is largely in the areas of overlapping ranges of leopard and speckled dace. Umatilla dace populations are mostly found in overall areas that thus also coincide with these other two dace species. This is very strong and uncommon support for the hypothesis that Umatilla dace evolved through hybridizations of leopard and speckled dace (Arnold 1997, Barraclough and Vogler 2000, Dowling and DeMarais 1993, Funk 1985, Stauffer et al. 1979, Stone 2000). Umatilla dace are presently clearly distinct from leopard and speckled dace, and exist in allopatry. However, their overall range even now remains wedded to the general distributions of leopard and speckled dace (Hubbs 1955, Miller 1961, Sorenson 1978, Stebbins 1959, Wiley and Mayden 1985).

The postglacial recolonization of Umatilla dace is also approximately intermediate to that of speckled and leopard dace (also see McPhail and Lindsey 1986). Leopard dace have postglacially recolonized the farthest, moving well into Canada up to near the top of the main Fraser River basin. They have also been found to undertake lengthy annual migrations in this region (Haas unpubl. data; also see Smith 1991, Winfield and Townsend 1991). Speckled dace are only found in the Kettle and Granby rivers, and Umatilla dace are found in the other lower portions of the Columbia River basin in Canada. This distance of postglacial recolonization into Canada is likely related to their specific flow tolerances, swimming abilities, and ecological preferences (Crawford et al. 1999, Dowling and DeMarais 1993, Herrera 1992, Pearson et al. 1988; also see Bisson and Bond 1971). These have been determined as intermediate for Umatilla dace (Chapters 5 and 9). The intermediate postglacial recolonization of Umatilla dace is once more supportive of the hypothesis for their original speciation and subsequent evolution as hybrids of leopard and speckled dace (Barraclough and Vogler 2000, Funk 1985, Sage and Wolf 1986, Smith 1992, Tauber and Tauber 1989).

Most Umatilla dace populations have also been found to more closely resemble either of their hypothesized parental species of leopard and speckled dace (Brust et al. 1998, Dowling et al. 1989, Larson 1989). This resemblance is seen in data from genetics (Chapters 4 and 7), ecological habitat preferences (Chapters 5 and 9), and morphometrics and meristics (Chapters 2 and 6). This phenomenon is discussed in detail in relation to each of these data sets in their pertinent Chapters. For their biogeography and distribution, this relatedness of Umatilla dace to speckled or leopard dace is clearly based on which of these two hypothesized parental species is in more general abundance at any particular location (Cathey et al. 1998, Crespin et al. 1999, Jordan 1991, Tauber and Tauber 1989).

In the USA, speckled dace in the USA predominate in most of the Columbia River basin, particularly in the southern and central portions (Lee et al. 1980). Leopard dace here are not common and unconfirmed as examined in this study. They are only definitively found in some abundance in north central tributaries to the Columbia River like the Similkameen River and at least formerly the Yakima River. Leopard dace and speckled dace are both found in the drainages to the Columbia River west of the Coastal Mountain range. In Canada, all three dace species are only found in allopatry, but the relatedness of Umatilla dace there is still to the downstream predominance of leopard or speckled dace. This makes sense since the Canadian populations of Umatilla dace must all be postglacial recolonizers from those same downstream areas. That these biogeographic and distribution patterns of abundance in speckled and leopard dace match those for their relatedness and resemblance to Umatilla dace is again very strong support of the hypothesis that Umatilla dace evolved as hybrids of these other two dace species (Buth et al. 1991, DeMarais et al. 1992, Dowling et al. 1989).

Umatilla dace in the Yakima River were related to speckled dace, but only distantly to their leopard dace (Chapter 4). Somewhat similarly, Umatilla dace in the Boise River appeared to be intermediate in their overall relation to speckled and leopard dace, and no leopard dace in fact were found there. In both rivers, there is a historic record of leopard dace in higher prevalence and abundance (Patten et al. 1970, Eigenmann and Eigenmann 1893). It is possible that such rivers are undergoing species displacement or shifts with respect to their leopard dace (Huxel 1999). Renewed hybridization is another possibility, but evidence for it was not definitively found in the molecular genetic data. The likely replacement appears to be Umatilla dace in each case (also see Hughes and Peden 1989, Peden and Hughes 1988). These two river systems are among the most heavily anthropogenically impacted in the generally heavily impacted Columbia River basin. It is possible that Umatilla dace are again doing particularly well there because of advantageous characteristics attributable to their past hybridizations and that were successful in perturbed systems (Arnold 1997, DeMarais et al. 1992, Dowling and DeMarais 1993). Disruption and modification of water flow are one of the variables most strongly impacted in the Boise and Yakima rivers.

The general distribution of Umatilla dace, or at least the main concentration of their populations and numerical abundance, also corresponds directly with the major downstream flooding and impact zones from Glacial Lakes Missoula and Bonneville (also see Hewitt 1996, Smith 1992). These huge lakes existed in the last Pleistocene glaciation when ice dams blocked portions of the Columbia River in particular (Chapters 1 and 11). These glacial lakes filled a vast area and then drained catastrophically, suddenly and repetitively with individual drainage periods estimated to be as short as two weeks. The subsequent floods are generally thought to have been the largest on earth since that geological time. The draining was caused

by the ice dams eventually being floated and thus after the lakes drained, the dams reformed and settled. The river was then blocked again and the filling and flooding cycle continued. Smaller glacial lakes formed under the same circumstances and with similar consequences in drainages clustered around the north central tributaries to the Columbia River.

This match of Umatilla dace to these areas strongly associated with these two glacial lakes, and particularly with Glacial Lake Missoula, are a direct fit to hypothesized mechanism for their evolution through hybridization between leopard and speckled dace (Dowling and Hoeh 1991; also see Barraclough and Vogler 2000, Zardoya and Doadrio 1999). The dace species segregate their stream environments using water flow. The breakdown of these flowing environments in or as a result of ponding in these glacial lakes is speculated to be what caused their hybridizations (Aspinwall et al. 1993a/b, Balon 1992, Butcher 1980, Hubbs 1955, Lens et al. 2000, Rieseberg et al. 1996). The harsh, fluctuating, and different environments found during and after this period in the last Pleistocene glacial epoch could also have been suited to ecological exploitation by a hybrid species (Arnold 1992, 1997, Dowling and DeMarais 1993, Kat 1985, Lewontin and Birch 1966, Tauber and Tauber 1989) that likely had characteristics favouring its survival there (Barton 1989, Bloom 1976, DeMarais et al. 1992, Hubbs 1955, Huxel 1999, Potts and Reid 1988). This is discussed in full detail in Chapters 1 and 11.

Chapter FOUR

Genetic Data

Materials and Methods

1. Cytochrome-b Region of the Mitochondrial DNA

1 - 1. Extraction of DNA

Tissue digestion was achieved by placing 20 mg of caudal fin clips in 562.5 mμL of 0.2M EDTA/0.5% sarcosyl buffer with 37.5 mμL of pronase (2mg/mL). Samples were incubated overnight at 37 °C. After complete digestion of tissue, 3 mμL of RNAse (10mg/ mμL) was added and samples were left at 37°C for one hour. Samples were rediluted to 600 mμL with TE (pH 8.0) if volume was lost over the course of this procedure.

Extraction of DNA was performed by a ten minute wash with 600 mμL of phenol, followed by 600 mμL of chloroform. Samples were then centrifuged for five minutes and the aqueous layer decanted. Isopropanol (0.6 volumes, 100%) was added to the aqueous phase followed with gentle overend turning to precipitate out DNA. If DNA did not 'fluff out', samples were placed in a 5° C refrigerator overnight. Samples were then centrifuged for five minutes and the isopropanol / aqueous phase was aspirated. DNA pellets were rinsed in 70% ice cold ethanol for one hour and centrifuged for three minutes. Ethanol was decanted and the pellet was left to dry at room temperature until all traces of alcohol were gone. DNA was resuspended in TE (pH 8.0) buffer and left to fully dissolve at room temperature. All samples were stored at -20°C.

1 - 2. Amplification of DNA using PCR

Analysis of variation was performed on a single, polymerase chain reaction (PCR) amplified, mitochondrial fragment. The primers, cytB2 (3' - CCC TCA GAA YGA TAT TTG TCC TCA - 5') and gluDG (5' - TGA CTT GAA RAA CCA YCG TTG - 3') (Palumbi 1996), yielded a 420 base pair (bp) fragment from the cytochrome-b gene which was used in sequencing.

PCR reactions were performed with 800 mμM dNTPs, 0.6 mμL of each primer, 4 mM MgCl2, 1X GibcoBRL Taq polymerase buffer, 8 units of Taq polymerase and 2 mμg of DNA template to a total volume of 150 mμL. Cycling parameters were 1 cycle of denaturation at 95 °C for 2 minutes, annealing at 45°C for 1 minute, and extension at 72 °C for 90 seconds. This was followed by 30 cycles of denaturation at 95 °C for 60 seconds, annealing at 45 °C for 60 seconds and extension at 72 °C for 90 seconds. Samples were then left for 5 minutes at 72 °C to ensure completion of strand synthesis.

1 - 3. DNA Sequencing

Sequencing of cytB2 / gluDG primed fragments was performed using dideoxy termination sequencing. An initial volume of 100 mμL of PCR product was purified using a Quiagen Quiaquick PCR Purification Kit. The purified DNA was then suspended in distilled water and its concentration measured using a spectrophotometer. Eighty-five nanograms of purified DNA was added to 0.16 mμM of gluDG primer, 1 mμL 0.05% DMSO, and a Taq/ddNTP mix obtained from the NAPS sequencing unit of UBC. Total volume of this reaction was 20 mμL. PCR was then run using the following parameters: 1 cycle of denaturation at 94 °C for 4 minutes, followed by 25 cycles of denaturation at 94 °C for 30 seconds, 55 °C annealing for 15 seconds, and 60 °C extension for 4 minutes. The finished reaction was purified using Centrisep Spin Columns to eliminate primers and excess ddNTPs. The purified DNA was then run on polyacrylamide gel and read by the NAPS automated sequencing unit at UBC.

1 - 4. Data Analyses

Cytochrome-b sequence from all individuals was aligned by eye, and comparisons were made among individuals and species based on the 306 bp common to all (positions 40 - 345 from the 5' end of the cytochrome-b gene). Genetic distances and phylogenetic relationships were calculated using the Molecular Evolutionary Genetics Analysis (MEGA) program Version 2 (Kumar et al. 2001). Genetic distances were estimated using Kimura's two-parameter distance (Kimura 1980) which accounts for the higher rate of transition substitutions observed in animal mitochondrial DNA relative to transversions. Phylogenetic relationships were inferred using the Neighbour-Joining method with statistical estimates of branch point validity presented as bootstrap confidence limits.

2. Internal Transcribed Spacer

2 - 1. Extraction of DNA

Former extractions from mitochondrial sequencing work were used.

2 - 2. Amplification of DNA using PCR

PCR and sequencing of specimens RF8, RO22, RO31, RU10, RU92, and RUB04 were performed at Canadian Department of Fisheries and Oceans West Vancouver Laboratory (WVL) and at the University of British Columbia (UBC). The ITS-1 region of all other samples was amplified and sequenced at the University of Windsor (UW)

At the WVL and at UBC, the forward primer KP-2 (5'-AAA AAG CTT CCG TAG GTG AAC CTG CG-3') and reverse primer 5.8S (5'-AGC TTG CTG CGT TCT TCA TCG A-3') (Phillips et al 1995) yielded a single band of approximately 480 bp. Sequencing was once again only possible in the forward direction as the reverse primer was not stringent enough to yield clean sequencing product. In order to sequence the ITS region further another forward primer KP-3

(5'-AACGAG TGG CGA GAG TTG CAA-3') was designed from the sequence obtained using KP-2. PCR with the primers KP-3 and 5.8S yielded two bands of approximately 450 and 400 bp. All PCR reactions contained 800 mμM dNTPs, 0.6 mμL of each primer, 4 mM MgCl2, 1X GibcoBRL Taq polymerase buffer, 8 units of Taq polymerase and 200 ng of DNA template to a total volume of 50 mμL. Cycling parameters were 1 cycle of denaturation at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing for 45 seconds, and extension at 72 °C for 90 sec. Samples were then left for 5 minutes at 72 °C to ensure completion of strand synthesis.

At UW, a PCR fragment approximately 700 bp in length was generated using the forward primer Tel-ITS-1-F (5'-GTA AAA CGA CGG CCA GTC CTT TGT ACA CAC CGC CCG TC-3') and reverse primer ITS-1-R (5'-CTG CGT TCG AAG TGT CGA TG-3'). The use of this forward primer generated more SSU sequence than did KP-3, and it gave cleaner PCR and sequencing results and still permitted sequencing of the entire ITS 1 region. Each 50 mμl reaction contained standard PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin), 0.2 mM dNTP (Saiki 1990), 50 pmol of each primer, and 1.25 units of Taq DNA polymerase (Gibco BRL). The reactions were run in a PTC-200 Thermocycler (MJ Research, Watertown, MA) for 35 cycles consisting of denaturation at 94 °C for 1 minute, primer annealing at 58 °C for 1 minute, and extension at 72 °C for 1 minute. The 35 cycles were preceded by an initial denaturation for 1 minute at 94 °C, and followed by a final 5 minute 72 °C extension. Negative controls, which used distilled water instead of template DNA, were included in each series of reactions to screen for possible contamination.

2 - 3. DNA Sequencing

At UBC, sequences of the ribosomal DNA fragments generated were obtained by manual P33 dideoxy chain termination sequencing. Sequences were run on 6% polyacrylamide / urea sequencing gel prepared with 15 mL 40% acrylamide solution (19 acrylamide : 1 bisacrylamide), 50 g of urea, 20 mL 5X TBE and 25 mL distilled water.

At UW, PCR products were sequenced using the OpenGene Automated DNA Sequencing System and Cy5 / Cy5.5 Dye Primer Cycle Sequencing Core Kit (Visible Genetics Inc., Toronto, Ontario) according to the manufacturer's instructions. The forward sequence used to generate the PCR product contained the M13f sequence and its 5'-end, thus allowing sequencing with dye-labelled M13f primers.

2 - 4. Data Analyses

The boundaries of the ITS-1 region were deduced by comparing the dace sequence with that from rainbow trout (*Oncorhynchus mykiss* (Walbaum)) (GenBank accession number AF308735), and the region was found to range from 295 bp to 309 bp in length. The sequence

could be read unambiguously in 11 individuals and was aligned by eye. Genetic distances and phylogenetic relationships were calculated in these individuals using the Molecular Evolutionary Genetics Analysis (MEGA) program Version 2 (Kumar et al. 2001). Phylogenetic relationships were inferred using the Neighbour-Joining method with statistical estimates of branch point validity presented as bootstrap confidence limits.

In another six individuals, only approximately 250 bp of sequence could be read. After this point, variations in the length of the PCR fragments appeared to produce multiple sequences superimposed one upon the other. Within the first 250 bp, some positions also showed multiple nucleotides at a single position, but this did not interfere with the ability to read further downstream. The sequence from these six individuals was combined with the same partial sequence from the above 11 individuals and the analysis was repeated as above. In a further four samples, as well as in the longnose dace (*Rhinichthys cataractae*) to be used as an outgroup, the sequences were repeatedly unreadable shortly after the beginning of the ITS region, again apparently due to length variations in the PCR fragments being sequenced. These samples were excluded altogether.

3. Ribosomal Region of the Genomic DNA

3 - 1. Extraction of DNA

Former extractions from mitochondrial sequencing work were used.

3 - 2. Amplification of DNA using PCR

Analysis of variation was performed on two areas of the ribosomal gene, the small subunit (SSU) and the internal transcribed spacer (ITS).

3 - 3. DNA Sequencing

The general forward primer 18e (5'-CTG GTT GAT CCT GCC AGT-3') and reverse primer 18g (5'-CGG TAC TAG CGA CGG GCG GTG TG-3') (Hillis and Dixon, 1991) amplified three bands of approximately 500 bp, 900 bp, and 1500 bp. The SSU is approximately 1500 bp long so the smaller fragments were most likely a result of the primers annealing internally.

In practice, two fragments were amplified of approximately 500 bp and 400 bp. The 500 bp fragment was sequenced from both ends and from this another forward primer, Rhin608 (5'-GCG TAT ACT AAA GTT GCT G-3'), and reverse primer, Rhin1200 (5'-AGG TTT CCC GTG TTG AGT-3'), were created. PCR using these primers was performed by the NAPS sequencing unit at UBC.

All PCR reactions contained 800 mμM dNTPs, 0.6 mμL of each primer, 4 mM MgCl2, 1X GibcoBRL Taq polymerase buffer, 8 units of Taq polymerase, and 200 ng of DNA template to a total volume of 50 mμL. Cycling parameters were 1 cycle of denaturation at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing for 45

seconds, and extension at 72 °C for 90 sec. Samples were then left for 5 minutes at 72 °C to ensure completion of strand synthesis.

3 - 4. Data Analyses

Long run sequencing gels tend to be hard to read as the probability of longer fragments forming in the sequencing reaction decreases as size increases. As well, polyacrylamide gel tends to break down if current is passed through for an extended period as this generates a certain amount of heat due to resistance. The reverse primer 18g is not useful for sequencing as it tends to amplify less stringently than 18e and therefore creates a lot of noise. To create a more manageable sized fragment and attempt to sequence from both ends, two new primers were generated. A forward primer, Rhin1f (5'-GCT AAT ACA TGC AAA CGA G-3'), was created using sequence obtained from the 18e primer. A reverse primer, Rhin2R (5'-CGA GAT CCA ACT ACG AGC-3'), was generated by comparing and looking for conserved areas of sequence between the SSU of coho salmon (*Oncorhynchus kisutch* (Walbaum)) and carp (*Cyprinus carpio* Linnaeus). From the carp and coho sequence, it was expected that these primers would yield a 494-bp fragment.

Results

The species' identifications for all the dace analyzed for DNA sequences are based on the morphometric and meristic data and results from Chapter 2. The sample sites were thus the same for morphometric / meristic data as for genetic data, and are specified in Appendix A. The sequence and alignment data for cytochrome-b are will be submitted to GenBank (Internet) for archiving and public access.

1. Cytochrome-b Region of the Mitochondrial DNA (mtDNA)

The mitochondrial DNA (mtDNA) sequence data from the cytochrome-b region (Fig. 19) strongly suggests that there are four groups of speckled dace (*R. osculus*), three groups of Umatilla dace (*R. o. umatilla*), and two groups of leopard dace (*R. falcatus*). The bootstrap values for these tree nodes are all greater than at least 87 % (Fig. 19) and there are sufficient significant base pair (bp) differences (Table 1) suggesting good discrimination of all these groups (Fig. 20). The Umatilla dace from the Similkameen River are identical to the leopard dace group from the Yakima and lower / upper Fraser rivers (Fig. 19 and Table 1). These Umatilla and leopard dace are thus treated as one combined group, but this does not imply sympatry between them. These groups are summarized starting at the bottom of the tree on Figure 19 and are presented on map Figure 20. Their sequence differences are given in the same format for the same groups in Table 1, and these bp differences are discussed in turn for

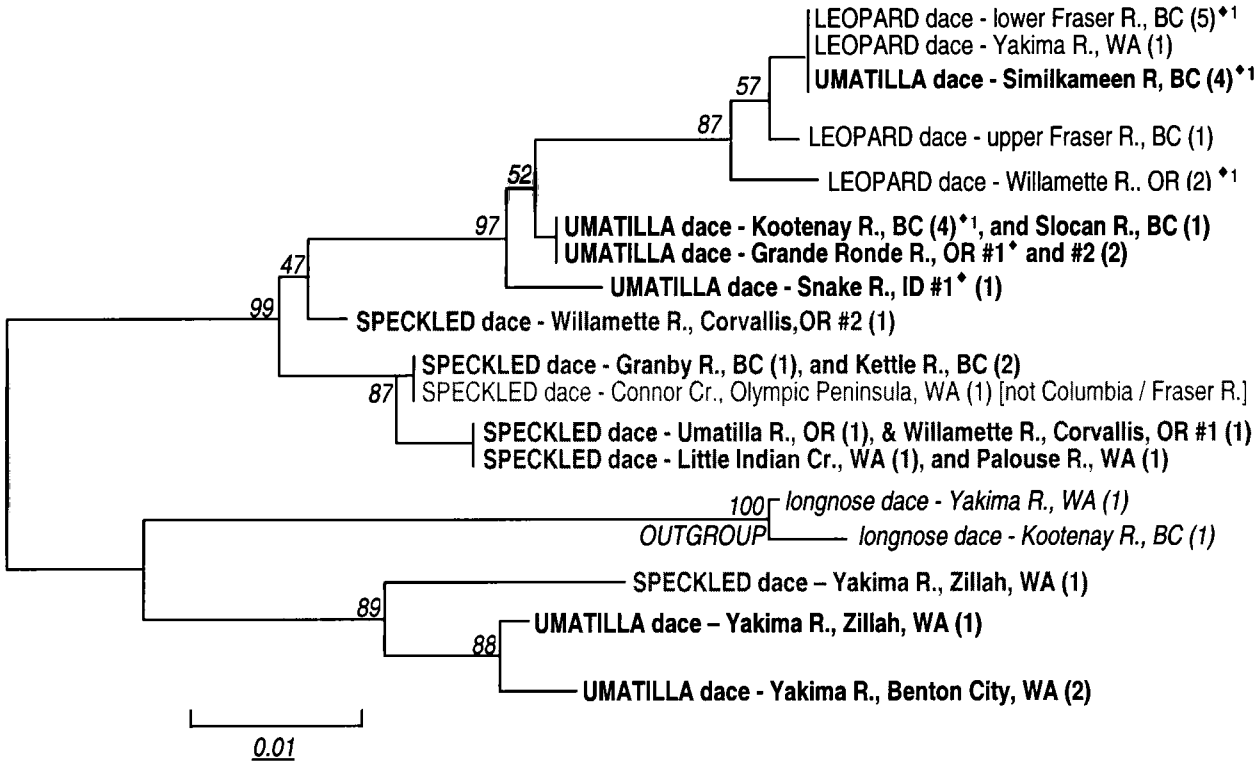


Figure 19. Tree (neighbour-joining) for mitchondrial DNA sequences for a 306 base pair segment of the cytochrome-b region starting at gene position 40. The data are for thirty-four dace from 19 samples (Table 1 and appendix A). The data for two longnose dace from two samples used as the outgroup in the analysis are also presented (Table 1). Leopard dace are presented in black font, Umatilla dace in bold black font, and speckled dace in bold dark grey font. Bootstrap values are based on 500 iterations and are presented in italic black font on the tree nodes. Five locations followed by a # sign designate specific dace that are different in the cytochrome-b mtDNA and ITS DNA sequence data or show samples with >1 fish that do not remain together as a group in all trees. Six sites followed by a diamond in superscript designate specific dace or part of its overall sample as common to the mtDNA sequence data set and at least one of the ITS data sets. If a sample site with a diamond superscript has more than one dace, the diamond superscript is followed by a number indicating how many of those dace are consistent between the mtDNA and ITS DNA sequence data sets. The sample number per site is in parentheses following the locality information. The specific groupings based on these data are shown and summarized starting at the bottom of this tree on map Figure 20.

DNA group	sample site location	S1	S2	S3	S4	U1	U2	L1	U3L2	long1
speckled dace *Group 1* **(S1)**	Yakima R., WA (1)	-	-	-	-	-	-	-	-	-
speckled dace *Group 2* **(S2)**	Little Indian Cr., WA (1) Palouse R., WA (1) Umatilla R., OR (1) Willamette R., OR #1 (1)	17	-	-	-	-	-	-	-	-
speckled dace *Group 3* **(S3)**	Granby R., BC (1) Kettle R., BC (2) Connor Cr., WA (1)	20	3	-	-	-	-	-	-	-
speckled dace *Group 4* **(S4)**	Willamette R., OR #2 (1)	19	2	1	-	-	-	-	-	-
Umatilla dace *Group 1* **(U1)**	Yakima R., OR (3)	6 8	16	17 19	16 18	-	-	-	-	-
Umatilla dace *Group 2* **(U2)**	Grande Ronde R, OR #1+2 (2) ♦1 Kootenay R., BC (4) ♦1 Slocan R., BC (1) Snake R., ID (1) ♦	20	6	9	8	18 20 24	-	-	-	-
leopard dace *Group 1* **(L1)**	Willamette R., OR (2) ♦1	23	8	11	10	22 24	6 8	-	-	-
Umatilla dace *Group 3* - *and* - leopard dace *Group 2* **(U3L2)**	**Umatilla dace:** Similkameen R., BC (4) ♦1 leopard dace: lower Fraser R., BC (5) ♦1 upper Fraser R., BC (1) Yakima R., WA (1)	23 24	8 9	11 12	10 11	22 23 24 25	5 6 7 8	2 3	-	-
outgroup – longnose dace *Group 1* **(long 1)**	Kootenay R., BC (1)	19	22	21	20	17 19 24	24	25	27 28	-
outgroup – longnose dace *Group 2* **(long2)**	Yakima R., WA (1)	20	23	22	21	18 20 25	25	26	28 29	1

Table 1. Table of the differences between the sequence groups for a 306 base pair segment of the cytochrome-b region of mitochondrial DNA (mtDNA) starting at gene position 40. The data are for thirty-four dace of all three species from 19 samples (appendix A). The data for two longnose dace from two samples used as the outgroup in the analysis are also presented. Some boxes in the table have multiple scores because these designated Groups are sometimes composed of several sample localities that clustered together (Fig. 19) but that also still show some minor sequence variation. Five locations followed by a # sign designate specific dace that are different in the cytochrome-b mtDNA and ITS DNA sequence data or show samples with >1 fish that do not remain together as a group in all trees. Six sites followed by a diamond in superscript designate specific dace or part of its overall sample as common to the mtDNA sequence data set and at least one of the ITS data sets. If a sample site with a diamond superscript has more than one dace, the diamond superscript is followed by a number indicating how many of those dace are consistent between the mtDNA and ITS DNA sequence data sets. The sample number per site is in parentheses following the locality information. These specific groupings match those shown and summarized on map Figure 20. The tree built on this mitochondrial DNA sequence data is Figure 19.

each group of each species. The overall patterns remain essentially the same under other jackknife data set trials, where sample(s) are deleted and the tree is generated again.

The speckled dace that form their Group 1 (Fig. 20) are from the Yakima River and cluster very distinctly from the other three speckled dace groups (Fig. 19). The other speckled dace groups are each distinct but also cluster as an overall entity. The Yakima River as a whole in fact clusters uniquely on its own and it is the only strict sympatric site found for all three dace species (Fig. 20 and appendix A). These Umatilla dace that form their Group 1 are again only from the Yakima River (Fig. 20 and Table 1), and also cluster very distinctly from the other Umatilla dace and all other samples of dace (Fig. 19). The speckled and Umatilla dace here cluster together on a unique tree placement, and in fact differ from all other dace by 16 – 25 bp (Table 1). The speckled and Umatilla dace from the Yakima River even differ from each other by 6 – 8 bp (Table 1). This pattern for the Yakima River is the same when it is analyzed by itself, and the overall patterns of the rest of the tree remain similar when its analysis is run without the Yakima River dace specimens (tree not shown). The Yakima River is also the only site of strict sympatry for all three dace species and for longnose dace too.

The leopard dace from the Yakima River are not distinct to just that drainage, but are indistinguishable from other leopard dace in the Fraser River (Fig. 19). The leopard dace in the lower and particularly in the upper Fraser River are geographically distant and in another watershed (Fig. 20; Fig. 16 in Chapter 3). The Yakima River leopard dace also cluster with the overall entity of leopard dace from the Fraser and Willamette rivers and with Umatilla dace from the Similkameen River (Fig. 19). The Umatilla dace from the Similkameen River are also geographically isolated and distant from both these leopard dace populations. Only the Yakima River dace sample is located south of the Canada / USA border. These three populations of two dace species are placed in the combined Umatilla Group 3 / leopard dace Group 2 (Fig. 20 and Table 1). The leopard dace from the Yakima River differ from the speckled and Umatilla dace there by 22 – 25 bp, with some other speckled and Umatilla dace groups from outside the Yakima River being less differentiated (Table 1).

The longnose dace from the Yakima River is also not as clearly unique to it as its speckled and Umatilla dace (Fig. 19). That longnose dace only differs by 1 bp from the leopard dace from the geographically distant and isolated eastern portion of the Kootenay River in Canada (Table 1 and Fig. 20). Both longnose dace differ from all other dace species and groups by 18 – 29 bp.

The two speckled dace from the Willamette River are significantly discriminated by more than one node on the tree (Fig. 19), even though this is represented by only two bp differences (Table 1). They are respectively placed in speckled dace Group 2 and as the only population in Group 4 (Fig. 20 and Table 1). These two speckled dace were further placed in two distinct

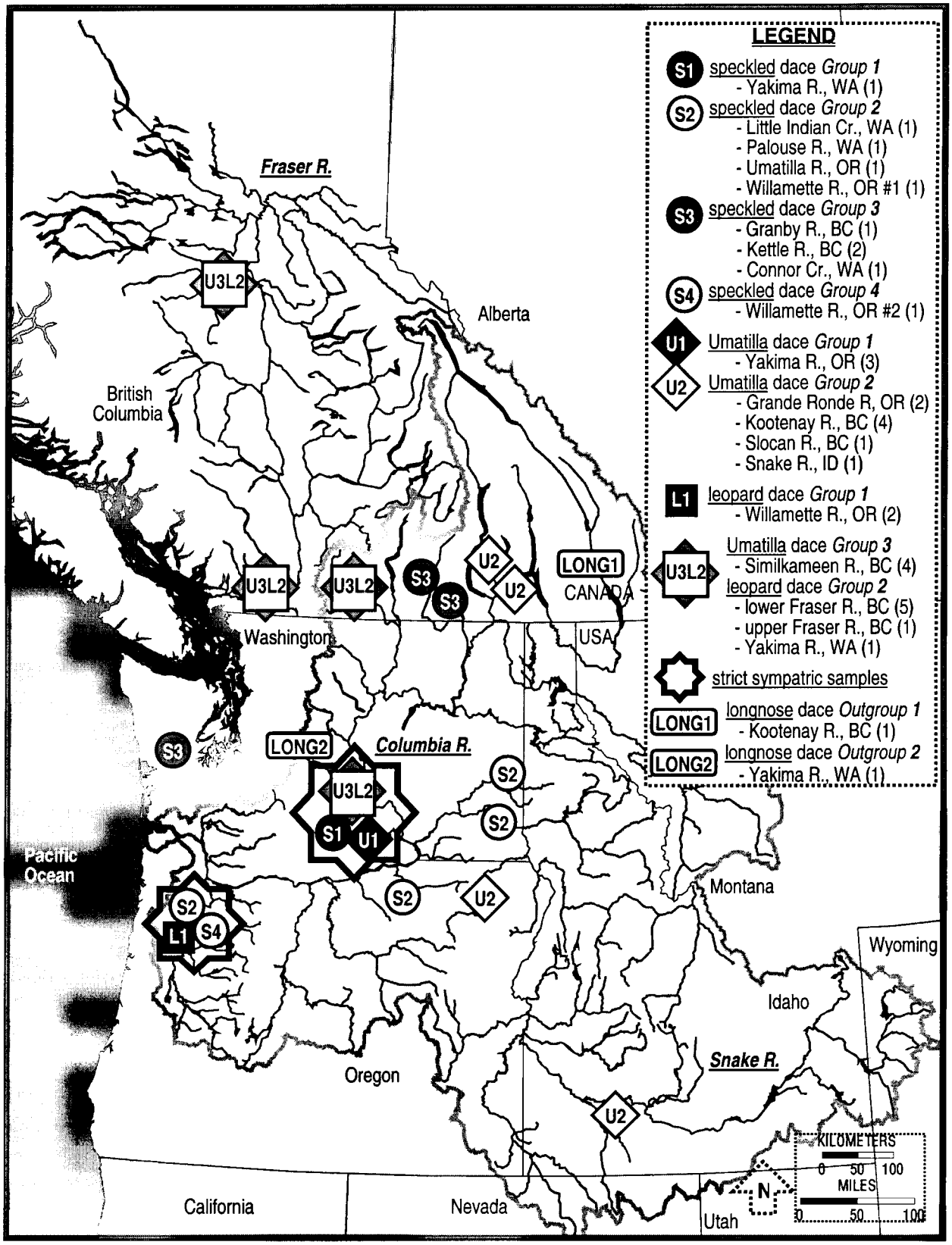


Figure 20. Map of distribution of DNA sequence groups from a 306 base pair segment of the cytochrome-b region of mitochondrial DNA (Fig. 19 and Table 1). These data are for 34 dace of all three species from 19 sample localities (appendix A). The data are also presented for two longnose dace from two sample localities used as the outgroup in the analysis (Fig. 19 and Table 1).

groups since they were strictly sympatric and because their differentiation remained similar and distinct throughout different jackknife runs. Speckled dace Group 2 also consists of several other Columbia River sample locations that are more geographically clustered and southerly, but more interior on the other side of the Coastal Mountain range as well (Fig. 20 and Fig. 16 in Chapter 3; appendix A). As well, the speckled dace in Group 2 are the only dace Group composed of multiple sample sites found exclusively and well south of the Canada / USA border.

Such within-site discrimination for speckled dace is also unique to the Willamette River. These two speckled dace were collected at the same sample site on the Willamette River, but interestingly from two different years. The only other speckled dace samples with more than one specimen are the Granby and Kettle river system, and their speckled dace showed no such within-site(s) differentiation (Fig. 19 and Table 1). It is perhaps possible that speckled dace are sometimes or maybe even often composed of more than one mtDNA sequence type at one locality, but this data cannot make that further determination. There are occasional indications of such within-site differentiation in the morphometric and meristic data for speckled dace, but these data also suggest this for some sample localities of leopard and Umatilla dace (appendix A – 'unusual' fish; Chapter 2).

Group 3 for speckled dace (Fig. 20) cluster distinctly (Fig. 19) and largely consist of the two most northerly locations in their range in the Columbia River system and in latitude (Fig. 16 in Chapter 3; appendix A). These sites are the Granby and Kettle rivers, which are also interconnected and isolated above a significant barrier at Cascade, BC. This Group 3 for speckled dace also includes the only dace analyzed that was not from the mutual range for these three dace species of the Columbia and Fraser river drainages. Umatilla and leopard dace do not occur outside these two river watersheds. This speckled dace was from the geographically distant and isolated Olympic Peninsula that also is on the other side of the Coastal Mountain range (Fig. 20; Figs. 16 and 17 in Chapter 3; appendix A). It was included to see if any associated differentiation might be noted for that region since it had a distinct refuge during the same last Pleistocene glacial period being discussed here in the evolutionary hypotheses for Umatilla dace (Armstrong 1981, Crandell 1965). Speckled dace on the Olympic Peninsula have been noted for their morphometric and meristic differentiation from nearby populations outside that region (McPhail 1967, McPhail and Lindsey 1986, Mongillo and Hallock 1997). There are only 1 – 3 bp differences between the speckled dace here in Group 3 and their associated Groups 2 and 4 (Table 1). There is no apparent uniqueness to the speckled dace from the Olympic Peninsula based on this mtDNA region and data.

Leopard dace in Group 1 consist of two specimens from only the Willamette River that are clustered differently and at a high bootstrap value from the only other leopard dace in the

combined leopard dace Group 2 / Umatilla dace Group 3 (Fig. 19). These other leopard dace are from the Fraser and Yakima rivers, and the Umatilla dace in that combined group are from the Similkameen River (Figs.19 and 20). The leopard dace in the Willamette River differ by two bp from these other leopard dace and by three bp from the Umatilla dace in the Similkameen River (Table 1). This level of discrimination and the tree position of the leopard dace from the Willamette River remained discrete and the same throughout other jackknife data runs. The leopard dace from the Willamette River occur in strict sympatry with both speckled dace Groups from there (Fig. 20; Fig. 16 in Chapter 3; appendix A). The leopard dace differ from the larger speckled dace Group 2 in the Willamette River by 8 bp and from the speckled dace Group 4 composed only of one Willamette River fish by 10 bp (Table 1 and Fig. 20).

Umatilla dace in Group 2 include the Snake River specimen even though the bootstrap value for its distinction is high in this instance (Figs. 19 and 20). Like the speckled dace from the Willamette River, their difference is only two bp from the other three sample localities in Umatilla dace Group 2 (Table 1). However, in the case of the Umatilla dace from the Snake River, only one of these bp is phylogenetically significant (Docker, pers. comm.). This differentiation is also across a single node on the tree, whereas it was across more than one for the speckled dace from the Willamette River. As well, the reasonably high bootstrap values differentiating this Snake River Umatilla dace drops as low at 53% in some jackknifed data sets and in some analyses using only a single set of each of the 13 cytochrome-b mtDNA types. Not including the Yakima River samples, the Umatilla dace in group 2 are more closely associated in sequence pattern (Table 1) with speckled dace (6 – 9 bp differences) than leopard dace (8 – 11 bp differences). The sample sites for Umatilla dace Group 2 are very similar for longitude, but the most geographically disparate of all the dace Groups in terms of latitude(Fig. 20; Fig. 16 in Chapter 3). All the Umatilla dace in Group 2 are found on the interior side of the Coastal Mountain range.

Umatilla dace from the Similkameen River in the combined group Umatilla Group 3 / leopard dace Group 2 are quite different from the main Group 2 of Umatilla dace (Figs. 19 - 20). They differ by 5 – 8 bp (Table 1). While not as different as the Umatilla (and speckled) dace from the Yakima River (18 – 25 bp), the Umatilla dace from the Similkameen River have still likely risen more independently and / or diverged considerably. The Umatilla dace from the Similkameen River are more closely associated with leopard dace (1 – 3 bp) than to non-Yakima River speckled dace (8 – 12 bp). This is opposite to the other Umatilla dace groups whose overall association is stronger with speckled dace, but this association is still more differentiated (6 – 9 bp) than that of the Umatilla dace from the Similkameen River to the other non-Yakima River leopard dace (1 – 3 bp). It might be casually suggested that the Umatilla dace from the Similkameen River are just leopard dace or that they have "recently" hybridized with leopard

dace (in particular, female leopard dace), but both other nuclear DNA data, and the morphometric / meristic data (Chapters 2 and 3), clearly indicate this is not the case. These genetic samples of Umatilla dace from the Similkameen River are all from British Columbia in Canada and above the Enloe Dam which sits on a former possible barrier site just below the Canada / USA border (Fig. 16 in Chapter 3; appendix A). This study further found no leopard dace upstream of that possible barrier in Canada, but Hughes and Peden (1989), Peden (1991) and Peden and Hughes (1988) morphologically identified leopard dace from just upstream of it. Their Canadian specimens were all very small with no adults (Peden 1991), but US adult specimens were identified in sympatry with Umatilla dace. These latter dace were not made available for examination and re-collection was unsuccessful. All their other leopard dace identifications from the Columbia River in Canada were deemed Umatilla dace by this study (appendix A; Chapters 2 and 3). Other leopard dace samples in dominant strict sympatry with Umatilla dace were confirmed here, but again only downstream of Enloe dam.

The clustering of the two longnose dace used as the outgroup is unusual. Outgroups usually look like the subsequent D3B analysis (Fig. 23), where the outgroup is placed clearly distinct and at the bottom of the tree. This unusual clustering of the outgroup for the cytochrome-b mtDNA is most likely caused by the unique Umatilla and speckled dace data from the Yakima River. These two dace species from the Yakima River are as or more genetically similar to these longnose dace from both the Yakima and Kootenay rivers as to other speckled, Umatilla, and leopard dace. Both these longnose dace still differ from speckled dace from the Yakima River by 19 – 20 bp, and the Umatilla dace from the Yakima River by 17 – 25 bp (Table 1). The speckled and Umatilla dace from the Yakima River differ from all three other dace species in all Groups by 16 – 25 bp. The longnose dace from the Yakima River are differentiated from the three other dace species in all Groups by 20 – 29 bp. These patterns also hold when this analysis is undertaken using only Yakima River dace and the longnose dace outgroup (tree not presented).

2. Internal Transcribed Spacer (ITS)

The data from both the partial and complete internal transcribed space (ITS) region examined are presented as an honest attempt at a reasonable compromise. The partial ITS is for a greater number of individuals, but for less sequence with occasional ambiguities at the level of more nucleotide heterozygosity. The complete ITS is unambiguous with no such concerns about data, but does not represent as many individuals and especially not as many sample locations for Umatilla dace (Fig. 21). The additional sites for Umatilla dace are also of particular interest with respect to the evolutionary hypotheses about their origin. The partial ITS data are thus acknowledged as sometimes more open to interpretation or speculation, but the complete ITS data alone does not accurately reflect all the available results. Furthermore, an

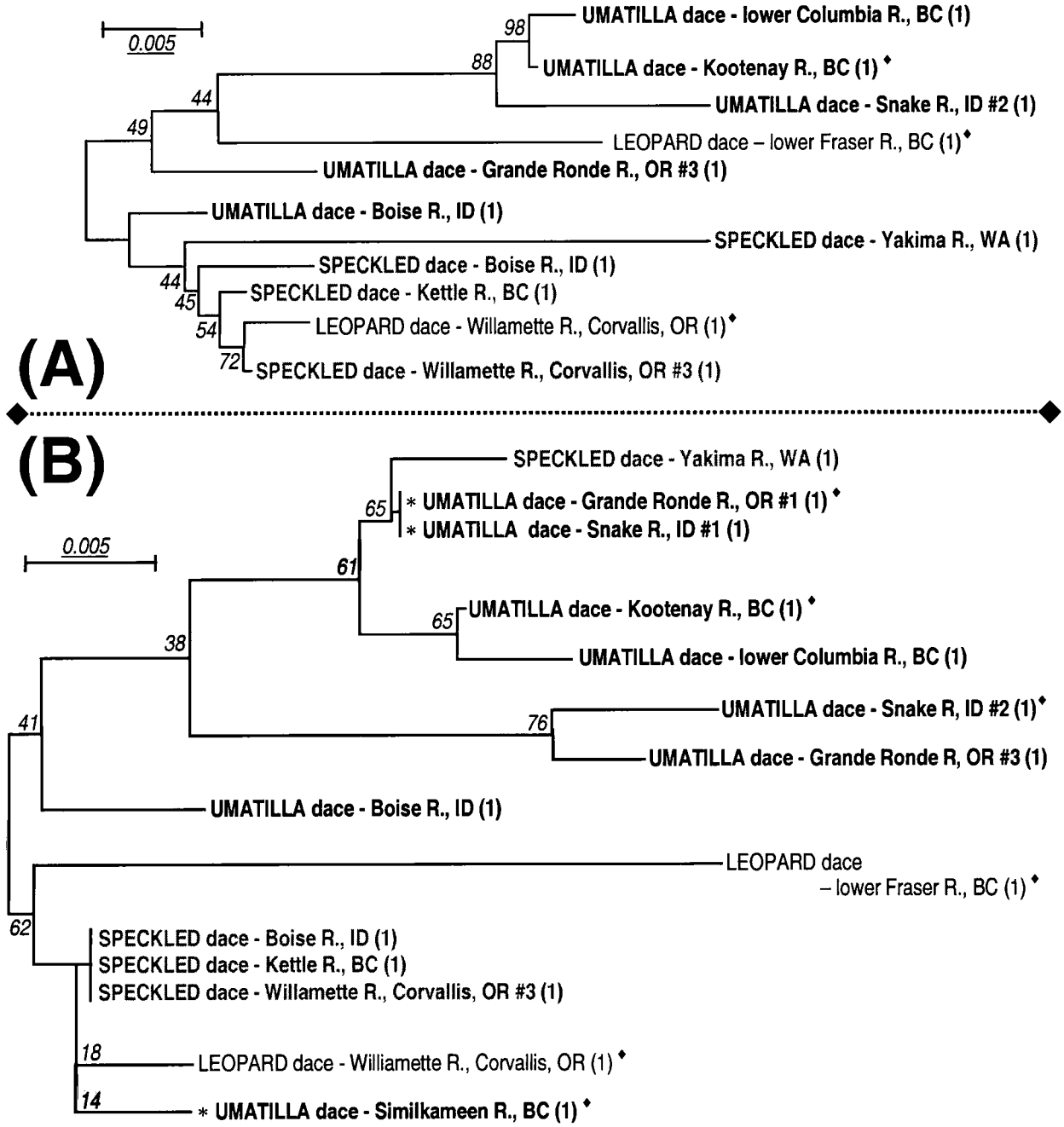


Figure 21. Trees (neighbour-joining) for DNA sequences from the internal transcribed spacer (ITS). Eleven dace from 11 samples (appendix A) had complete ITS data (A), meaning an unambiguous 316 base pair (bp) sequence was obtained without finding unreadable length variation. Fourteen dace from 12 samples (appendix A) could be similarly examined as partial ITS data (B) based on a smaller unambiguous 255 bp sequence. Three dace marked with an asterix (*) on the partial ITS tree (B) had somewhat ambiguous DNA sites or multiple bases, but no length variation. Five other dace from five samples are not presented as they had sufficient length variation to render their data unreadable (appendix D). Leopard dace are presented in black font, Umatilla dace in bold black font, and speckled dace in bold dark grey font. Bootstrap values presented in italic black font on the tree nodes. Locations followed by a # sign designate specific dace that are different in ITS DNA and cytochrome-b mtDNA sequence data sets or show samples with >1 fish that do not remain together as a group in all trees. Sample sites followed by a diamond in superscript designate specific dace as common to the mtDNA sequence data set and at least one of the ITS data sets. The sample number per site is in parentheses following the locality information. The specific groupings on the partial ITS tree (B) are shown and summarized starting at the bottom of this tree on map Figure 22. The complete ITS tree (A) gives very similar results except for two Umatilla dace groups that are further differentiated. This is specified for those two Umatilla dace groups on the map Figure 22 legend by designation of a further [A and B] subgrouping. A further analysis of the partial ITS data using only those individuals which also had complete ITS data gave the same overall pattern and similar specific results.

analysis of the partial ITS sequence region from only those specimens that had complete ITS data resulted in the same general pattern for both ITS data sets (no tree presented). For these reasons, both ITS partial and complete data are presented. Their complete sequences including insertions, deletions and ambiguous bp are also thus given in Appendices C and D, and not summarized in a table of differences as for cytochrome-b mitochondrial DNA (mtDNA). Such actual sequence information is more important and pertinent to ITS as well.

The ambiguity in the partial ITS data is due to variation within individuals among multiple copies of the genes. This variation was occasional nucleotide substitutions that had minimal or moderate impacts. These did not impair overall reading of the sequence but did result in the presence of an ambiguous bp. There were five additional dace from five samples that had sequences that could not be interpreted at all (appendix D). In these cases, the variation within individuals among multiple copies of the genes was substantial. These latter five sequences had sufficient length variations among multiple copies of the genes that their sequence could not be properly aligned. These five additional dace were thus necessarily left out of the ITS data sets and trees (Fig. 21 and appendices C - D).

It is possible to get around the latter substantial length variation problem through cloning. Polymerase chain reaction (PCR) products are inserted into bacteria which can harvest and sequence each gene copy individually. The result of then sequencing several of these different individual copies with length variations is that insight can be gained into what they individually look like and what phylogenetic patterns they might be attributed to. This was beyond the scope of, and funding for, this study.

The five additional fish from five samples that had uninterpretable ITS sequences due to substantial length variation were three Umatilla dace, one leopard dace and one of the outgroup longnose dace (appendix D). One of the Umatilla dace, the leopard dace, and the longnose dace also all came from the Yakima River. In the Yakima River, only the one speckled dace sample from the Yakima River was legible and usable (Fig. 21). Unfortunately, this meant that the unique cytochrome-b mtDNA variability observed for the Yakima River, and its status as the only strictly sympatric site found for all three dace species, could not be completely examined.

There were three dace in the partial ITS data that had readable sequences with a few somewhat ambiguous DNA sites or with multiple nucleotide substitutions, but with no large length variation (Fig. 21 and appendix D). These were all Umatilla dace specimens. Two of these Umatilla dace were also from sites represented by two specimens, and the second Umatilla dace in both cases had complete ITS data with no unambiguous sequences. All other Umatilla dace had unambiguous and complete ITS data (appendix C).

The partial ITS data suggest that there are two groups of speckled dace, five groups of Umatilla dace, and two groups of leopard dace (Fig. 21B). The complete ITS data gives overall

similar results (Fig. 21A) with two differences. The first is a significant separation with high bootstrap percentages for two of the Umatilla dace groups based on the partial ITS data. The second change is three populations make a transition or definitive move in terms of group clustering. These are Umatilla dace from the Boise River, leopard dace from the lower Fraser River, and speckled dace from the Yakima River. This second set of differences are not supported by good bootstrap values even in comparison to the overall lower bootstrap percentages observed for the ITS data. These overall patterns for both ITS data sets do nonetheless remain similar under jackknife data set trials, where sample(s) are deleted and the tree is generated again. The overall groups for the ITS data are thus discussed in terms of the partial ITS information with acknowledgement of the further discrimination present in the complete ITS data set, particularly that more significant differentiation for the two Umatilla dace groups (Fig. 22 and appendix C). The groups for all three dace species are summarized starting at the bottom of the partial ITS tree and are presented on map Figure 22.

The bootstrap values for these ITS groups are all lower than for the cytochrome-b mitochondrial DNA data (Fig. 19) and the D3B 28s ribosomal DNA (Fig. 23), and all still partly distinguish the individual fish in each clustered end group. None of the bootstrap percentages for the discrimination of these groups are above 61 – 76 %. One set of bootstrap levels for the complete ITS are higher and at a level deemed significant (88 and 98%). These partial ITS groups are nonetheless all distinctly clustered and do represent the complete ITS cluster with the high bootstrap values. They also have sufficient significant bp differences (appendices C and D) to suggest their group integrity and their differentiation from other groups.

The Umatilla dace from the Similkameen River have the tightest cluster with the leopard dace group from the Willamette River. The bootstrap values for their within-group differentiation are only 14 and 18 %. These two populations are separated by a substantial distance and latitude, and by the Coastal Mountain range. These Umatilla and leopard dace are thus subsequently treated as one combined group (Fig. 22 and appendices C and D). That group is Umatilla dace Group 1 / leopard dace Group 1. Such combined groups do not imply sympatry between their species. The Umatilla dace from the Similkameen River are also one of the three populations of Umatilla dace that had some minor sequence ambiguity due to multiple nucleotide bases. They are also the most unique Umatilla dace.

The Umatilla dace from the Grande Ronde (fish #1) and Snake (fish #1) rivers that only have partial ITS data (i.e. some sequence ambiguity) cluster together with speckled dace from the Yakima River. Their within-group bootstrap delineation is the more typical here at 65 %. This combined group all occur on the interior side of the Coastal Mountain range. The latter speckled dace and two Umatilla dace are also treated as one combined group called speckled dace Group 2 / Umatilla dace Group 5. The two populations of Umatilla dace are other the two

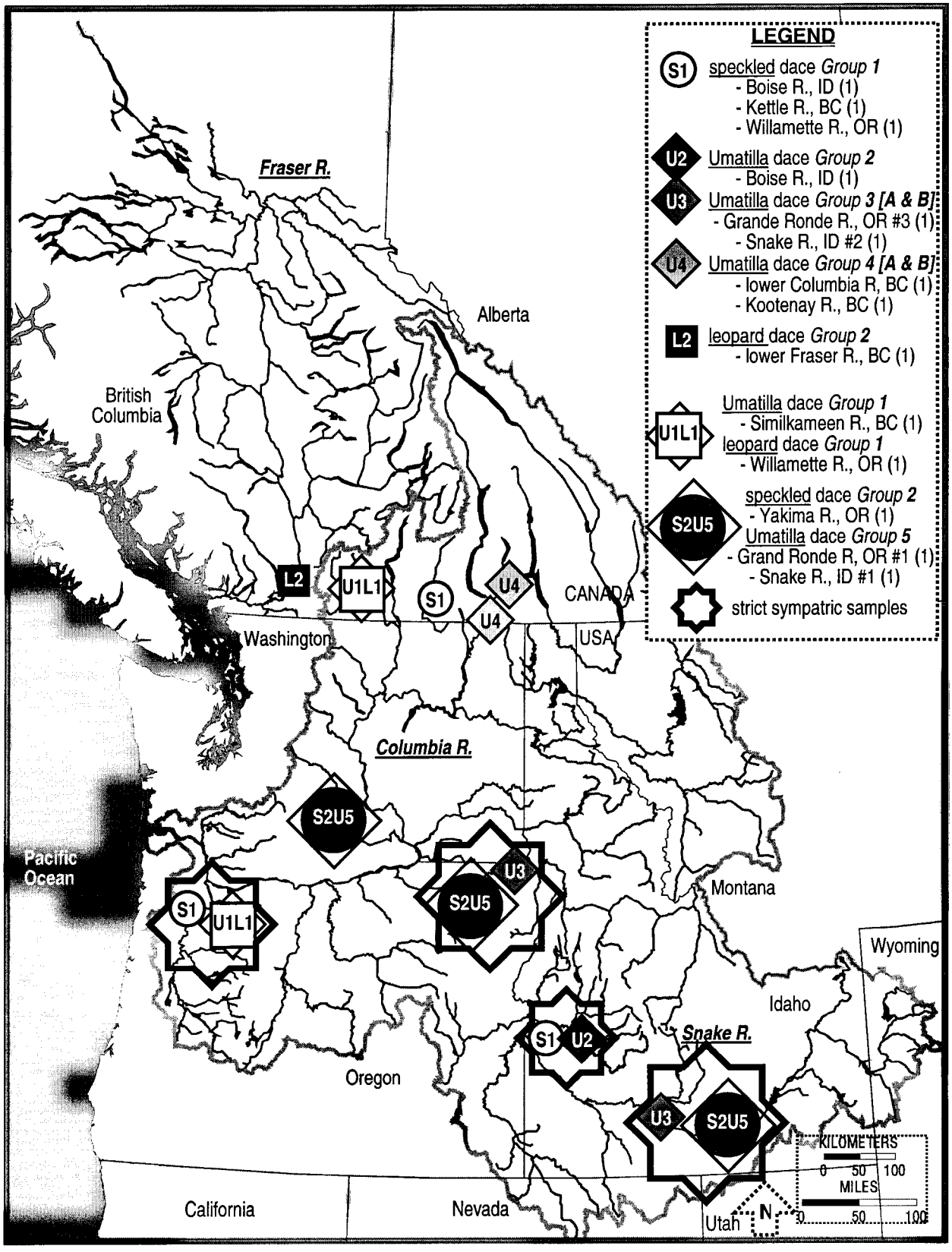


Figure 22. Map of the distribution of DNA sequence groups from the internal transcribed spacer (ITS). This is based on the larger sample size of 14 dace of all three species that had data for the smaller 255 unambiguous base pairs of the partial ITS sequence (Fig. 21B and appendix A). The complete ITS data (Fig. 21A) gave very similar results, but with further separation for two Umatilla dace DNA groups. These are specified here on the legend as DNA subgroups *[A and B]* for Umatilla dace DNA groups 3 and 4.

samples of Umatilla dace that had some minor sequence ambiguity due to multiple nucleotide bases.

These speckled dace from the Yakima River are very and significantly distinct from all other speckled dace using the partial ITS data. They still are somewhat different in the complete ITS data, but nowhere near as much and at greatly reduced bootstrap percentages. The speckled dace from the Yakima River are the first aforementioned population to make a transition or definitive move in terms of group clustering, but one that is does not have strong bootstrap support. These speckled dace are associated with the Umatilla dace using the partial ITS data and with the other speckled dace using the complete ITS data. The Umatilla dace that they are associated with using the partial ITS data are two fish that had minor sequence ambiguity problems.

The two other Umatilla dace from the same Grande Ronde (fish #3) and Snake (fish #2) river sites, but that have complete ITS data, cluster distinctly on the partial ITS tree from the Grande Ronde (fish #1) and Snake (fish # 1) river samples that only have partial data. As a consequence, their Umatilla dace Group 3 only occurs in strict sympatry with the Umatilla dace from the combined group called speckled dace Group 2 and Umatilla dace group 5. Umatilla dace Group 3 is one of the two sets of Umatilla dace that are further separated in the complete ITS data where they show at high bootstrap values. Their bootstrap percentages are also the highest of the reduced value range found in the partial ITS data. However, there again are very few bp changes between these two sample sites in either the partial or complete ITS data and the two populations of Umatilla dace in this Group 3 also both had minor sequence ambiguities due to multiple nucleotide bases.

Umatilla dace Group 4 is represented by the Kootenay and lower Columbia River sites. This group is both geographically isolated from the other Umatilla dace and in the closest proximity to each other as compared to any of the populations or groups for all the three dace species. These sites are both in Canada, and are freely connected with a separation of about 50 km. The two populations are the other set of Umatilla dace that are further separated in the complete ITS data where they show the strongest differentiation based on the highest bootstrap values. Their bootstrap percentages are also at the high end of the reduced value range found in the partial ITS data. Once more, there are very few bp changes between these two sample sites in either the partial or complete ITS data supporting this distinction.

Umatilla dace group 2 is from a single population from the Boise River and clusters in a reasonably distinct position in relation to the other Umatilla dace groups and somewhat in between the groups for the other two dace species. While still more associated with other Umatilla dace populations, it is separated from them across multiple nodes on the tree with one node at least having a reasonably high bootstrap value. When its position is analyzed in the

complete ITS data, it is the second of the three populations that make a transition or definitive move. The Umatilla dace from the Boise River now cluster distinctly with the speckled dace groups using the complete ITS data. This is unlike all the other Umatilla dace. Both these features using the complete ITS data have low bootstrap percentages.

Speckled dace from the Boise, Kettle, and Willamette rivers form speckled dace group 1. These speckled dace are the most spread out geographically in terms of distance, latitude, and longitude. They also occur on both sides of the Coastal Mountain range. These represent all the speckled dace populations with ITS data except for the Yakima River. These speckled dace remain a similar entity if examined using the complete ITS data, and then are much more associated with the speckled dace from the Yakima River. The speckled dace in the Boise River are strictly sympatric with Umatilla dace there represented by Umatilla dace Group 2. Their species separation in the partial and complete ITS data is across more than one node on the tree and has reasonably good bootstrap support.

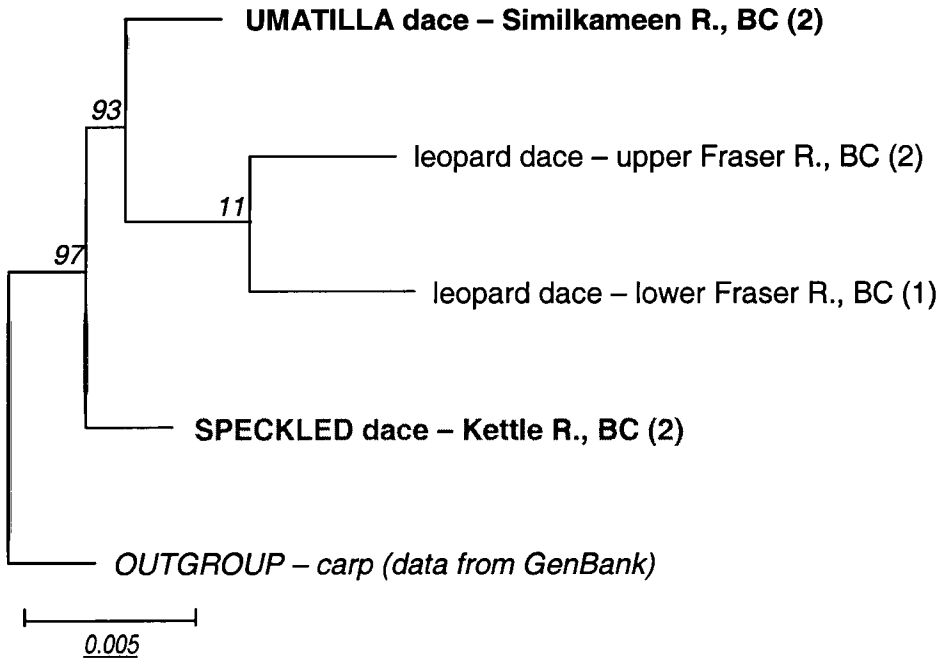
Leopard dace from the lower Fraser River are the only sample examined from a distinct watershed outside the Columbia River basin and only in Canada. They are the only population in leopard dace Group 2. They cluster in a unique and reasonably significant central location similar to, but still separated from, Umatilla dace from the Boise River. They are nonetheless still definitely associated with the three speckled dace populations in speckled dace Group 1, and with the combined group of Umatilla dace from the Similkameen River, and with the other leopard dace population from the Willamette River. The leopard dace from the Fraser River also are the third of the three aforementioned populations to make a transition or definitive move using the complete ITS data. These dace move to be clustered with Umatilla dace in the complete ITS data, but this relationship does not have particularly good bootstrap support.

While the leopard dace from the Fraser River are clearly associated with the only other leopard dace sample from the Willamette River, it is obviously not as closely related to the leopard dace in the Willamette River as are the Umatilla dace from the Similkameen River or all the speckled dace not from the Yakima River. This is in spite of the similar species status between the leopard dace in the Fraser and Willamette rivers, and conversely of the geographic proximity of the leopard dace in the Fraser River to the Umatilla dace in the Similkameen River. The sequence differentiation between these two leopard dace samples is still lower than that seen within either of the other two dace species. At the same time though, the leopard and speckled dace from the Willamette River have as much sequence in common as these two leopard dace samples.

3. Ribosomal Region of the Genomic DNA

The D3B 28s region of ribosomal DNA shows complete separation of the three dace species (Fig. 23). There are two separate sample locations for leopard dace and these are

(A)



(B)

leopard dace	
upper Fraser R., BC	TTGCACGTCA GAATCGCTGC GGTCCTCCAC CAGAGTTTCC
lower Fraser R., BC (1)	....
Umatilla dace	
Similkameen R., BC (2)	.... .G.C...... T.G...... ....
speckled dace	
Kettle R., BC (2)	.... .G.C...... ..G...... ....

leopard dace	
upper Fraser R., BC (2)	TCTGGCTTCA CCTTCGCAAG CATAGTTCAC CATCTTTCGG
lower Fraser R., BC (1)	....
Umatilla dace	
Similkameen R., BC (2)	.........G .G.G.C..G. ....
speckled dace	
Kettle R., BC (2)	.........G ...G.C..G. ....

Figure 23. Neighbour-Joining tree (A) and sequence data (B) for ribosomal DNA of an 80 base pairs segment of the D3B 28s region. The data are for seven dace from 4 samples (appendix A). The data presented for carp are taken from GenBank and used as the outgroup. Carp (*Cyprinus carpio*) are in the same minnow Family Cyprinidae as the dace species. D3B data was not available for longnose dace which were the outgroup in the other DNA analyses. Leopard dace are presented in black font, Umatilla dace in bold black font, and speckled dace in bold dark grey font. Bootstrap values are given in smaller italic black font on the tree nodes. The sample number per site is in parentheses following the locality information. No associated map detailing only the specific distribution of these few samples was made. The general sample site locations are the same as on map Figures 20 and 22, but none of the specific D3B dace specimens were used in any of the other DNA analyses (cytochrome-b mtDNA or ITS).

somewhat distinguished on the tree (Fig. 23A). However, there is no sequence variation between these two leopard dace samples (Fig. 23B) and the bootstrap value that distinguishes these groups is accordingly very low.

Umatilla dace share seven bp with speckled dace, none with leopard dace, and have two unique bp from both these other dace species (Fig. 23B). This population of Umatilla dace, while clearly distinct, is more closely related to this population of speckled dace using this DNA sequence data. This is the opposite relationship to both the cytochrome-b mitochondrial DNA (mtDNA) and the ITS, where Umatilla dace from the Similkameen River are more closely related to leopard dace, albeit not always leopard dace from the Fraser River basin (Figs. 19 – 20 and 21 – 22). None of these specific D3B dace specimens were used in any of these other two DNA sequence analyses. It must be noted though that none of these specific D3B dace specimens were used in any of these other two DNA sequence analyses.

The leopard dace populations differ from the one for speckled dace by seven bp. The amount of sequence variation for a reasonably short 80 bp segment of genomic DNA for three closely related species is quite high. While not directly comparable, the within-species sequence variability found for the D3B region is higher than that for either the cytochrome-mtDNA or the ITS complete data (Table 1 and appendices C and D). This is the case even though the D3B populations are not as geographically disparate and all were completed covered by ice during the last Pleistocene glaciation.

While the discrimination is clear and significant for species status, it is unfortunately not further possible to look at the large within- and between-species variabilities that existed for the cytochrome-b mtDNA and the ITS data since essentially only a single population of each of the species was analyzed here. With particular regard to Umatilla dace, evidence of multiple origins could not be determined.

No associated map was made detailing the specific distribution of these D3B data all from sample sites in Canada. The general sample site locations are the same as for the cytochrome-b mtDNA and ITS data shown on map Figures 20 and 22, and on Figure 16 in Chapter 3. The leopard dace from at least the lower Fraser River site are in a separate watershed but geographically proximal to the Umatilla dace from the Similkameen River. The Similkameen River also formed part of one of two water connections between the Columbia and Fraser rivers at the end of the last Pleistocene glaciation. The speckled dace from the Kettle River are the most geographically distant of these three samples and are completely isolated above a barrier at Cascade, BC from the rest of the Columbia River system.

Discussion

Systematics

The results from all three sequence data sets based on both mitochondrial (mtDNA) and ribosomal DNA support the existence of each of the three dace species, and of longnose dace as well (for general comparisons in Family Cyprinidae see Buth et al. 1991, Child and Solomon 1977, Jenkin and Gold 1992). The genetic data thus mirror and support the species identifications and protocols determined in Chapter 2 with morphometric and meristic data (Campton 1987, Dowling et al. 1989, Meagher and Dowling 1991). The three dace species can be taxonomically summarized for the mtDNA sequence data as having more or at least as much sequence differentiation and species separation in different combinations of strict sympatry than these species have in allopatry. There is no indication that their uniqueness as species is broken down through (further) hybridization or other processes where at least two of the species co-occur (Arnold 1997, Buth et al. 1991, Wilson et al. 1974).

In particular, Umatilla dace warrant status as a distinct species separate from leopard and speckled dace based on the genetic data (Rieseberg and Linder 1999). A key criterion within most species concepts, but particularly within the biological species concept, is that a species must retain its genetic integrity where it co-occurs and could interbreed with other related species (Mayr 1963, 1965; for fish see Nelson and Hart 1999). This criterion is easily met by Umatilla dace, and by the other dace species. Umatilla dace retain their distinctiveness in allopatry as well.

There has been particular interest in Canada as to the uniqueness and conservation status of the three dace species (Cannings and Ptolemy 1998, Haas 1998, Hughes and Peden 1989, Peden 1991, Peden and Hughes 1984). Umatilla dace are differentiated within their distribution in Canada and from other Umatilla dace in the USA. The Umatilla dace in the Similkameen River are particularly distinct. Leopard dace in Canada show some minor differentiation between the upper and lower Fraser River in some of the genetic data, but are discriminated from leopard dace in the Columbia River basin in the USA. The only speckled dace in Canada are in the Kettle and Granby rivers that are isolated from the rest of the Columbia River system by a large barrier at Cascade, BC. These speckled dace show some evidence of meristic distinction from the other speckled dace in the Columbia River drainage (Chapter 2; also see Peden and Hughes 1984). However, there is no support from any of the three types of DNA sequences examined here that these two river systems are differentiated or notable.

Cytochrome-b Region of the Mitochondrial DNA (mtDNA)

The sequence of this cytochrome-b region of the mitochondrial DNA (mtDNA) subdivides the three dace species into clusters of genetic groups with some sequence differentiation

between them. The allopatric samples and groups show less overall sequence differentiation within-species than between-species (Campton 1987).

The minor exception to this general pattern of increased between species variation occurs within the Yakima River, which clearly is a unique system and also is the only site of sympatry for all three dace species. The speckled and Umatilla dace in the Yakima River are far more respectively differentiated from all other speckled and Umatilla dace populations than from each other. While leopard dace in the Yakima River are also strongly distinct from its speckled and Umatilla dace, they remain more similar to other leopard dace populations. While the Yakima River is a distinctive system in which the overall pattern of reduced within-species differentiation does not quite hold for Umatilla and speckled dace, the three dace species in the Yakima River are still as or better differentiated than anywhere else. These good or increased species' distinctions within the Yakima River also exist under conditions of their co-occurrence. Longnose dace are also much more differentiated between-species where they occur with the three other dace species in the Yakima River than they are between the two populations examined. Longnose dace are also by far the most unique overall of these four dace species when compared to Umatilla, leopard, and speckled dace without or only within the Yakima River (also see Becker 1962, Bisson and Reimers 1977, Child and Solomon 1977).

The other site of strict sympatry is the Willamette River where leopard dace co-occur with two genetic types of speckled dace. The leopard dace are quite distinct even though they are being compared to both types of speckled dace at that location. The leopard and speckled dace from the Willamette River are both respectively more closely clustered with other leopard and speckled dace populations. Their between-species rather than within-species sequence differentiation is much higher for leopard dace and at similar levels for speckled dace. This comparison does now again not include the Yakima River. Leopard and speckled dace in sympatry in the Willamette River thus also retain their uniqueness as species, and at enhanced levels for leopard dace.

Another possible, but somewhat 'reversed', test for assessing species status is an examination of co-occurring sequences and not necessarily species or populations. This would be a situation where two or more dace species from geographically disparate locales have essentially identical mtDNA (Arnold 1997, Buth et al. 1991, Martin and Simon 1990). Such data would imply that each of these populations of dace species has independently evolved the same sequences in this mtDNA segment while still maintaining their distinct identities. Umatilla dace from the Similkameen River essentially have the same cytochrome-b mtDNA sequence to leopard dace from the Fraser and Yakima rivers. These locations are on opposite sides of the Coastal Mountain range in western North America and in completely distinct drainage basins. Yet, these two dace species in these three locations are clearly differentiated using

morphometric, meristic (Chapters 2 and 3) and some DNA data from ITS. The leopard dace from the Fraser River and the Umatilla dace from the Similkameen River have also demonstrated their species distinctiveness through tests of their ecological preferences and tolerances to water flow regimes (Chapters 5 and 9) and through controlled laboratory cross rearing and viability experiments (Chapters 6 and 8).

Internal Transcribed Spacer (ITS)

As with the mtDNA, the DNA sequences of both the internal transcribed spacer data sets subdivides the three dace species into clusters of genetic groups with some sequence differentiation between them. Where the sample localities of these subgroups are the same in both the ITS and mtDNA data, there is strong congruence for their overall patterns of association. For both ITS and mtDNA data, each of these recognized groups of the three dace species are further not all strongly related to geographic proximity, barrier or watershed boundaries. However, their between-group differentiation is structured that way to some degree (Buth et al. 1991, DeMarais et al. 1992, Dowling et al. 1989, Dowling and Hoeh 1991). The groups of the three dace species are each somewhat more discrete and geographically structured, particularly with respect to latitude, in the ITS data. The allopatric samples and groups also continue to show less overall sequence differentiation within-species than between-species too.

Two of the three dace species furthermore continue to form discrete overall clusters and groups in an examination of both entire ITS data sets, while the third does not for seemingly explicable reasons. Umatilla and speckled dace are each discrete species overall in the complete ITS data, and also in the partial ITS data with the respective exceptions of the Similkameen and Yakima rivers. These two river systems have consistently been different in the mtDNA data as well. The two leopard dace samples are together much less discrete a clustered entity in the ITS data, but they also are the only species sampled here from the two distant populations in entirely separate watersheds, the Columbia and Fraser rivers.
The distinct situation in the Yakima River is again seen here as such, but just in the partial ITS data and not as uniquely as in the mtDNA data. In the partial ITS data, only speckled dace in the Yakima River did not have such substantial problems with DNA sequence length variation as to render it illegible. These speckled dace in the Yakima River are very distinct from the other speckled dace, but unlike with the mtDNA these speckled dace are not uniquely placed by themselves but rather with Umatilla dace from the Grande Ronde and Snake rivers. In the complete ITS data, the speckled dace in the Yakima River are well associated with the other speckled dace populations. In the Yakima River, it is also Umatilla and not leopard dace that are most closely related to these speckled dace that is the only dace species there to have a stable ITS sequence pattern.

There are two sites of strict interspecific sympatry and two sites of strict intraspecific sympatry. The two strict interspecific sympatric species and sites are leopard dace and speckled dace (also now one fish) in the Willamette River, and Umatilla and speckled dace in the Boise River. The two interspecific sympatric sites represent co-existence between some combinations of all three dace species and in both situations each dace species maintains its distinctiveness. The two strict intraspecific sites are for Umatilla dace in the Grande Ronde and Snake rivers.

In the partial ITS data for strict interspecific sympatry in the Willamette River, the speckled dace are clustered and somewhat more associated with the other speckled dace samples, but there nonetheless remains some overall relationship between the two species at this site. The speckled dace from the Willamette River are definitely somewhat discrete and related to speckled dace overall, but the leopard dace are not. However, the two populations of leopard dace are again those from distant populations in the completely separate Columbia and Fraser river systems. The stronger between-species than within-species relationship of leopard dace in the Willamette River is confirmed and more definitive in the complete ITS data. The speckled dace are also now more closely clustered with the leopard dace from the Willamette River. The DNA sequences and cluster of these leopard and speckled dace in the complete ITS data does also still differentiate these two species. Overall, leopard and speckled dace in sympatry in the Willamette River thus retain their uniqueness as species, but not as strongly as in the mtDNA data.

In the partial ITS data for strict interspecific sympatry, the speckled and Umatilla dace in the Boise River are both more clearly related to their respective species than was the case in the Willamette River. In the complete ITS data, the Umatilla dace are still very discrete from the speckled dace in the Boise River. However, the Umatilla dace in the Boise River are more associated, albeit with weak support, to speckled dace overall than to the other Umatilla dace populations. Overall, speckled and Umatilla dace in the Boise River thus strongly maintain their species distinctiveness in strict sympatry, and more so than leopard and speckled dace did in strict sympatry in the Willamette River.

The two strict intraspecific sympatric populations in the Grande Ronde and Snake rivers each involve the subdivision of Umatilla dace. Umatilla dace at each of these locations are clearly differentiated into two groups, but only for the partial ITS data. Their second set of individual dace specimens showed sufficient ambiguous DNA sites due to minor variation from multiple nucleotide bases to not provide enough readable sequences for the complete ITS data. The individual dace specimens from the Grande Ronde and Snake rivers that did have both partial and complete data show similar relationships in both the partial and complete ITS data, but are more associated with each other in the partial ITS data. Both sets of individual specimens of

Umatilla dace from these two sites thus each separately show their populations to be strongly related as discrete groups, but these both also remain within the overall distinct grouping of Umatilla dace that is largely separated from leopard and speckled dace. As well, there is some support for intraspecific variability in Umatilla dace at both these locations.

In the partial ITS data only, there are two sets of co-occurring sequences between geographically distinct populations of some combinations of all three dace species. This implies that each of these dace species has independently evolved very similar sequences in this partial ITS segment while still maintaining their distinct identities (Arnold 1997, Avise et al. 1975, Buth et al. 1991, Martin and Simon 1990). These independent evolutions also are all geographically distant within the two sets of locations where this DNA sequence similarity has occurred (DeMarais et al. 1992, Dowling and Hoeh 1991). In the first instance, Umatilla dace from the Similkameen River have almost the same partial ITS sequence to leopard dace from the Yakima River. Their locations are quite latitudinally distant, on opposite sides of the Coastal Mountain range in western North America, and respectively drain into the Columbia River from a north and south direction. In the second case, Umatilla dace from the Grande Ronde and Snake rivers have very similar partial ITS sequence to speckled dace from the Yakima River. These locations are again quite geographically distant between their most disparate two populations. As well, the Yakima River is a direct Columbia River tributary draining from a northwesterly direction whereas the Grande Ronde is southwesterly tributary to the Snake River which is the major, and a more southeasterly, tributary to the Columbia River itself. In spite of all this, each pair of dace species within the two sets of locations where this DNA sequence similarity has occurred remain clearly differentiated on the basis of morphometric, meristic and some mtDNA data (Chapters 2 and 3). The Umatilla dace from the Similkameen River have also demonstrated their species distinctiveness through tests of their ecological preferences and tolerances to water flow regimes (Chapters 5 and 9) and through controlled laboratory cross rearing and viability experiments (Chapters 6 and 8).

Umatilla dace might also be considered to be overall related as a species through the levels of minor ambiguity and significant length variation seen in a few of the ITS DNA sequences. Umatilla dace are the only dace species to show the minor, but still legible, partial ITS sequence ambiguities that resulted from nucleotide substitutions. Similarly, Umatilla dace compose the majority of specimens that had such substantial ITS sequence problems with length variation that their sequences were rendered unreadable. The only other one of the three dace species that had illegible ITS sequence difficulties was a leopard dace. That leopard dace, and one longnose dace that had the same sequence problem, both also came from the particularly and consistently distinct Yakima River. The uniqueness of the Yakima River may be at least partly

and similarly explained by these same "heteroplasmies" since only its speckled dace sequences were not completely unreadable due to major length variations.

Ribosomal Region of the Genomic DNA

The D3B 28s region of ribosomal DNA shows complete separation of the three dace species (Fig. 23). These patterns of species distinction are congruent with those for the mtDNA and ITS data. Umatilla dace are more like speckled than leopard dace, but remain unique in their D3B DNA sequence. The leopard dace are the most differentiated of the three dace species. While the discrimination is clear and significant for species status, it is unfortunately not possible to further look at within- and between-species variabilities since essentially only a single population of each of the species was examined here.

Evolution

Umatilla dace not only show strong and definite evidence of differentiation from leopard and speckled dace, but also of multiple origins involving the hybridization of these latter two dace species (Martin and Simon 1990, Mukai et al. 1997, Taylor and Bentzen 1993). These multiple and hybrid origins are consistent with the hypothesis here for their role in the speciation and evolution of Umatilla dace (Arnold 1997, Buth et al. 1991, DeMarais et al. 1992, Dowling et al. 1989). Support for these interpretations comes from both mtDNA and nuclear DNA sequence data. The discordancies between the mtDNA and nuclear DNA data seen here are also often discussed as the most typical and reliable evidence for historical hybridization between two species (Arnold 1997, Dowling et al. 1989, Forbes and Allendorf 1991, Normak and Lanteri 1998).

In the mtDNA data, the groups of Umatilla dace are more disparately clustered and not found in as discrete entities as the other two species. Only Umatilla dace have groups that each cluster and have sequence patterns associated more closely with the overall grouping of speckled or leopard. The recognized groups of Umatilla dace are always more closely related to either speckled or leopard dace (DeMarais et al. 1992, Dowling and Hoeh 1991, Dowling et al. 1989, Martin and Simon 1990). This is supported by the D3B ribosomal DNA sequences as well. These patterns are the case only without collectively examining the three dace species from the Yakima River, but they nonetheless hold within the Yakima River too. In the Yakima River, Umatilla and speckled dace are more closely related with leopard dace being very differentiated from both other dace species. The salmon (*Oncorhynchus* spp.) species in the Yakima River are well and much better studied and also largely considered somewhat distinct.

The recognized groupings of Umatilla dace, except in the Similkameen River, occupy an intermediate position between the overall species groupings for speckled and leopard dace (DeMarais et al. 1992). Intraspecific mtDNA sequence differences between populations of Umatilla dace are also larger than for leopard or speckled dace, except as aforementioned for

the Yakima River (Arnold 1997). Leopard dace are the most cohesive overall species in the mtDNA data. Furthermore, Umatilla dace retain about the same level of within-species sequence variation between their populations in the mtDNA data at allopatric and strict sympatric sample sites. The leopard and speckled dace populations are more distinctive in strict sympatry showing reduced within-species mtDNA sequence variation.

Umatilla dace even have an intermediate number of three recognized groups based on 15 fish from six populations whereas leopard dace have two groups based on nine fish from four populations and speckled dace have four groups based on ten fish from nine populations. This latter intermediate group number could easily be a function of the sample size and population number analyzed, but these data for this data set do not support that easy dismissal.

In the ITS data, there again are strong, but not identical, indications of past multiple hybridizations in Umatilla dace. In some instances like the Yakima River, there is evidence of considerable hybridization, which includes leopard dace and interestingly even longnose dace at this one unique location. Even at this Yakima River site, there further are still no good signs of very recent or ongoing hybridization. Speckled and leopard dace also coexist under present natural conditions in the Willamette River without evidence of ongoing hybridization.

More indications of past hybridization in Umatilla dace are that the two strict sympatric sites of intraspecific variation in the partial ITS data set both involve Umatilla dace (Hillis and Dixon 1991). Similarly, the strict sympatric location in the Boise River has Umatilla dace somewhat shifting their species association, with it being more to speckled dace in the complete ITS data and more to Umatilla dace in the partial ITS data. The strictly sympatric speckled dace from the Boise River do not alter their specific or overall relationship to the other speckled dace populations in either ITS data set.

Umatilla dace also represent all but one of the ITS sequences that were illegible due to major length variation and all the partial ITS sequences that gave some ambiguity resulting from more minor nucleotide substitutions. Furthermore, within-species sequence variation is in general and overall highest for Umatilla dace, followed by non-Yakima River speckled dace, and then leopard dace. There is again more than one distinct recognizable group of Umatilla dace which are once more usually associated more strongly with either leopard or speckled dace.

A summation of these nuclear DNA data also run somewhat counter to the general theoretical expectations of concerted evolution (Arnheim 1983, Buth et al. 1991, Hillis and Dixon 1991). Multiple copies of ribosomal RNA are thought to be evolving in concert and not independently, and that this process of concerted evolution keeps these copies more or less homogenous. If there are differences between individuals, these are supposed to be sequence length variations within the non-transcribed spacer (NTS) regions, which were not examined

here. However, differences and sequence length variations do still exist between individuals in this study for regions outside the NTS, and they do so here largely for, and most strongly in, Umatilla dace.

Chapter FIVE

Ecology - Water Flow Data

Materials and Methods

Flow Preference Data - 'Wild'

Water velocity measurements were taken for each of the three dace species in the 'wild', meaning under natural conditions in the streams. This was done to assess any preferential site selection by individuals of each dace species based on water flow conditions. The data were all collected in Canada, and are from 60 speckled dace (*Rhinichthys osculus*) from four allopatric sites, 57 Umatilla dace (*R. o. umatilla*) from five allopatric sites, and 56 leopard dace (*R. falcatus*) from four allopatric sites (appendix A). Each allopatric site consisted of at least ten flow measurements. The DNA sequence data for the multiple allopatric flow sites for any one species indicated that the dace from them were identical (Chapter 4).

The sampling protocol was identical for all of the three dace species. Data collection was over three consecutive years and necessarily all undertaken in the fall when water flows were lowest. This was the only time when these streams and dace could be accurately, safely, and reasonably abundantly sampled in the 'wild'. All electrofishing and fish spotting was done while wearing polaroid glasses to enhance the accuracy of locating the dace. All sampling and data collection was also done only by me to ensure the same methodology was consistently followed. All measurements are biased to the minimal extent that this work could only take place in water no deeper than the top of rubber chest waders necessarily worn for the electrofishing work.

Allopatric sites were electrofished to specifically and accurately locate the exact benthic position from which individual dace were 'turned over' by their inherent attraction to the electric field. Flow measurements were then immediately made at those precise positions. In some later instances, a rock that had been spray-painted a highly visible blaze orange was placed at the exact location and the flow measurements were made there at the end of determining a sufficient number of specific dace locations. The rocks were picked up after the measurements had been completed.

It would have been desirable to collect a body size (length) measurement for each of these individual dace sampled to assess any possible impact on flow preferences, but this was not feasible. The main rationale was that concentration on spotting the exact benthic locations of each dace was deemed most important. The collection of a body size measure would have additionally involved trying to catch each particular dace and this was felt to likely jeopardize its precise benthic placement. This was particularly true since the majority of the flow

measurements taken in the 'wild' were done while working alone. The collection of yet more data would have been quite difficult. Size was qualitatively assessed during this field work and no obvious effect was noticed. All fish on which flow data was collected also were mature individuals and probably fairly similar in length as a result, as was determined in the laboratory component of this study. Only the youngest, likely just hatched, dace were sometimes found in slow water on edges of the main stream channels, with all other older life-history stages being found in similar habitats to that studied here.

The flow data was collected with a Marsh McBirney model 2000 flow meter. This type of flow meter works on electromagnetic principles and is thus more highly accurate and less influenced by small-scale localized turbulent or non-laminar flow conditions than traditional propeller-type flow meters. The flow meter was calibrated by the manufacturer. The flow meter was set to provide one final flow measurement data point based on an average of 12 taken over the period of one minute (i.e. mean of flow measurements taken every five seconds for one minute). The flows were taken to an accuracy of one cm/sec water velocity.

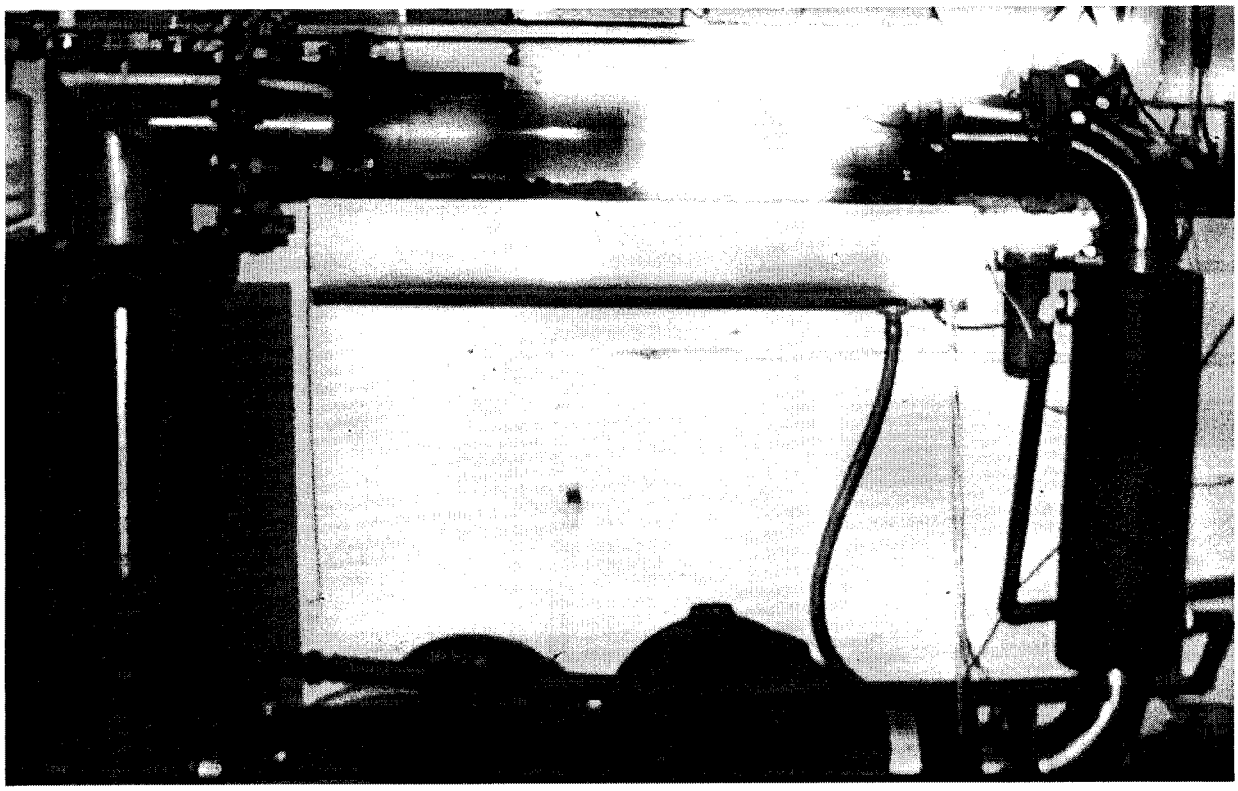
Two sets of flow measurements were collected at the exact benthic locations of each dace individual. Water flow at 60 % mean depth was taken because it is a common standard in fisheries work, and is often used to establish legal and regulatory flow conditions. Water flow at the bottom of the stream was collected since dace are a benthic fish and that is where all sampled individuals were found. The benthic measurement was presumed to be more biologically realistic. Total depth of each sample location was also measured.

The bottom flow measurement was made as near to the actual surface of the stream bottom as possible, but the sensor for the flow meter was necessarily attached to a measuring rod that usually placed the sensor very slightly (two to five cm) above the true bottom. The difficulty of assessing the actual surface of the stream bottom was directly related to any increase in size of the substrate being sampled. If the substrate consisted of larger materials such as boulders then the difficulty of both locating the precise position of the dace and the bottom was increased. Each situation was handled as best as possible within the limitations of such field work.

The water flows at the bottom and at 60% mean depth are visually compared using linear least-squares regression. This is to establish that a valid difference exists between the flow measurements taken at these two depths under natural conditions, and also to compare them to the results from the experimental flow tube which sets up a more artificial but fully controlled laboratory test environment.

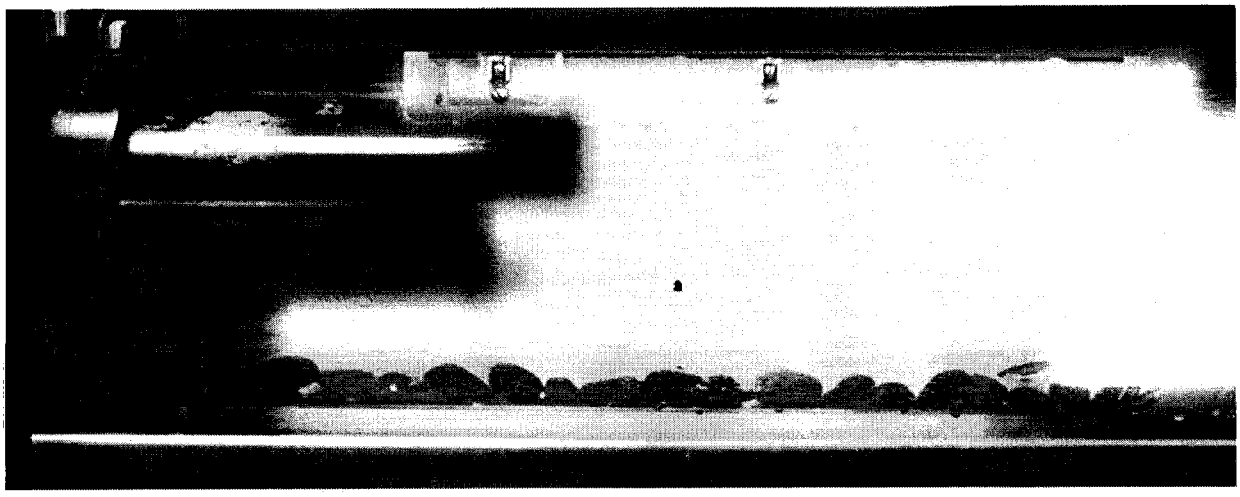
Flow Tolerance Data - Laboratory

Controlled tests of water velocity tolerances of each of the three dace species were undertaken in the closed system recirculating flow tube (Fig. 24) belonging to Dr. D. Randall's



(A) Experimental Flow Tube

- fish sit in enclosed clear plexiglass cylinder chamber
- note - coarse gravel bottom for dace
  - net and then mildly electrified screen at back
- water circulates clockwise



(B) Enclosed Plexiglass Cylinder Chamber

- note - 60mm dace on bottom at back near net
  - mildly electrified screen not visible behind net
  - coarse gravel substrate for dace
- water circulates left-to-right

Figure 24. Photographs of a lateral view of the entire experimental flow tube (A) and a closeup of its enclosed plexiglass cylinder chamber (B) with the substrate modifications for dace.

laboratory at UBC. This was done to assess any differences between individuals of these species in their ability to hold their benthic positions under regular increased water velocities that reflected the natural water flow data regime collected in the 'wild'. The type of flow preference data collected in the 'wild' could not be readily evaluated with this apparatus. Depth measurements were also not taken since they would not be valid.

The laboratory data were all collected on mature dace reared from crosses of allopatric parentals of each species. These laboratory dace were kept under identical and no water flow conditions, and thus had no prior exposure or predilection to water velocity. Their body lengths were similar and ranged from about 50 to 70 mm. The parentals came from the same allopatric sites in Canada that the flow measurements were made in the 'wild'. The data were from 50 speckled dace from two allopatric sites, 50 Umatilla dace from one allopatric sites, and 50 leopard dace from two allopatric sites. No fish was used more than once. The parental collection sites for speckled dace was the Kettle River, for Umatilla dace was the Similkameen River, and for leopard was the Nicola / Coldwater and Fraser rivers (Fig. 16 in Chapter 3; appendix A). A complete description and discussion of the details, methodology, and protocol for the data and the making and rearing of the crosses is available in the 'Materials and Methods' section of Chapter 6.

Since a length measurement was easily collected on the laboratory fish, their flow tolerances could be adjusted for any effects of body size. This was probably not critical for the laboratory fish since most were of similar size due to their common rearing conditions and maturity status. Proper standardization to size is still desirable if possible though as it makes full comparisons more realistic. Such standardization also did not matter for purposes of comparison to 'wild' data collected under natural stream conditions because that field data was for flow preferences and not tolerances. Only the overall flow patterns and trends are compared between the 'wild' and laboratory.

A standard methodology for size correction is conversion of the laboratory water flow data to 'critical holding velocity' (CHV). CHV is calculated, in terms of body lengths per second based on the fish sizes, as follows:

$CHV = Vi + (t_i / t_{ii} \times V_{ii})$, where: V_i = highest water velocity tolerance;

V_{ii} = water velocity increments;

t_i = time (mins.) each fish held at highest water velocity;

t_{ii} = time period at each velocity increment (15 mins).

The curved concave bottom of the inside of the plexiglass cylinder on the flow tube was not a suitable surface on which to test the flow tolerances of dace since they are strongly benthically and substrate oriented. Small coarse gravel was epoxied to a sheet of coarse grit sandpaper that was then placed on the bottom of the experimental chamber of the flow tube

(Fig. 24). This gave a standardized and more realistic benthic surface that could be removed and replaced without alteration. A series of short flow tube trials with dace not used in the actual flow tolerance experiments established that this artificial substrate surface did not provide any large or overt flow refugia that might have biased the results. This is why gravel rather than a larger substrate component was originally selected. These short trials also demonstrated that the entire artificial substrate would remain in place at flows sufficient to test the flow tolerances of these dace.

Water is automatically re-oxygenated in the flow tube. Water temperature could be fully controlled in the flow tube and was set to 18 °C. This was the same water temperature that the dace were being maintained at in the laboratory. This temperature was largely selected because it was that at which the dace in the laboratory came into their best reproductive condition and it reflected those temperatures found under natural conditions. This was felt to represent the temperature at which much natural spawning might take place and at which the most critical habitat segregation of the three dace in terms of species evolution would occur. Water velocity is a reasonable surrogate for overall habitat in this case. The segregation of the three dace species by different flow tolerances at spawning time permitted a further assessment of their species statuses under the criterion of coexistence in the biological species concept (Mayr 1963, 1969, Nelson and Hart 1999) largely being employed here (see Chapters 2 and 3). The other reason for selecting 18 °C as the temperature for these experiments is that the dace were simply most active so probably would have the best opportunity to exhibit realistic flow tolerances (Cunjak and Power 1986, Haas unpubl. data).

The protocol for testing in the laboratory flow tube was identical for all the three dace species. The experimental flow tube trials were run on ten dace of one species at a time. Each complete trial on ten fish took approximately three to four hours. All trials were conducted over a two week period to avoid any changes in the fish. Each set of ten dace were not fed for 24 hours prior to their experimental trial in order to avoid affecting their overall body shape, largely through the ventral distension associated and noted with eating. These 10 were first acclimated to the flow tube apparatus and my presence by placing them in the flow tube at no flow for one hour with me seated next to it and them for that time. This seemed to work and be adequate since the dace were all benthically positioned and had ceased to swim pelagically as they usually did when first placed into the flow tube.

The water flow in the flow tube was then turned on to its lowest setting which was about 0.17 cm/sec at the bottom and 0.22 cm/sec at 60% mean depth. The was increased by one incremental flow tube setting every 15 minutes for the rest of the experimental trial. This meant an increase every 15 minutes in benthic flow ranging from 0.06 to 0.12 cm/sec (mean = 0.1 cm/sec) and in flow at 60% mean depth from 0.04 to 0.08 cm/sec (mean = 0.06 cm/sec). The

reason for the minor variability in flow increases was it is regulated by incremental settings that cannot be adjusted. The range of the means of the incremental flow increases is roughly equivalent to 0.5 body length per second. The actual flows at the bottom and at 60% mean depth were taken with the same flow meter as used and discussed for the natural 'wild' data.

The flow tube works by having an enclosed experimental chamber that has a net followed by a mildly electrified screen at the back (Fig. 24). Dace eventually come onto the net as they are moved out of their benthic holding positions by increased water flows and fatigue. The mild electric current behind the net is uncomfortable and repels the dace. The dace attempts to avoid the net and electric current and re-establish its holding position. Eventually the dace fatigue and can no longer avoid the net and remains pushed onto it by the water current. My experimental protocol involved waiting for five seconds to see if the dace could come off the net on its own. If it could not, then the fish was gently prodded three times with a rubber tipped probe at five second intervals to see if it could be so coaxed off the net. The dace was given another five seconds to come off the net after the final third prodding. If the dace could not come off the net at this point, it was taken out of the flow tube with as minimal disturbance and movement as possible so as not to affect the other dace still in the experimental trial. The removed dace was then scored for its final flow increment tolerance and for amount of time (minutes) spent in it, and was also measured for length and wet weighed prior to placement in a recovery tank with no flow.

There was some minor bias noted at the higher flows in these experiments. It was more difficult to gently prod and remove dace from the net at the back of the flow tube under high water velocities. This very occasionally spooked another dace out of its holding position and onto the net. Once that other dace was unintentionally displaced it was very difficult for it to re-establish its holding position at such high water velocities. Sometimes this unintentionally displaced dace could not get back into a holding position and had to be prematurely removed from the experiment. These instances were infrequent, but most involved leopard dace since they had the highest flow tolerances of these three species. Leopard dace were also found to be the most skittish and more susceptible to accidental displacement. The leopard dace results may thus be somewhat downward biased in terms of flow tolerance.

The water flows at the bottom and at 60% mean depth are compared using linear least-squares regression. This is to establish whether a valid difference exists between the flow measurements taken at these two depths in the more artificial environment of the experimental flow tube, and also to compare them to the results taken in 'wild' natural streams.

Results

Flow Preference Data - 'Wild'

All the following differences in flow preferences between each dace species are statistically significant (Figs. 25A-B). Leopard dace had the highest flow preferences at bottom and 60% mean depths in 'wild' conditions in natural streams. Their median bottom flow was 0.2 cm/sec and median 60% mean depth flow was about 0.45 cm/sec. Speckled dace had the lowest flow preferences ranging from a median bottom flow of approximately 0.03 cm/sec to a median flow at 60% mean depth of 0.15 cm/sec. Umatilla dace were intermediate to speckled and leopard dace in flow preference, with their median for bottom flow at about 0.11 cm/sec and their median 60% mean depth flow at 0.25 cm/sec. Umatilla dace have flow preferences at both these depths that overlap more with leopard than speckled dace.

Hughes and Peden (1989), Peden (1991), and Peden and Hughes (1988) have speculated that Umatilla dace prefer faster water to leopard dace, but not on the basis of any rigorous or specific data or tests. Furthermore, their identifications of leopard dace on which these comparative speculations are based are suspect or at least unconfirmed (Chapter 3).

The relationship between water velocity at the bottom and at 60% mean depth of natural streams is correlative but not completely tight (Fig. 25C; $r = 0.73$). This suggests there likely are real differences in water flow in the 'wild' at the bottom and at 60% mean depth of natural streams. These two water flow data are clearly related and correlated, but the actual water flow preference readings are smaller at the bottom, and this difference is statistically significant. The reduced flow at the bottom makes intuitive sense since there is frictional and laminar reduction of water velocity at the surface of the bottom substrate. Also, the majority of fluvial fish are recognized to hold positions near the bottom if any real water flows are present in the stream, likely to minimize their energy expenditure.

Speckled dace preferred the shallowest water depths, and only their depth preferences are statistically significantly different from Umatilla and leopard dace (Fig. 25D). The median depth preference for speckled dace is about 30 cm. Umatilla dace have depth preferences that overlap more with leopard than speckled dace. Umatilla dace have a higher data quartile range for depth preference, but leopard dace have a higher full data range. These slight differences between Umatilla and leopard dace are not statistically significant. Their median values for depth preference are almost identical at about 50 cm. If their depth preferences are only viewed as tendencies or trends, then Umatilla dace overall prefers slightly deeper water than leopard dace, but leopard dace can be found in both deeper and shallower water (for leopard dace also see Gee and Northcote 1963).

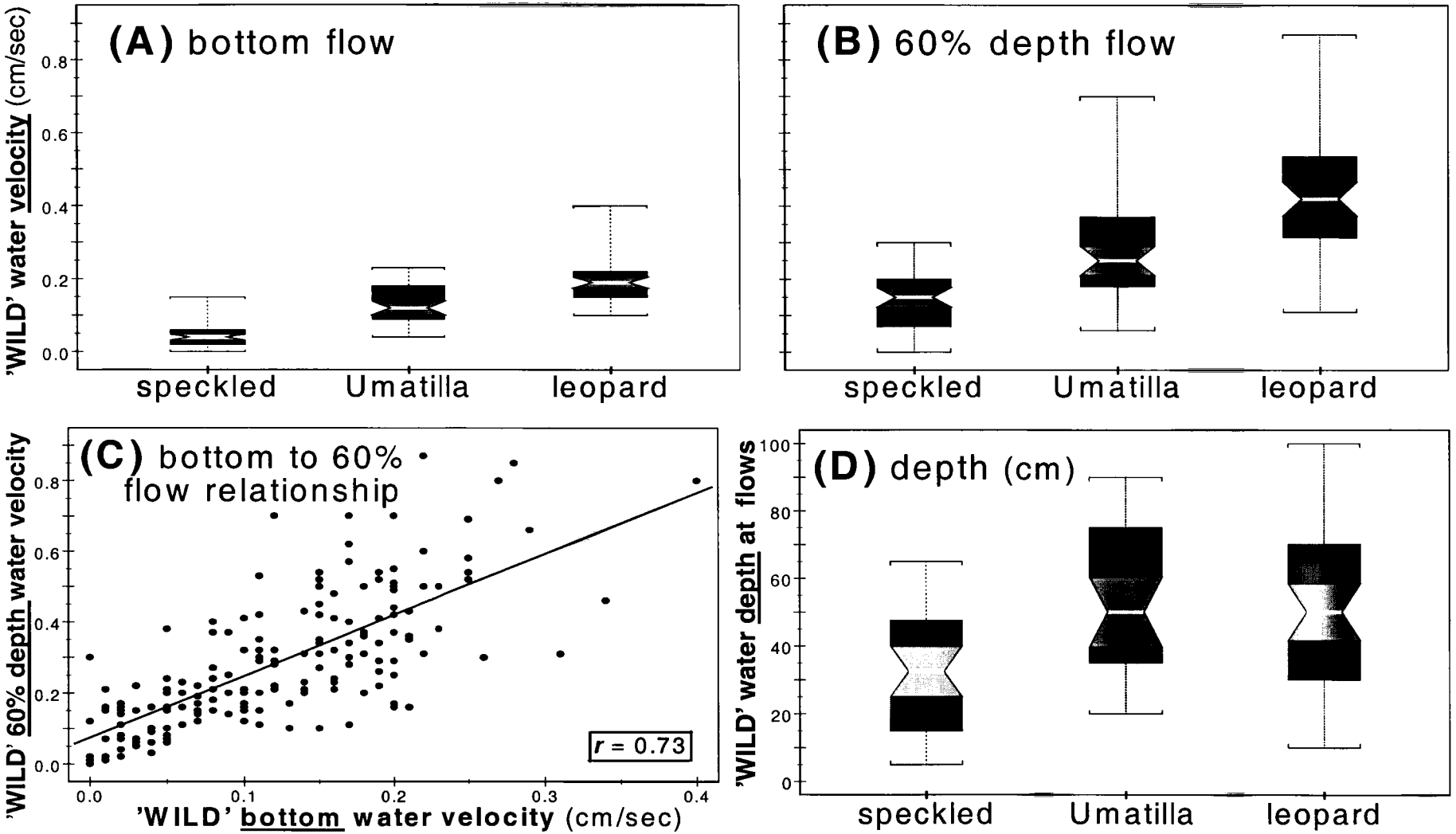


Figure 25. Boxplots of water velocity (A-B) and depth (D) where individuals of each species were 'precisely' found in the wild in allopatry. Boxplot (A) is for flow at the stream bottom and boxplot (B) is for the common fisheries standard 60% mean depth. Scatterplot (C) is the relationship between the natural stream flows at these two depths (A and B). That relationship is fit by linear least-squares regression. These data are from 60 speckled dace from four allopatric sites, 57 Umatilla from five allopatric sites, and 56 leopard from four allopatric sites (appendix A). For the boxplots, the horizontal line in each box is each species' data median, the boxes are their data quartiles, and the vertical lines from each box are their respective full data ranges. Nonoverlapping grey notched areas between species' boxes indicate statistically significant differences at a rough 5% (Chambers et al. 1983).

Flow Tolerance Data - Laboratory

Speckled dace have the lowest water flow tolerance at bottom and 60% mean depths in the laboratory experimental flow tube tests (Fig. 26A-B). They have a median bottom flow tolerance of approximately 0.4 cm/sec and a similar median flow tolerance at 60% mean depth of 0.45 cm/sec. Only these water flow tolerances for speckled dace are different at a statistically significant level from Umatilla and leopard dace. The differences in flow tolerance between leopard and Umatilla dace are slight and not statistically significant. The tendencies and trends of the laboratory flow tolerance data for Umatilla dace nonetheless remain intermediate to speckled and leopard dace. Umatilla dace have similar median values for flow tolerance that are consistently lower than leopard dace by about 0.05 cm/sec. Leopard dace still have higher values for flow tolerance with their median bottom flow tolerance about 0.68 cm/sec and median 60% mean depth flow about 0.75 cm/sec. The actual slightly reduced medians for Umatilla dace at bottom flow are about 0.65 cm/sec and at 60% mean depth flow are 0.70 cm/sec. Leopard dace are also found in a larger range of flow tolerances than Umatilla dace. Leopard dace exist in faster water ranges at bottom flows, and in both faster and slower water ranges at 60% mean depth. These overall patterns and trends of flow tolerance for the three dace species match the flow preference data taken in the 'wild' under natural conditions.

The relationship between water velocity at the bottom and at 60% mean depth in the flow tube is very strong (Fig. 26C; $r = 0.99$), and much more so than that found under natural stream conditions (Fig. 25C; $r = 0.73$). This suggests that, unlike in the 'wild', there are no real or statistically significant differences in water flow at the bottom and at 60% mean depth in the artificial environment of a completely round and narrow experimental flow tube. Only a very small, yet consistent, difference of about 0.05 cm/sec exists in terms of flow tolerances in the flow tube for the three dace species between water velocity at its bottom and at its 60% mean depth. Natural streams have higher water velocities at 60% mean depth too (Fig. 25A-B), but their flow differences between bottom and 60% mean depth are much more substantial and statistically significant. These differences between the flow tube and natural measurements must also at least partly be the result of the flow tube being of limited diameter and being simply somewhat less realistic. The slightly reduced flow at the bottom of the flow tube still makes intuitive sense since there is frictional and laminar reduction of water velocity at the surface of the bottom substrate. Dace also continued to hold position on the bottom in the flow tolerance experiments likely due to this reduced flow, but also probably from natural tendency.

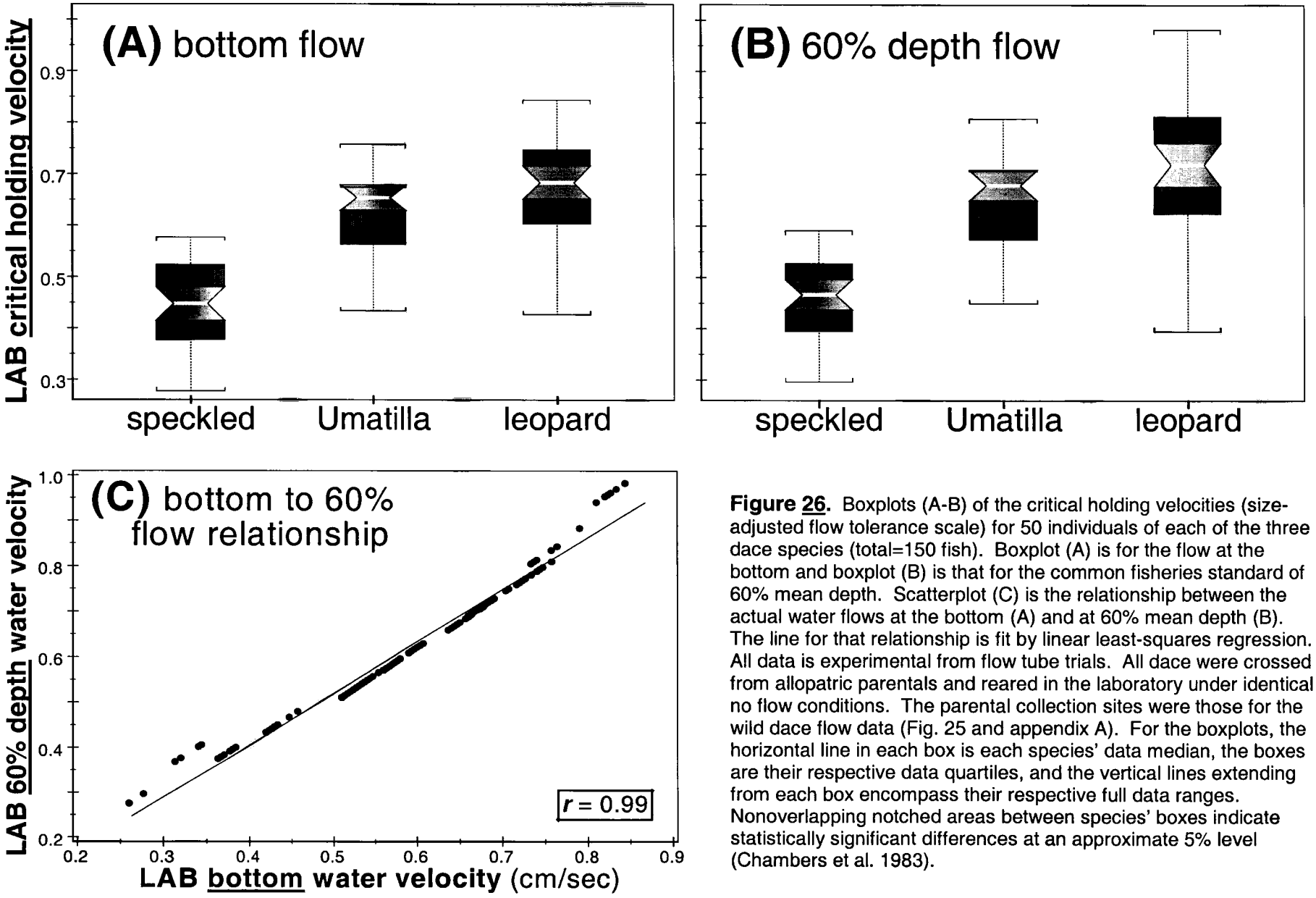


Figure 26. Boxplots (A-B) of the critical holding velocities (size-adjusted flow tolerance scale) for 50 individuals of each of the three dace species (total=150 fish). Boxplot (A) is for the flow at the bottom and boxplot (B) is that for the common fisheries standard of 60% mean depth. Scatterplot (C) is the relationship between the actual water flows at the bottom (A) and at 60% mean depth (B). The line for that relationship is fit by linear least-squares regression. All data is experimental from flow tube trials. All dace were crossed from allopatric parentals and reared in the laboratory under identical no flow conditions. The parental collection sites were those for the wild dace flow data (Fig. 25 and appendix A). For the boxplots, the horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical lines extending from each box encompass their respective full data ranges. Nonoverlapping notched areas between species' boxes indicate statistically significant differences at an approximate 5% level (Chambers et al. 1983).

Discussion

Systematics

The ecologies of the three dace species are distinctive based on water flow preference data taken under natural conditions (also see Hughes and Peden 1989, Peden 1991, Peden and Hughes 1988) and on water flow tolerances taken under laboratory experimental conditions. These differential water flow preferences and tolerances were also exhibited in allopatry where this was not potentially influenced by the co-occurrence of another of the dace species. This is in concert with general theoretical expectations and with a large amount of study data that species should have and choose their own specific habitats (eg. Schluter 1995, Schluter and McPhail 1993). This expectation is generally based on the perceived need of a species to limit its interaction with other related species in order to optimize its life-history (Endler 1989). This phenomenon is usually studied as feeding segregation and / or as reproductive isolation. Reproductive isolation is deemed critical or best for the maintenance and evolution of a species, and is the most basic and critical tenet behind the biological species concept (Mayr 1963, 1969; for specifics in fish also see Nelson and Hart 1999).

Each of the three dace species demonstrated similar differential laboratory flow tolerances at temperatures and times of reproduction (also see Bartnik 1972, Mills 1991). This is strong support for the recognition of Umatilla dace as a distinct species, and of specialization by all three of these three dace species to water flow as a primary source of their maintenance of this distinction (Persson 1991). Reproductive isolation due to water flow could be a particularly influential species' segregation mechanism because it would so on a pre-, during, and post-mating basis (Butlin 1989, Cracraft 1989, Diehl and Bush 1989, Nelson 1968, Rao and DeBach 1969, Rice 1984, 1987). Water flow preferences and tolerances may actually be surrogates or correlates to other factors of importance to their ecological and reproductive specialization and isolation, but if so water flow seems to be a good and valid representation, and maybe particularly for dace (Bartnik 1972, Mills 1991, Raney 1940a).

Water flow preferences and differentiation of species has been noted in some other dace field studies and species as well (Bartnik 1970a, Gee 1968, Gee and Bartnik 1969, Gee and Northcote 1963, Johannes 1958, Gryska et al. 1998, Marchetti and Moyle 2001, Mullen and Burton 1998, Persson 1991). Dace in some of these other natural situations were found to have an adaptation to specific water velocities through swim bladder length, volume and buoyancy adjustment (Gee 1968, 1972, Gibbons 1971, Gibbons and Gee 1972). Natural water flow characteristics have also been found to permit the coexistence of some unisexual fish species with their bisexual parentals (Collares-Periera 1989, Dawley and Goddard 1988,

Echelle and Echelle 1997, Schenk and Vrijenhoek 1986, Vrijenhoek 1978, 1979). These unisexual species are triploid females of hybrid origin from two bisexual closely related species.

Further support for species status is that many of the morphometric and meristic body form and allometric distinctions noted for the three dace species could be easily related to their existence in different water flow regimes (Lauder 1990, Persson 1991, Schaefer 1991; for drawings and photographs see Figs. 14-15 in Chapter 2, and in Peden and Hughes 1988). Such relationships between water flow, habitat, and within- and between-species variabilities have been determined for other similar benthic fish species (Daniels 1987, Garner 1996, 1997, Harding et al. 1998, Schaefer 2001, Welsh and Perry 1998). Umatilla dace further show an overall difference in mouth width and particularly eye size. These two characters could have additional correlation to species specific habitat use, particularly with respect to feeding (Lammens and Hoogenboezem 1991, Persson 1991, Sibbing 1991, Turner and Grosse 1980, Tyler 1993). Umatilla dace were again distinct on a statistically significant level from leopard and speckled dace in the majority of these univariate / bivariate measures and always overall in their multivariate analyses (Figs. 2, 3, 6 and 7 in Chapter 2).

Finally, it is possible that the populations of the three dace species listed as 'unusual' in aspects of their specific morphology and general appearance (appendix A and Chapter 2) may look this way due to unusual or altered habitat and water flow conditions further exacerbated by sympatric interactions with other dace or introduced non-native fish and aquatic fauna (Balon 1992, Baltz et al. 1982, Krupka and Holcik 1976).

Evolution

Umatilla dace have natural water flow preferences and experimental laboratory water flow tolerances that are intermediate to those of leopard and speckled dace. This is a very likely expectation for a species that is hypothesized here to have evolved through hybridizations of speckled and leopard dace (Arnold 1992, 1997, Blouws 1998, Brust et al. 1998, Dowling et al. 1989, Greenfield and Greenfield 1972). Once again, these water flow preferences and tolerances were exhibited under natural conditions of allopatry or by dace of each species reared under no flow conditions from the same allopatric populations. This means that their water flow preferences are not potentially influenced by the co-occurrence of another of the dace species.

Many of the morphometric and meristic differences and their allometric relationships between Umatilla, speckled, leopard could also be attributed to their preferences and tolerances in water flow (Lauder 1990, Persson 1991, Schaefer 1991; for drawings and photographs see Figs. 14-15 in Chapter 2, and in Peden and Hughes 1988). Umatilla dace are again intermediate to speckled and leopard dace for most of these univariate / bivariate measures and always for the multivariate analyses. Of likely particular relevance here is that Umatilla dace

have intermediate caudal peduncle depth, tail falcation, dorsal and anal fin lengths, and pelvic fin lengths and stays. All of these differences are statistically significant, and have immediately obvious and strong correlates to swimming and holding abilities. Several of these measures have actually been demonstrated as being important in other studies on intra- and inter-specific variabilities in shape and water flow / habitat selection of different benthic fish species (eg. Garner 1997, Persson 1991, Schaefer 1991). Umatilla dace also show a mild trend towards an intermediate mouth size that might be further significant in terms of their species differentiation through diet (Lammens and Hoogenboezem 1991, Sibbing 1991, Tyler 1993). As well, barbels are usually implicated as having a sensory function for feeding and these too are generally intermediate in size for Umatilla dace (Figs. 2 and 3 in Chapter 2).

The geological scenario and timeframe under which the hypothesis of the evolution of Umatilla dace through hybridization takes place is during the breakdown of natural flow environments as a result of ice cover and their blockage impacts during the last Pleistocene glaciation (Chapter 1). That leopard and speckled have particular water flow tolerances and preferences supports this possible mechanism, although not necessarily its timing. The blockage and disruption of flowing water during this or another glacial or geological event could have easily impacted the natural reproductive isolation of leopard and speckled dace (Aspinwall et al. 1993a/b, Balon 1992, Butcher 1980, Geist et al. 1996, Izyumov et al. 1998). This could have provided the conditions for their hybridization and the opportunity for the speciation and evolution of Umatilla dace (Herrera 1992, Lens et al. 2000, Pearson et al. 1988). Water flow preferences and tolerances may thus not only be associated with the maintenance and evolution of each of the three dace species, but may also be part of the mechanism of the formation of them and particularly of Umatilla dace.

Fisheries and Habitat

Bottom flow is probably a much better estimate of the true water flow preferences for these three dace species under natural conditions and of the water flow tolerances under laboratory experimental flow tube tests. The standard fisheries and habitat measurement of 60 % mean depth can nonetheless still accurately determine specific habitats for these three dace species on a statistically significant basis, but can only do so in the 'wild' under natural conditions. In the field, the 60 % mean depth measure of water flow will be higher as a natural consequence of taking it away from the benthic surface, and will be a much less realistic representation since these dace are benthic fish. This is most likely the case for most benthic fish and even other species and is worthy of some reconsideration in terms of which water flow measurements to take and use in setting legal and regulatory water conditions for them.

The water flow preferences of the three dace species under natural conditions are also related at some level to the relatively common fisheries and habitat designations of streams into

the coarse flow and depth categories of pools, glides, and riffles (Bisson et al. 1981; for minnow example see Schaefer 2001). The names for these categories are largely visual onomatopoeia, and their appearance can be imagined as such, particularly if one has spent any time working or even looking at flowing water. Pools have the deepest and slowest water, riffles have the fastest and usually although not necessarily shallower, and glides are largely intermediate to pools and riffles. Pools will generally look like they have no flow and are deep, glides will have a smooth surface with a shallower look, and riffles generally have a broken surface appearance that is caused by some proximity to the stream bottom or objects on it. Each of these three coarse stream categories would also have microhabitats within them on finer mosaic scale (Harrison and Rand 1989) that similarly correspond to fast, intermediate, and slow water flows.

It could be said then that speckled dace are generally found in stream habitats resembling pools, that Umatilla dace are usually found in glide situations, and that leopard dace are indeed often found in riffle environments. Both sets of water flow data correspond to these designations, but the depth preferences of the three dace species are at least at first look not as supportive. Speckled dace prefer shallower water on a statistically significant basis, and leopard and Umatilla dace both exhibit preference trends for deeper water. However, these depths were collected in different streams for each species where their perspective interpretations could very well also differ as a result of the different natural conditions. As well, the change in depth preference from speckled to leopard and Umatilla dace is only 20 cm over a 50 cm depth range.

The three dace were sometimes, at least initially, difficult to collect especially when sufficient numbers were needed in simultaneous reproductive condition for the laboratory crosses (Part III). It was incidentally determined that more specimens could be more readily collected in stream reaches where a few dace had been collected before by constructing small-scale stream 'berm' habitat resembling and enhancing these pool, riffle and glide conditions. This was done simply by moving large rocks around, and was often remarkably successful. Such habitat manipulation would presumably also work in any future efforts for the conservation of these and probably other dace or benthic fish species should such work become necessary (Piller and Burr 1999).

PART III - TESTS

Chapter SIX

Tests - Morphometric and Meristic Data

Materials and Methods

Laboratory Crosses

Three separate laboratory crosses each of speckled dace (*Rhinichthys osculus*), Umatilla dace (*R. o. umatilla*), and leopard dace (*R. falcatus*) were made from allopatric populations from the Columbia and Fraser river drainages. These sites were necessarily in Canada due to the permissions, legalities, and ethics of transporting live fish. Three laboratory crosses of each of the two possible hybrid combinations of speckled and leopard dace were also made from the same allopatric populations. These two artificial hybrid combinations were speckled dace females × leopard dace males and leopard dace females × speckled dace males. The allopatric sites for speckled dace were the Kettle and Granby rivers, for Umatilla dace was the Similkameen River, and for leopard dace was the Nicola / Coldwater and lower Fraser rivers (Fig. 16 in Chapter 3; appendix A). Where two allopatric sites were used for the crosses involving any one dace species, the DNA sequence data was identical for those two locations (Chapter 4).

Each cross was made 'dry' with a separate male and female since the egg number and milt output was never large, and the eggs are very adhesive and easily damaged if subdivision was attempted. All laboratory crosses were made in May or June. The crosses were made by constantly examining males and females several times every day for their degree of ripeness or readiness. Most dace came into best condition after at least 1600 hours. The eggs for each cross were separately incubated in gently aerated 500 ml glass jars at a constant temperature of 18°C. This temperature was the same that the adult dace used for the crosses were kept. The full spectrum light regime was controlled by electronic timers and appropriate for late spring or early summer in southern British Columbia, Canada (~49° N). This was just prior to or at the time of year when it was known that these three dace naturally spawned in the wild (Haas, unpubl. data). The egg densities were very similar at the beginning and end of rearing since very few eggs died once the correct technique for crossing had been determined.

Hatching of the crosses occurred at roughly the same time over a two day period (also see Kaya 1991). The hours to hatching ranged from about 140 to 170. This translates to degree-days of 105 to 122. After hatching, the fish were briefly kept in their rearing jars and fed live brine shrimp nauplii and an infusoria culture. Once they were swimming freely and eating well and properly, they were transferred to a separate 100 litre aquarium for each cross. These rearing aquaria were closed systems and had no flow. The feeding of live brine shrimp nauplii

and infusoria culture continued until all the dace in the tank were eating ground Tetramin tropical fish food. The dace were maintained at 18°C. To maximize their growth, the dace were fed to satiation several times a day with all excess food and waste material siphoned off at least daily. Mortality was unbiased and negligible, so rearing densities remained very comparable between all the crosses.

Morphometric and Meristic Data

The dace in the laboratory crosses were raised to sexual maturity, which occurred at about age two at the 18 °C temperatures they were reared at. Their body size at this stage represented a standard length measurement ranging from about 50 to 70 mm (also see Peden and Hughes 1981). They were then humanely euthanized, preserved, and measured as fully described and detailed in the 'Materials and Methods' section Chapter 2. Tissue samples consisting of a small fin clip were removed from a subset of each cross, preserved in 95% ethanol, and placed in a refrigerator.

Ten dace from each of the three crosses for each of the five cross combinations were measured and counted. This meant that morphometric and meristic data were collected on 30 dace of each species and of each of the two hybrid crosses. The total number of laboratory reared fish measured was 150. The morphometric data set utilized here consisted of 27 variables. Twenty-four characters came from a lateral truss network and three variables came from non-truss measurements (Fig.1 in Chapter 2; appendix B). This reduction in character number from those data used in Part II was necessary since the number of fish measured for each of the five cross combinations was lower. It was statistically desirable to keep the number of fish in each cross (30) higher than the number of characters (27) analyzed in multivariate statistics. The meristic data collected were the score index for barbels and pelvic fin stays developed in Chapter 2 (Figs. 2 and 3).

Analyses

The meristics of barbel and pelvic fin stay scores were independently and separately examined for all the crosses. As with the dace collected in the wild (Chapter 2), Q-mode principal components analysis (PCA) was similarly used to objectively verify and define the typological species statuses of the three dace and the two hybrid combinations of speckled and leopard dace. This PCA was based on a covariance matrix of the $\log_{10}$ transformed 27 morphometric characters (appendix B; Fig. 1 in Chapter 2).

A Q-mode linear discriminant function analysis (DFA) was also similarly undertaken on the same morphometric data and separately on the 'wild' and laboratory dace data. This was done to determine if its *a priori* group designation and its analytical maximization of between-group in relation to within-group variation would provide a different and / or better species discrimination.

PCA does not use *a priori* group designation or maximize within-group variability. The DFA was legitimate since all the groups were indeed absolutely known in advance for these laboratory crosses. The morphometric data were corrected for body size through division with fork length prior to the DFA. This was done so the maximum discrimination on the first discriminant function axis (DF1) would represent shape and not be biased and / or confounded by size. Size will generally also simply not be as strong a factor in DFA when the body sizes of its groups are similar as they are here in each of the laboratory and 'wild' dace data sets.

The PCA and DFA on the laboratory crosses were also used for direct comparisons to a separate but identical PCA and DFA done on wild dace specimens. This comparison involved the same morphometric and meristic characters. The wild dace specimens consisted of 30 fish of each species that had been randomly selected from their sample sizes of 50 fish per species in the 'original 150 fish' sample category in Chapter 2 (appendix A). These wild dace specimens for the three species came from the same allopatric sites in Canada as the dace used for the laboratory crosses.

All the necessary information for the morphometric and meristic data and their analyses are fully presented and detailed in the 'Materials and Methods' and 'Results' sections of Chapter 2. All the tests for data characteristics and for statistical assumptions of the analyses discussed and presented in Chapter 2 were also undertaken for the data used here in Chapter 6. The morphometric and meristic data in this Chapter 6 gave the same overall results.

In particular regard to the data in Chapter 6, the reduced sample sizes of 30 dace per laboratory cross were again found to be sufficient and there were no problems with ipsative measures, matrix singularity, or Rao's paradox. Only the first two eigenvectors and principal components (PCs) were significant under all tests and guidelines, with the first PCA vector again strongly related to body size and the second PCA vector representing body shape. The morphometric data were homoscedastic, which again enabled the analysis of total data matrices in subsequent multivariate statistics, rather than of the pooled within-group matrices sometimes suggested. This was further verified by PCAs run as checks using pooled within-group data and by the DFA that necessarily requires *a priori* group designation. Jackknife tests on the PCAs were also again used to examine the effects on their output of different subsets of data.

The PCAs on pooled within-group data, the jackknife tests of the PCA, and the pooled within-group procedures of sheared PCA and of DFA all produced very similar results to the PCA. A direct analysis of the total data matrix was justified. Such decisions on analyses of total versus pooled within-groups could have been important because PCA was specifically selected here since it does not require *a priori* grouping. Prior group designation can be subjective, is better used for discrimination than description, and assumes there is only one

taxon per group that can be completely distinguished (Humphries et al. 1981, Pimentel 1979, Thorpe 1976, 1980). The basis of statistics involving *a priori* group designation also is group ordination rather than individuals, which was not necessarily desirable here. DFA was used only as a further assessment.

Results

Laboratory versus 'Wild'

The PCAs show the crosses of the three dace species that were made and reared in the laboratory to be virtually identical to their respective species of 'wild' dace collected from the same natural allopatric populations that acted as the source of parentals for the laboratory crosses (Figs. 27A-B). All three dace species in both the laboratory and 'wild' data are clearly and very similarly distinct. In all cases, Umatilla dace and any measures of central tendency for its data are consistently intermediate to speckled and leopard dace. The group distinctions in the PCAs are solely based on the second PC that has been demonstrated here and in Chapter 2 to represent multivariate shape. An examination of only the shape component of the scores for the morphometric data in the PC2 shape vectors (Fig. 27C) provide identical or very similar results to the overall PCAs (Figs. 27 A-B) and between each other. This is also the case for the DFAs and their shape data in DF1 (Fig. 27D).

The species' centroids and their medians, quartiles, and ranges (Fig. 27C) show the same patterns in the PCAs, with the centroids for the laboratory and 'wild' data having particular congruence. There even is a strong association between the directionality of their within-species group scatter patterns. This is in spite of the laboratory crosses accounting for much less variation on the PC1 size axis. This reduction in size variation is most probably a result of the greatly reduced size ranges that necessarily existed and could be looked at in the laboratory dace (Fig. 27A) due to their rearing at the same time under identical conditions.

The DFAs do not provide as strong an association between the laboratory and 'wild' specimens, with that association being statistically significant only for Umatilla dace (Fig. 27D). The DFAs still show the same overall strong relationships between the laboratory and 'wild' specimens for all three dace species. Each of the three dace species also continues to have a closer relationship between its own laboratory and wild specimens than to those for the other two species. Umatilla dace remain intermediate to leopard and speckled dace.

These multivariate results from the PCAs and DFAs are also mirrored by the two univariate meristic scores examined (Fig. 28). The PCA and DFA here then further provide an objective, independent, and multivariate corroboration of the use of these univariate characters for species assignment (Fig. 13 in Chapter 2). The barbel and pelvic fin stay score indices are both

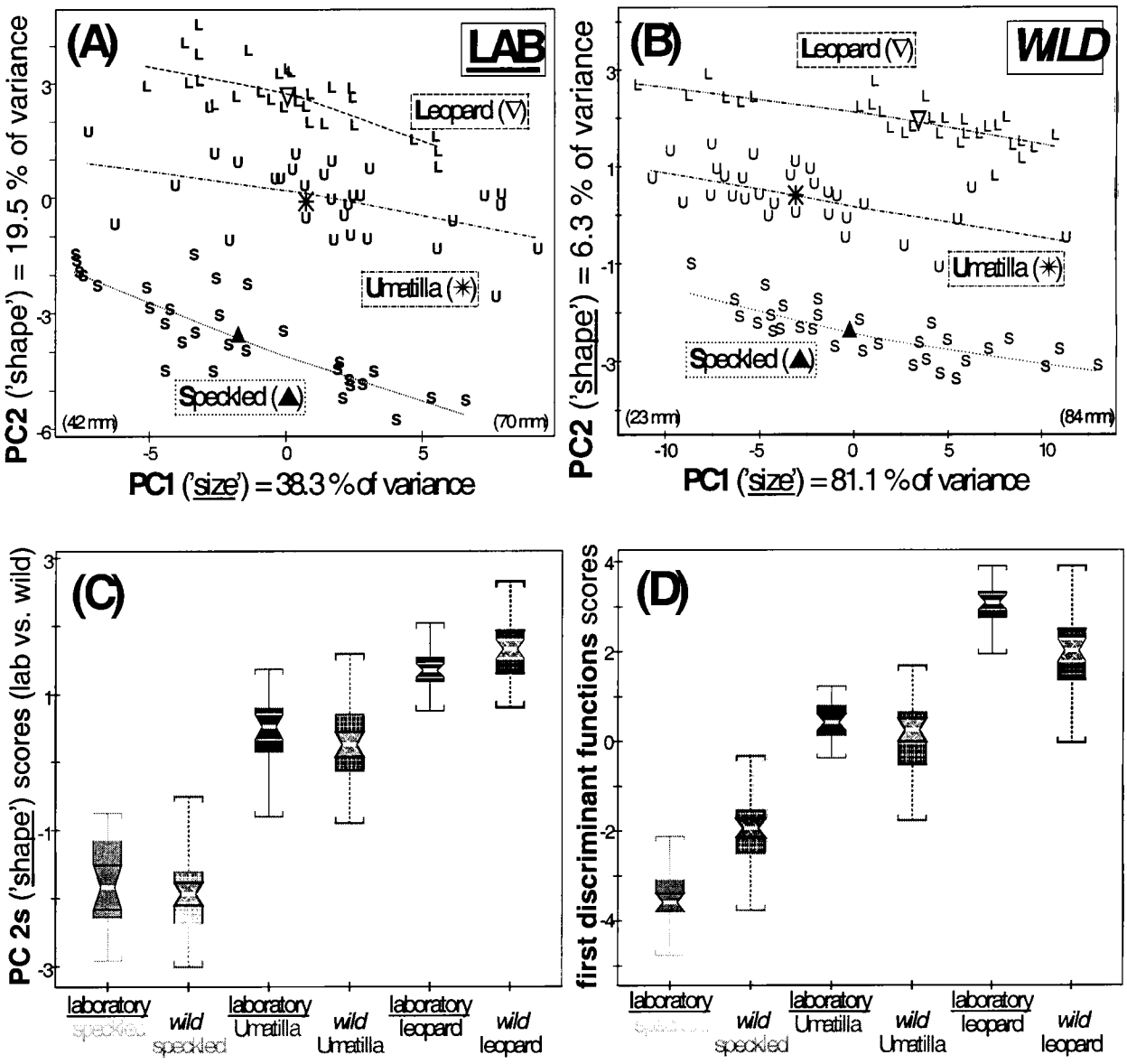


Figure 27. Scatterplots A-B of principal component analyses (PCA) of the three species analyzed separately as (A) laboratory made and reared dace (identical conditions) and as (B) wild dace (natural conditions) from the same respective allopatric collection sites (appendix A). The species are denoted by a symbol for their centroids and by a regression line from a robust local weighting procedure (Cleveland 1979). Boxplot C is of the PC2 scores from scatterplots A-B. Boxplot D is of the scores from similarly separate linear discriminant function analyses (DFA) on each of these same lab reared (DF1 = 55.4 % of variance) and wild (DF1 = 52.5 % of variance) dace groups. Boxplots C-D have the scores on their y-axes 'scaled' so the two groups can be equivalently compared, with those on the PCA scatterplots A-B retaining and showing the natural distribution of the unscaled scores. In the boxplots, speckled dace are plotted in light grey, Umatilla dace in dark grey, and leopard dace in black. Using these colours, lab reared samples for each species are plotted in a solid pattern and wild collected samples in a hatched template. The white solid horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical lines extending from each box encompass their full data ranges. Nonoverlapping notched areas between species' boxes within the lab reared and within the wild groups indicate statistically significant differences at a rough 5% level (Chambers et al. 1983). In every case, there are 30 fish for each species in the lab reared (total = 90) and in the wild collected (total = 90) groups. The analyses are all based on the same lateral body truss 27 measurement data set (Fig. 1 and appendix B), which are all size adjusted for the DFA through division by fork length.

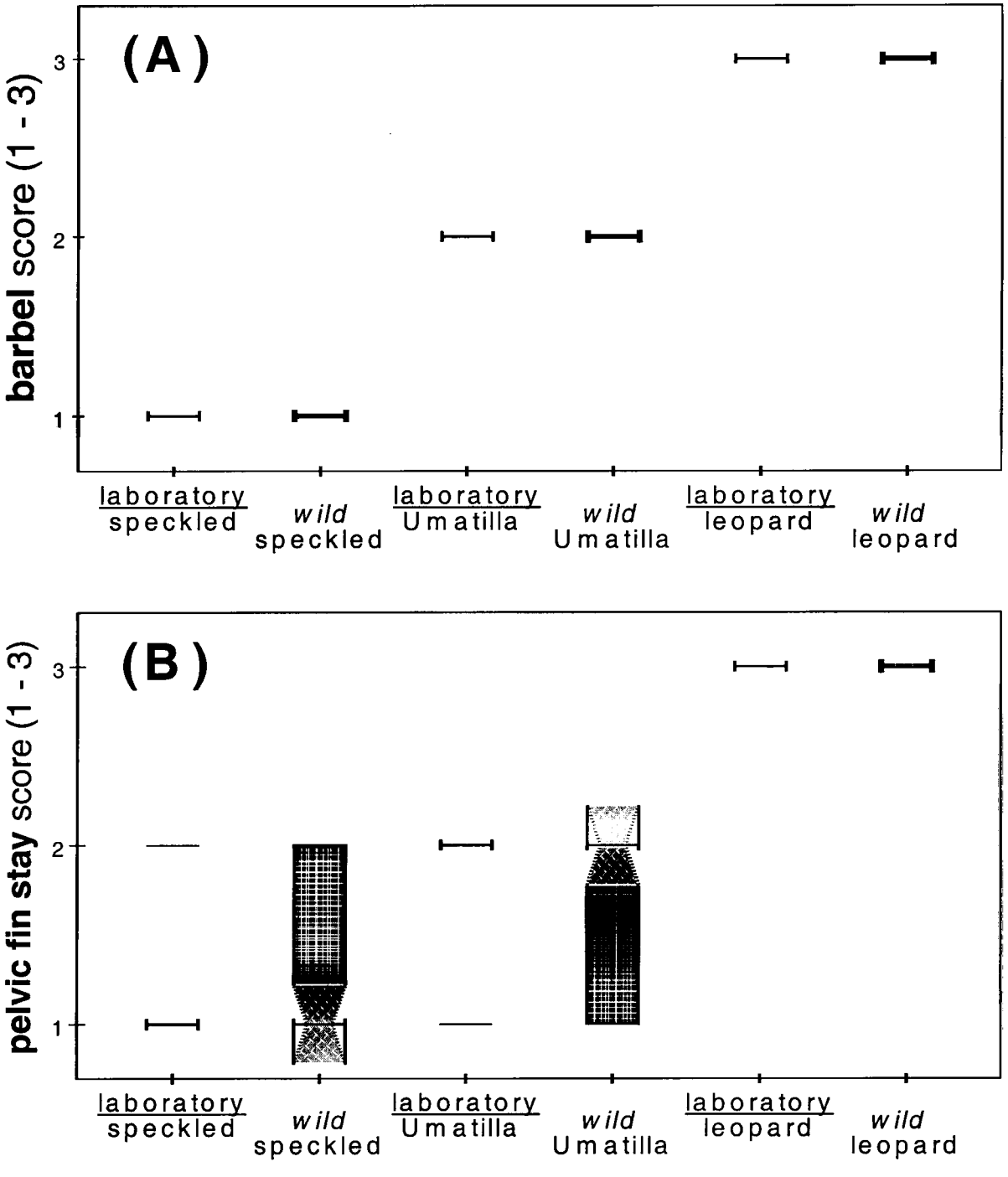


Figure 28. Boxplots of barbel (A) and pelvic fin stay scores (B) for the three dace species. Laboratory crosses and wild collections came from the same respective allopatric sites (appendix A). In box plots without boxes, the lab dace are represented by thin black lines with vertical bars at each end and the wild dace similarly by a thick black line. These single lines instead of boxes indicate that species has only one score. In the box plots with boxes, the lab dace are in solid black and the wild is in a hatched pattern. The white horizontal line in each box or the separate black horizontal line with vertical bar ends is each species' data median. Separate black horizontal lines without bar ends are statistical outliers, but also represent the full data ranges. The boxes are their respective data quartiles, and the vertical lines extending from samples with boxes are their full data ranges. Notched areas that do not overlap between species' boxes within the lab reared and the wild groups indicate statistically significant differences at a rough 5% level (Chambers et al. 1983). In every case, there are 30 fish for each species in the lab reared (total = 90) and in the wild collected (total = 90) groups.

very similar between the laboratory and 'wild' dace specimens (also see Peden and Hughes 1988). Each of the three dace species is differentiated using the barbel scores and largely separated using the pelvic fin stay scores. There is slightly more overlap in the pelvic fin stay scores between speckled and Umatilla dace in the 'wild' than in the laboratory. Umatilla dace are again intermediate to leopard and speckled dace in the 'wild' and laboratory. The consistent similarities in results from multiple analytical approaches strongly suggest that the multivariate and univariate results are realistic.

The scores for the 'wild' specimens of the three dace species show more variation than the laboratory specimens on the PC2 and DF1 shape vectors (Figs. 27C-D). The species discrimination in the DFAs is also stronger for the laboratory than 'wild' dace specimens of each of the three dace species (Fig. 27D). These are both again the likely consequence of the laboratory fish having been reared at and for the same time under identical conditions. This decreased variation is particularly pronounced in leopard dace in the PCA. Leopard dace also show the only, but still marginally, statistically significant differentiation between laboratory and 'wild' specimens. In the DFA, this reduction in variability is greatest for Umatilla dace, and only Umatilla dace show a statistically significant relationship between their 'wild' and laboratory specimens. For these populations, the Umatilla dace are more closely associated with leopard dace than speckled dace in the PCAs and DFAs of both laboratory and 'wild' specimens. Speckled dace are the most differentiated of the three dace species.

Other noteworthy PCA results are that the Umatilla dace reared in the laboratory also show a slightly larger multivariate size, and thus presumably higher growth rate, than leopard dace and particularly speckled dace (Fig. 27A). Also, the separation of the three species in the 'wild' and laboratory data are still slightly reduced (Fig. 27) in comparison to the original PCA on 50 dace of each species using 42 morphometric characters (Fig. 7 in Chapter 2; appendix B). This is most likely caused by the smaller data set of 27 morphometric variables and / or the random selection of 30 fish from their original data set of 50 dace of each species.

The shape scores for PC2 and DF1 on the boxplots (Figs. 27C-D) were 'scaled' so that species from the laboratory and 'wild' could be equivalently and properly compared. This 'scaling' was done simply by separately taking the largest and smallest PC2 or DF1 scores from both PCAs and DFAs and then respectively dividing the largest to the smallest data by the largest difference in PC2 or DF1 scores from the two PCA and DFA data sets. This brought both PC2 and both DF1 ranges in line with each other through the respective use of a single constant scalar. The PCA scatterplots continue to show the unscaled and natural distribution of these same data scores. These boxplots were only intended to assist in the interpretation of the data rich PCA scatterplots, and are for the discrimination rather than description of the three

dace species. Since no other biological interpretations are being made here, the boxplot representations through scaling for are much less potentially problematic.

Laboratory Hybrids

The three dace species are demonstrated to be similar in the 'wild' and laboratory data, with Umatilla dace intermediate to leopard and speckled dace. These latter data for the two laboratory hybrid crosses of leopard and speckled dace can also be related to 'wild' dace in natural conditions.

The PCA on the laboratory reared dace show both laboratory hybrid crosses of leopard and speckled dace to be most closely related to Umatilla dace and intermediate to leopard and speckled dace (Figs. 29A-B). The species' centroids and their medians, quartiles, and ranges (Fig. 27C) show the same patterns in the PCA. There even and once more is a strong association between the directionality of their within-species group scatter patterns. The group distinctions in the PCA are solely based on the second PC that has been demonstrated here and in Chapter 2 to represent multivariate shape. An examination of only the shape component of the scores for the morphometric data in the PC2 shape vector (Fig. 29B) provides identical or very similar results to the overall PCA and between each other. This is also the case for the DFA and its similar shape data in DF1 (Fig. 27D).

The PC2 relationships of Umatilla dace from this population are only statistically significant with the laboratory hybrid cross made from a leopard dace female. The relationship of Umatilla dace to the laboratory hybrid cross made with a speckled dace female is not statistically significant, but is still clearly most strongly associated with and similar to Umatilla dace and the other laboratory hybrid cross. Furthermore, the two laboratory hybrid crosses are more differentiated on a statistically significant basis from leopard and speckled dace. The two laboratory hybrid crosses also show a maternal effect. Both laboratory hybrid crosses are more closely and statistically significantly associated with the species that provided their female gametes (Fig. 29B).

DFA provides the same overall results for discrimination of the three dace species and the two laboratory hybrids as the PCA (Fig. 29C). As would be analytically expected, DFA provides an even stronger species discrimination than the PCA, which itself still fully discriminates the three dace species. Of more importance, is that the DFA was not able to fully discriminate the two laboratory hybrid crosses from Umatilla dace and not much from each other. The two laboratory hybrid crosses are both statistically significantly different from all three dace species but are clearly still more closely associated with Umatilla dace than leopard or speckled dace. The two laboratory hybrid crosses are not statistically significantly different and are basically placed in the same group by the DFA results, even though they were separately designated as distinct groups in its data input. Unlike the PCA, DFA places both laboratory hybrid crosses as

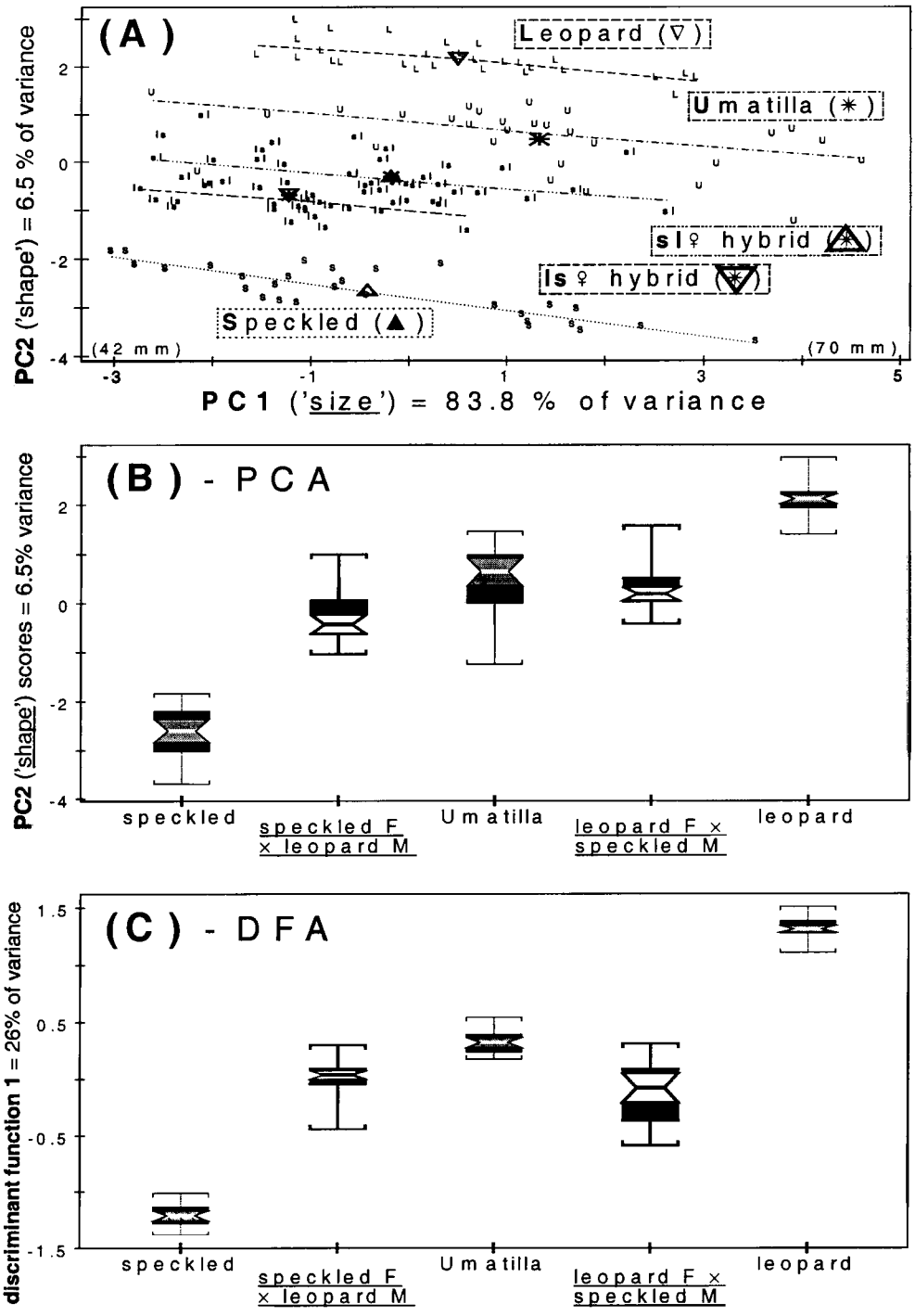


Figure 29. Scatterplot (A) of a principal component analysis on the three species and the artificial hybrids of speckled and leopard dace all made and reared in the laboratory under identical conditions. The species are denoted by a symbol for their centroids and by a regression line from a robust local weighting procedure (Cleveland 1979). Boxplot (B) is of the PC2 scores from scatterplot (A). Boxplot (C) is of the scores from a linear discriminant function analysis (DFA) on the same data, but first divided by fork length. The data consist of 27 measurements from a lateral body truss set (Fig. 1 and appendix B). In each plot (A-C), there are 30 dace for each species or hybrid (total = 150). In the boxplots (B-C), both hybrid combinations are distinctly plotted in white for their inner quartiles and have thicker lines than for the three dace species. The white or black solid horizontal line in each box in all the boxplots (B-C) is each species' data median, the boxes are their respective data quartiles, and the vertical lines extending from each box encompass their full data ranges. Nonoverlapping grey or white notched areas between species' boxes indicate statistically significant differences at a rough 5% level (Chambers et al. 1983).

more similar to speckled dace when only comparing them to speckled and leopard dace. However, in DFA this placement is not based on the more realistic or unbiased multivariate description produced by PCA, but rather on deliberate species discrimination that maximizes between-species in relation to within-species variation.

The addition of the morphometric data from the two laboratory hybrid crosses into the PCA has also resulted in improved differentiation of the three dace species. The improved discrimination is now more similar again to that based on the original PCA on 50 dace of each species using 42 morphometric characters (Fig. 7 in Chapter 2; appendix B). It has also caused the PC1 size vector to account for a more typical level of variation. This is because the two laboratory hybrid dace are smaller in size to the three dace species, even though they were reared at and for the same time under identical conditions.

The two univariate meristic scores examined (Fig. 30) also mirror these multivariate results from the PCA and DFA. The PCA and DFA here then also provide an objective, independent, and multivariate corroboration of the use of these univariate characters for species assignment (Fig. 13 in Chapter 2). The consistent similarities in results from multiple analytical approaches strongly suggest that the multivariate and univariate results are again realistic. The barbel and pelvic fin stay score indices are very similar between the two laboratory hybrids and between them and Umatilla dace. The two laboratory hybrids are more different from speckled and leopard dace, yet again intermediate to those two dace species along with Umatilla dace. Each of the three dace species is differentiated using the barbel scores (Fig. 30A) and very nearly separated using the pelvic fin stay scores (Fig. 30B). The two laboratory hybrid dace show a very few outlying barbel scores more in common with speckled than leopard dace and unlike Umatilla dace. The two laboratory hybrid dace show some outlying pelvic fin stay scores in the speckled dace range as well, but in this case that is an exact match with the Umatilla dace.

Discussion

Systematics

The three dace species' crosses made and reared at and for the same time period under identical conditions in the laboratory produce very similar and consistent species differentiation as measurements on their 'wild' specimens collected from the same allopatric sites in natural conditions. The natural data for the three dace species from the 'wild' was also similarly discriminated in analyses of the total data set of 500 dace (e.g. Fig. 7 in Chapter 2). Each of the three dace species in the laboratory crosses data remains distinctive. In particular, Umatilla dace are still and almost identically differentiated from leopard and speckled dace.

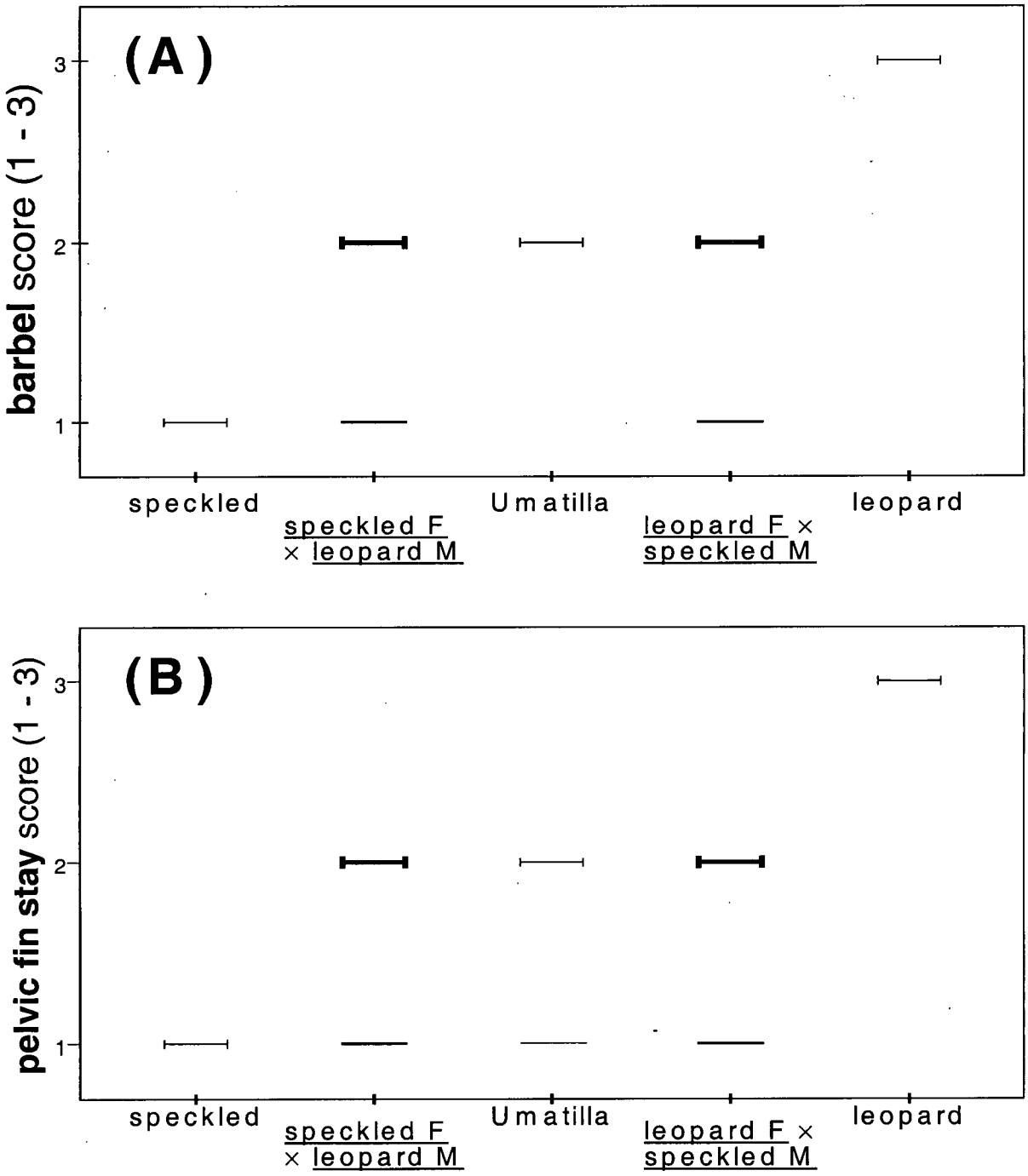


Figure 30. Boxplots of barbel (A) and pelvic fin stay scores (B) for the three species and the artificial hybrids of speckled and leopard dace all made and reared in the laboratory under identical conditions (for score definitions see Fig. 2 and appendix B; also see 'wild' dace data in Fig. 3). All parental fish came from allopatric sites (appendix A). The horizontal lines with vertical bar ends are the respective species' data medians, and separate horizontal lines without vertical bar ends are statistical outliers that also represent the full data ranges. Nonoverlapping horizontal lines with vertical bar ends indicate statistically significant differences at better than a rough 5% level (Chambers et al. 1983). These single lines instead of boxes indicate that species has only one score. In each plot, there are 30 fish for each species or hybrid combination (total = 150).

This is very strong support and endorsement for the recognition of Umatilla dace as a distinct species from speckled and leopard dace (Brust et al. 1998, Haas and McPhail 1991, Jordan 1991, Stone 2000). Each of the three dace species would also have been distinguished using the key developed previously (Fig. 13 in Chapter 2). These laboratory cross data also give full corroboration of that identification protocol.

Evolution

Umatilla dace crossed and reared in the laboratory for and at the same time under identical conditions retain their distinctiveness as a species. More importantly, Umatilla dace remain intermediate to leopard and speckled dace (for drawings and photos see Figs. 14 and 15 in Chapter 2, and in Peden and Hughes 1988). The two laboratory hybrid crosses of speckled and leopard dace are also intermediate to these two parental species (Figs. 31-32). Most importantly, the two laboratory hybrid crosses thus have the same overall association, and the only statistically significant relationship, with Umatilla dace (Figs. 33-34). The two laboratory hybrid dace are much more similar to Umatilla dace than leopard or speckled dace. This relationship between the two laboratory hybrid and Umatilla dace is even reflected in some subtle nuances such as data outliers specific only to these three groups.

This is very strong evidence for an association of hybridization originally in Umatilla dace and between leopard and speckled dace (also see Arnold 1997, Brust et al. 1998, Child and Solomon 1977, DeMarais and Minckley 1992, Dowling et al. 1989, Greenfield and Greenfield 1972, Jordan 1991). The hypothesis that Umatilla dace speciated and evolved from past hybridizations of leopard and speckled dace is well supported (Figs. 31-34). The no flow conditions in the laboratory that the three dace species were reared under also match the environment that is hypothesized to have been present during, and the cause of, the original hybridizations of leopard and speckled dace (Chapters 1 and 11).

Umatilla dace from this population in the Similkameen River (Fig. 16 in Chapter 3; appendix A) used for the laboratory crosses are also more like leopard than speckled dace. This is even substantiated by a maternal effect on the multivariate morphometric shape of the two laboratory hybrid dace crosses (Cathey et al. 1998, Crespin et al. 1999). These Umatilla dace are more similar to the laboratory hybrid cross involving the leopard dace female than the speckled dace female. The closer relationship of this population of Umatilla dace to leopard dace is also seen in the genetic (Chapters 4 and 7), water flow preference / tolerance (Chapters 5 and 9), and overall morphometric (Chapter 2) data. This will be further and summarily discussed in the concluding General Discussion and Summary Chapter (Chapter 11).

Umatilla dace are the largest of the three dace species in the laboratory data, which could be translated as them having increased growth. This size and growth increase may give them advantages in certain circumstances and situations to leopard and speckled dace (Huxel 1999,

Larson 1989, Lewontin and Birch 1966, Potts and Reid 1988, Stebbins 1959). It may also be related to their specific environments in that body size and shape are presumably somewhat correlated to the substrates found at the water flows differentially preferred and tolerated by the three dace species (Chapters 5 and 9). The two laboratory hybrid dace show reduced size and growth, but this could be a first generation effect or the result of leopard and speckled parental dace being from very disparate locations in two distinct watersheds (Goodfriend and Gould 1996, Turner 1971). The latter geographic and biogeographic situation would not have been the case in the hypothesized original hybridizations of leopard and speckled dace. These hybridizations would presumably have largely occurred with leopard and speckled dace more proximally located in the same general watershed (Chapters 1 and 11).

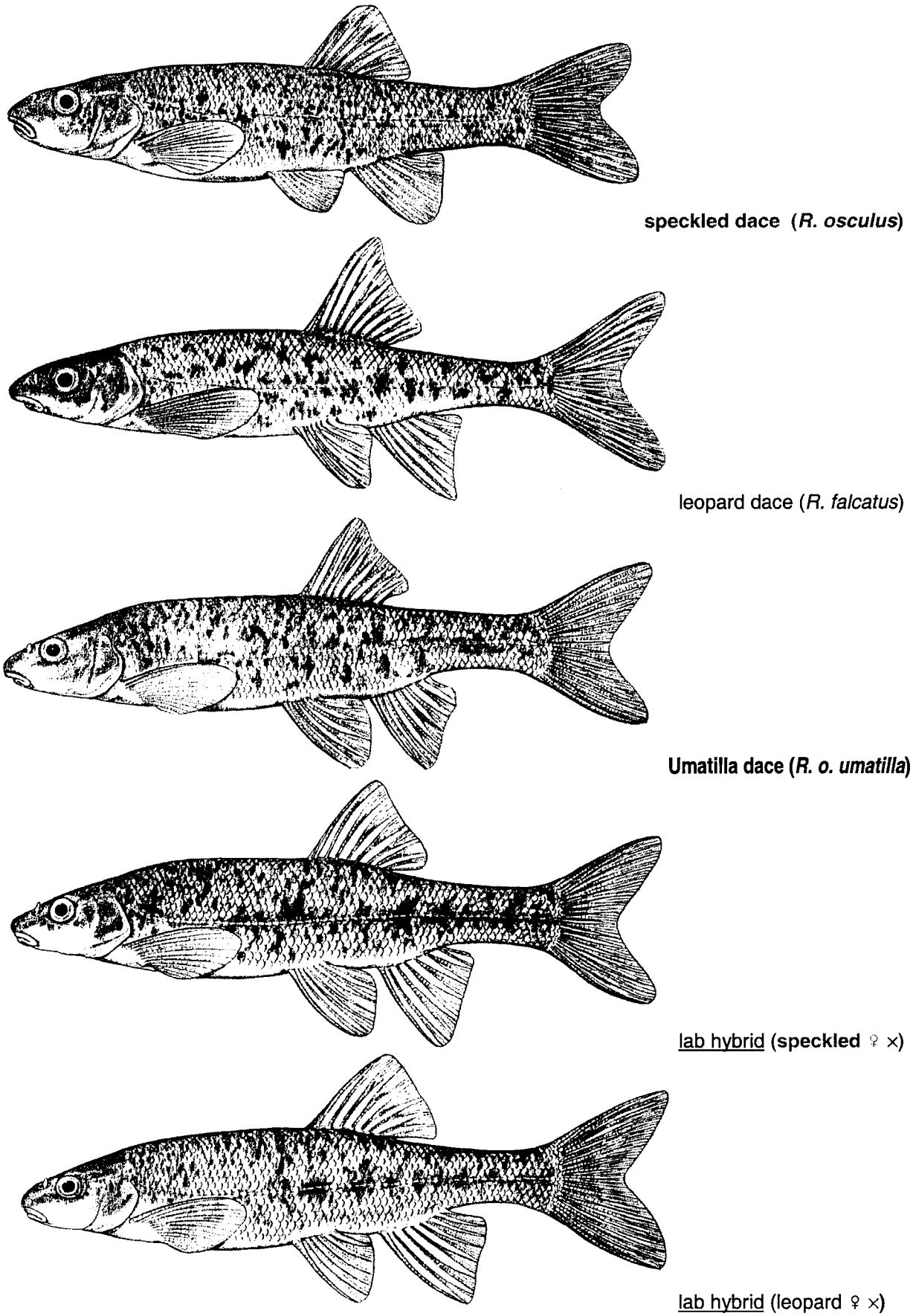


Figure 31. Composite drawings at maturity (drawn at ~ 70 mm) of the three dace (*Rhinichthys* spp.) species and of both possible artificial laboratory hybrid crosses of speckled and leopard dace. The drawings were done for me by Karen Klitz.

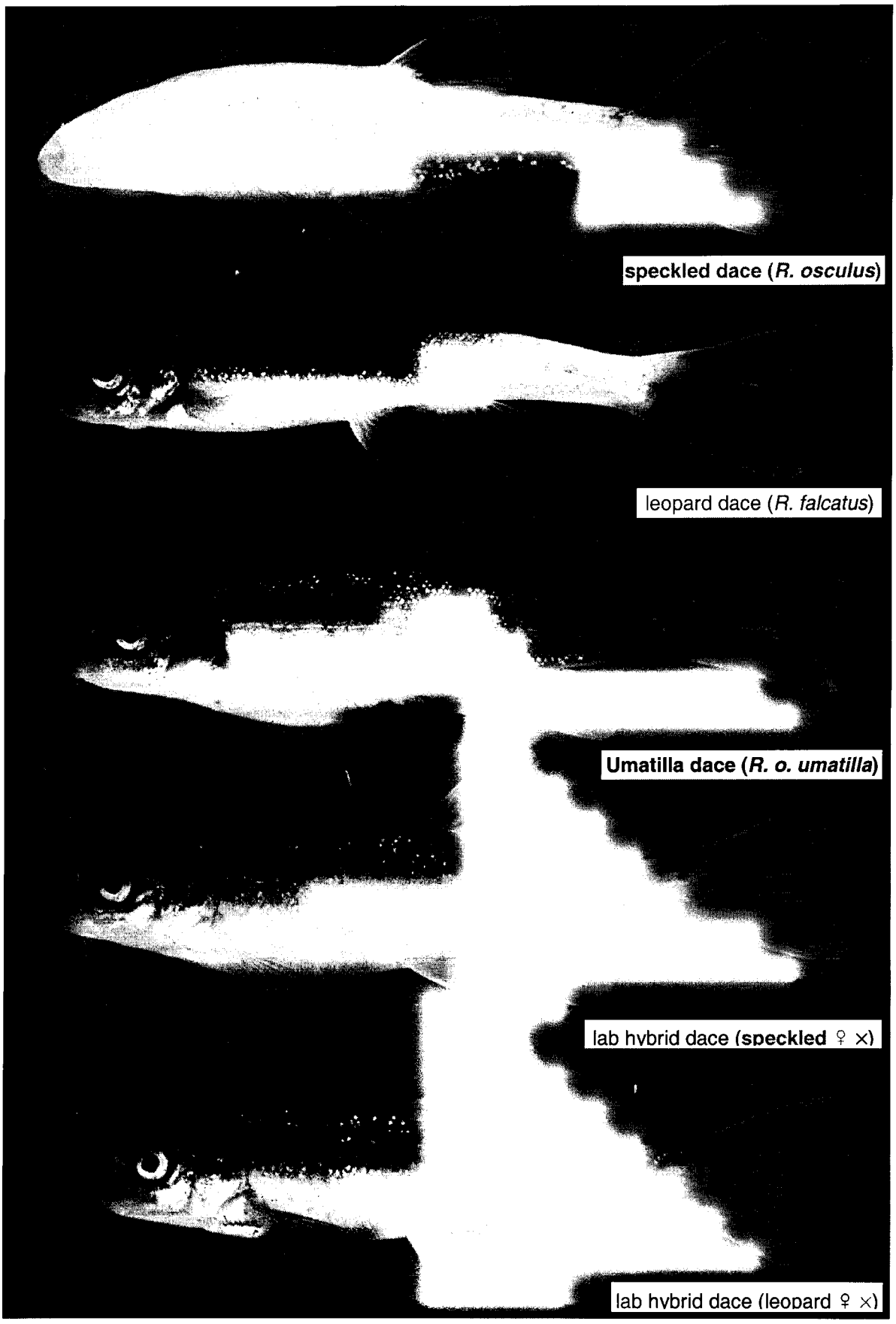
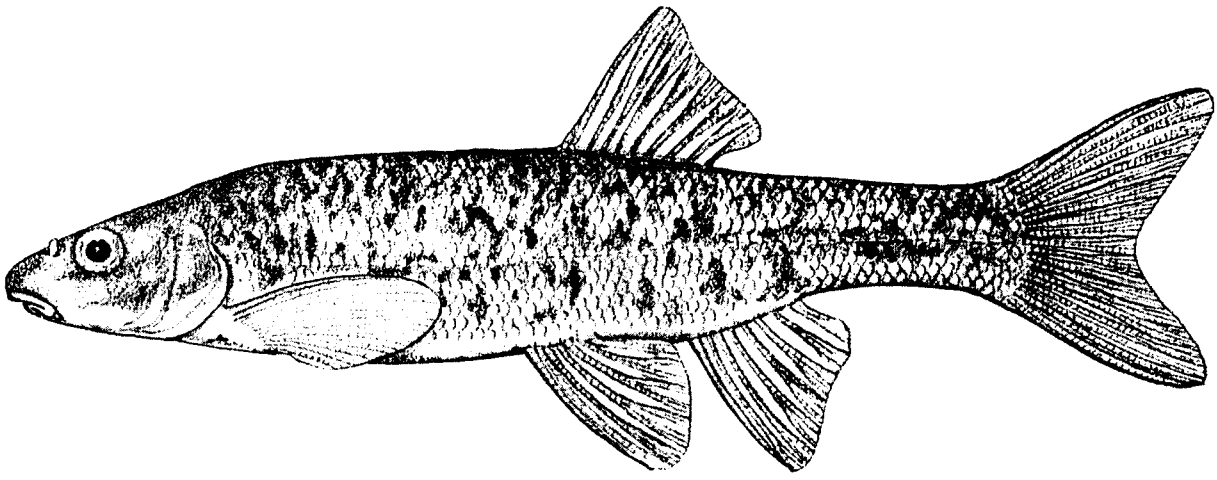
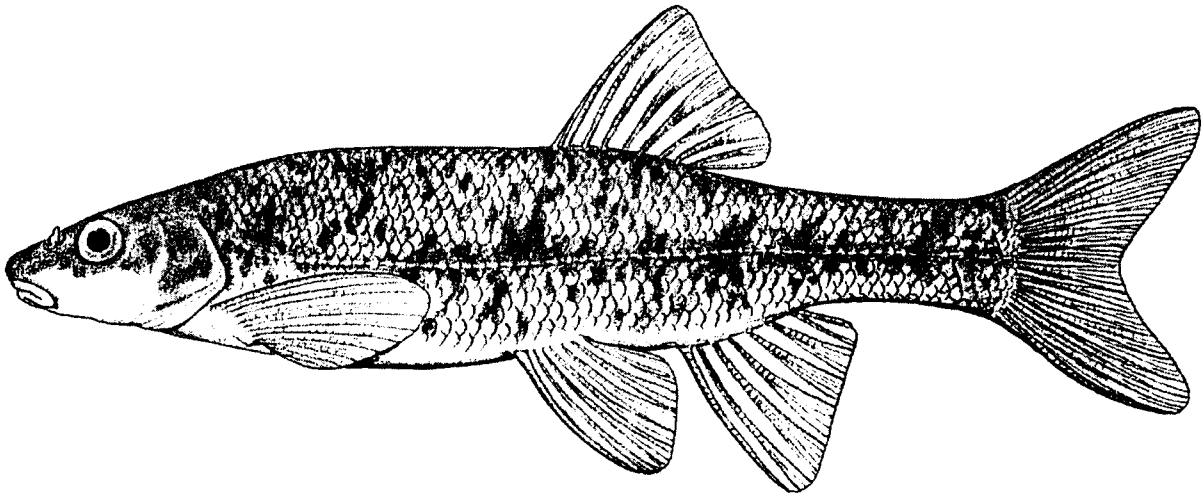


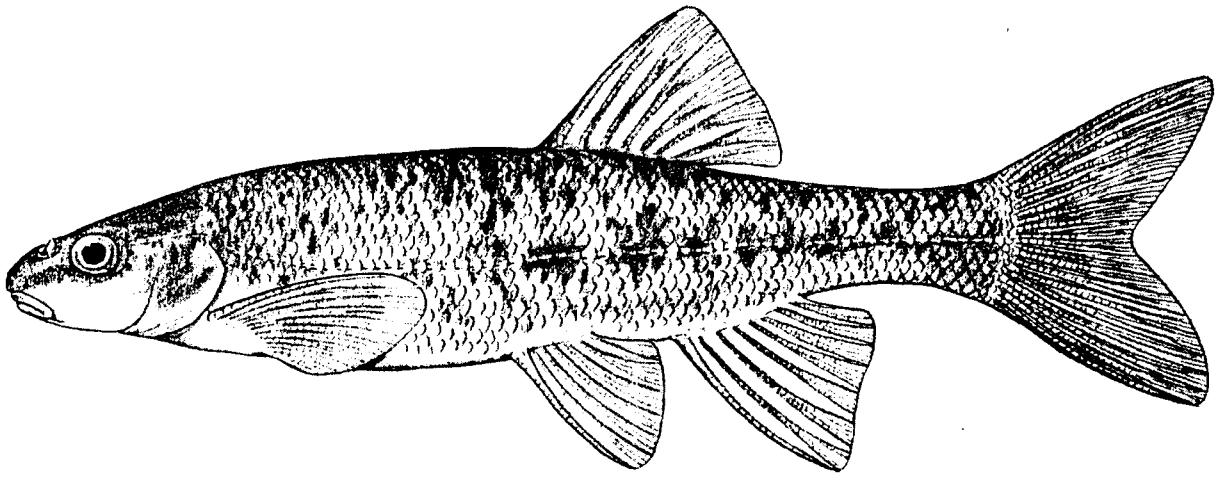
Figure 32. Photograph of laboratory reared mature specimens (~70mm) of the three dace species and of both possible artificial laboratory hybrid crosses of speckled and leopard dace.



Umatilla dace (*Rhinichthys o. umatilla*)



laboratory hybrid dace (**speckled dace** ♀ ×)



laboratory hybrid dace (leopard dace ♀ ×)

Figure 33. Larger composite drawings at maturity (drawn at ~70mm) of only Umatilla dace and both possible artificial laboratory hybrid crosses of speckled and leopard dace. The drawings were done for me by Karen Klitz.

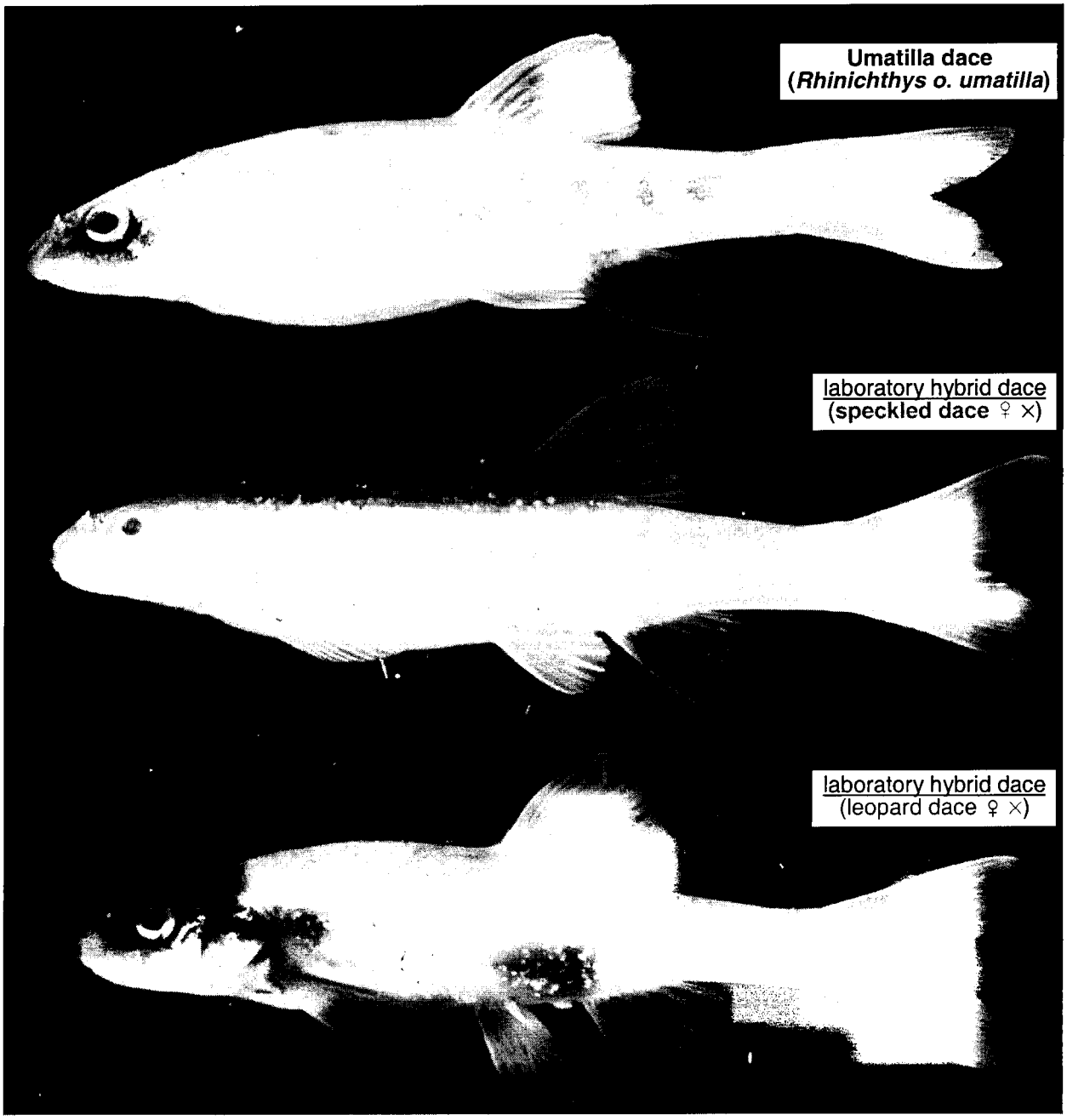


Figure 34. Photograph of laboratory reared mature (~ 70 mm) specimens of only Umatilla dace and both possible artificial laboratory hybrid crosses of speckled and leopard dace.

Chapter SEVEN

Tests - Genetic Data

Materials and Methods

The cytochrome-b region of the mitochondrial DNA (mtDNA) and the Internal Transcribed Spacer (ITS) were assessed on laboratory hybrid crosses of speckled (*Rhinichthys osculus*) and leopard (*R. falcatus*) dace. The crosses involved both possible hybrid combinations: speckled dace females × leopard dace males and leopard dace females × speckled dace males.

These laboratory hybrid crosses and all the necessary information for the DNA sequencing and are fully presented and detailed in the 'Materials and Methods' and 'Results' sections of Chapter 4. The ribosomal DNA D3B region of the genomic DNA that was examined in Chapter 4 was not assessed for these laboratory hybrid crosses.

Results

Cytochrome-b Region of the Mitochondrial DNA (mtDNA)

DNA sequence for the cytochrome B region of the mitochondrial DNA (mtDNA) was successfully obtained for one fish from each of the two possible hybrid combinations. Each of the hybrid crosses groups out as it should with the parental female (Fig. 35), and no sequence divergence was present between them. The laboratory hybrid created from a leopard dace female from the lower Fraser River clusters with the overall leopard dace sample from the lower Fraser River. The other hybrid combination made with a speckled dace female from the Kettle River clusters with the overall speckled dace sample from the Kettle River. The overall and specific pattern of relationships in the tree is unaffected by the addition of these two samples of hybrids except for a few very small changes of 1 – 3 % in bootstrap values (compare to Fig. 19 in Chapter 4).

The hybrid cross involving the female leopard dace also clustered with Umatilla dace from the Similkameen River, with leopard dace from the Yakima River, and in general with the more distinct leopard dace sample from the Willamette River (map – Fig. 20 in Chapter 4). The hybrid cross involving the female speckled dace clustered with speckled dace from the Granby River and Connor Creek, and with more distinct speckled dace samples from Little Indian Creek, Palouse River, Umatilla River, and two differentiated samples from the Willamette River. Furthermore, the speckled dace aside from the Yakima River sample and now this hybrid cross involving the female speckled dace are all more closely associated (Table 1 in Chapter 4) with

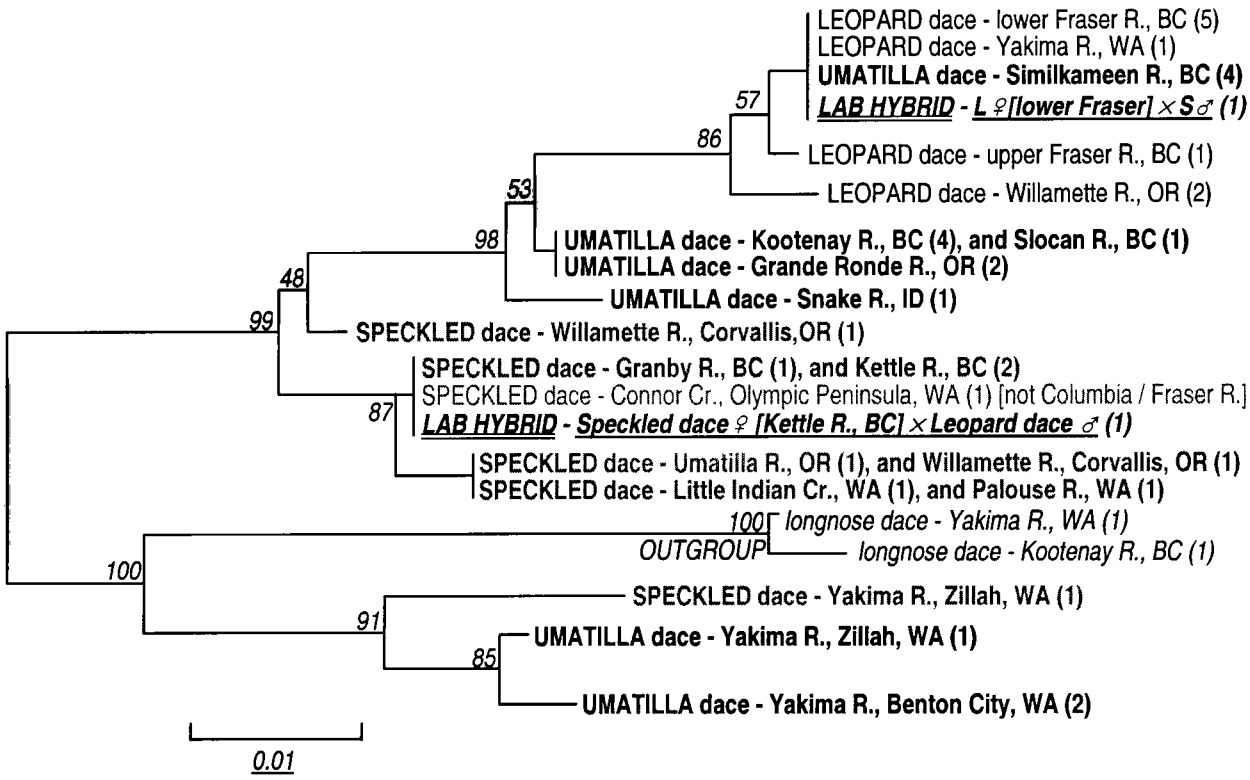


Figure 35. Tree (neighbour-joining) for mitchondrial DNA sequences for a 306 base pair segment of the cytochrome-b region starting at gene position 40. The data are from the same thirty-four dace from 19 samples analyzed earlier (Fig. 19 and appendix A), but also now include the data from one fish each of both possible artificial laboratory crosses of speckled dace and leopard dace. The data for two longnose dace from two samples used as the outgroup in the analysis are also presented. Leopard dace are presented in black font, Umatilla dace in bold black font, speckled dace in bold dark grey font, and the laboratory hybrids in bold italicized font. Bootstrap values are based on 500 iterations and presented in italic black font on the tree nodes. The sample number per site is in parentheses following the locality information. The specific groupings from the original cytochrome-b tree are also shown and summarized on map Figure 20.

the Umatilla dace (6 – 9 bp differences) from the Grande Ronde, Kootenay, and Slocan rivers. These Umatilla dace are more divergent from the leopard dace samples (8 – 11 bp differences), and represent all the Umatilla dace specimens except for the Similkameen and Yakima rivers. The Yakima River is a related but still unique situation, and no similar laboratory crosses were tried from that location. The Yakima River samples are fully discussed in Chapter 4.

Internal Transcribed Spacer (ITS)

DNA sequence was derived for two individuals of the hybrid cross speckled dace female × leopard dace male and for one fish of the hybrid cross leopard dace female × speckled dace male (appendix D). Their DNA sequences for the internal transcribed spacer (ITS) was only for the 'partial' data based on 255 base pairs (bp) rather than the 'complete' ITS data where 316 bp could be examined (Fig. 21 in Chapter 4; appendices C and D).

A full explanation of the complete and partial ITS data sets is in the 'Results' section of Chapter 4. Briefly, the partial ITS data has some possible ambiguity due to variation within individuals among multiple copies of the genes. This variation consists of occasional nucleotide substitutions that had minimal or moderate impacts on the data. These substitutions did not impair overall reading of the sequence but did each result in the presence of an ambiguous base pair (bp). They also gave a shorter overall region that could be interpreted.

There was more substantial variation in five other non-hybrid dace samples and these could not be interpreted at all (appendix D). These five sequences had sufficient length variation among multiple copies of the genes that their sequences could not even be properly aligned. These five additional dace were thus necessarily left out of both ITS data sets and trees. These five severe unreadable cases of length variation consisted of three Umatilla dace (*R. o. umatilla*), one leopard dace, and one longnose dace (*R. cataractae*).

All three hybrid fish thus had 255 bp sequences that were legible with a few somewhat ambiguous DNA sites or with multiple nucleotide substitutions (appendix D and Fig. 36). There were three other dace in the original analysis of partial ITS data for non-hybrid dace that had similar ambiguities, and all were Umatilla dace. The overall and specific pattern of relationships of the original partial ITS data are unaffected by the addition of these two samples of hybrids except for changes of 3 – 5 % in bootstrap values (compare to Fig. 21 in Chapter 4).

The data from the single laboratory hybrid made using female leopard dace clusters most closely with leopard dace from the Willamette River, but also with Umatilla dace from the Similkameen River and with speckled from the Boise, Kettle and Willamette rivers (Fig. 36). The male speckled dace in this hybrid cross came from this Kettle River sample site. This laboratory hybrid also clusters overall with the leopard dace from the Fraser River where the female for this hybrid cross came from, but the relationship of this laboratory hybrid is still closer to the leopard dace from the Willamette River. This laboratory cross is then more closely

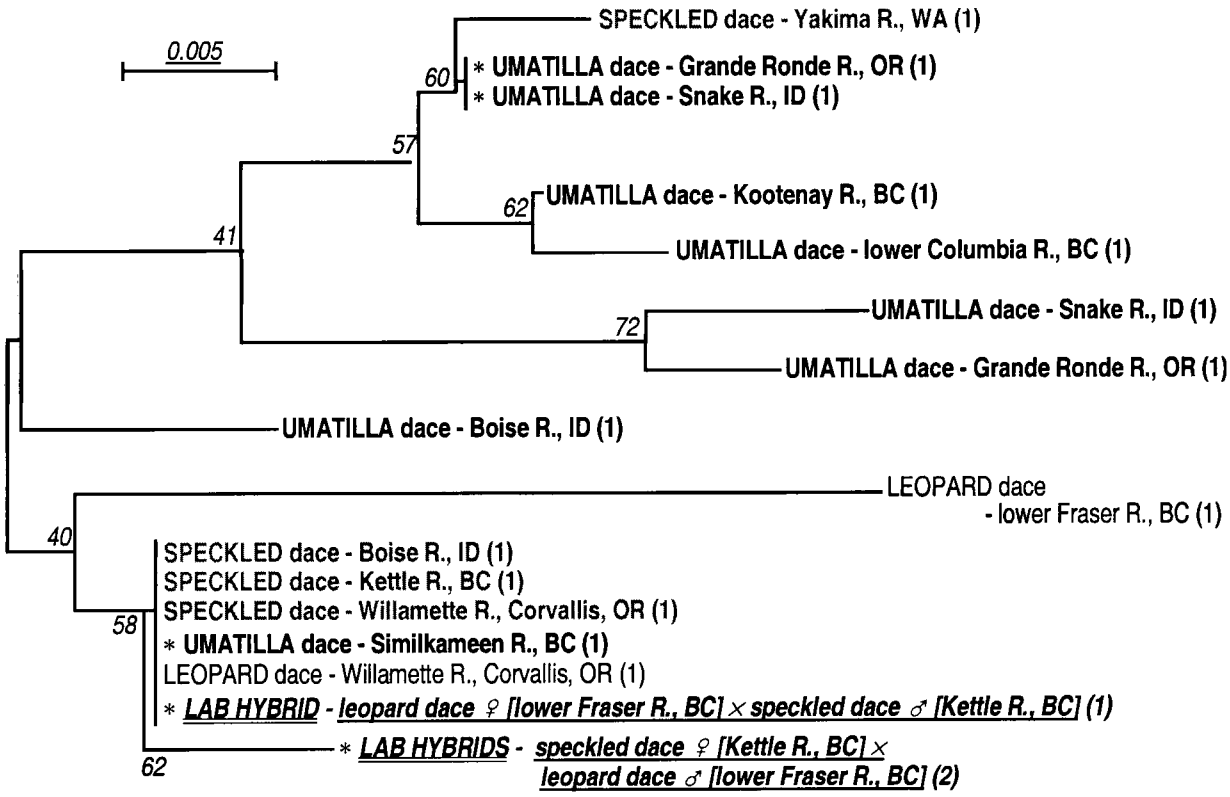


Figure 36. Tree (neighbour-joining) for DNA sequences from the internal transcribed spacer (ITS). This is for the same 14 dace from 12 samples analyzed earlier (Fig. 21B and appendix A), but also now includes the data from three individuals of both possible artificial laboratory crosses of speckled dace and leopard dace. The data are from the partial ITS based on unambiguous 255 base pair sequences. The six dace marked with an asterix (*) had somewhat ambiguous DNA sites or multiple bases, but no length variation. Six other dace from five samples are not presented as they had sufficient length variation to render their data unreadable (appendix D). The complete ITS data set of 316 unambiguous base pairs (Fig. 21A) could not be presented here as no laboratory hybrids produced that additional legible ITS sequence. Leopard dace are always presented in black font, Umatilla dace in bold black font, speckled dace in bold dark grey font, and the artificial laboratory hybrids in bold italicized black font. Bootstrap values are based on 500 iterations and presented in italic black font on the tree nodes. The sample number per site is in parentheses following the locality information. The specific groupings from the original partial ITS tree without the laboratory hybrid data are also shown and summarized on map Figure 22.

associated with its paternal parent population from the Kettle River. This latter association may not be overly meaningful due to the inheritance mechanisms of genomic DNA.

The data from the two laboratory hybrids made using female speckled dace cluster distinctly from the other laboratory hybrid and its relationships just described (Fig. 36). They also form a new branch on the tree. The bootstrap values supporting this (and other) distinction on this tree are not that significant in a general sense but are in the high range here where the bootstrap percentages are reduced overall. These two laboratory hybrids are still overall associated with the other laboratory hybrid and its same aforementioned relationships with speckled, leopard, and Umatilla dace.

All three hybrid crosses are not closely associated with the other Umatilla dace samples except those from the Similkameen River and to a lesser degree those from the Boise River. The Umatilla dace from the Boise River occupy a somewhat intermediate position on the partial ITS tree, but are still more closely related to the speckled and leopard dace than to the other Umatilla dace (Chapter 4). The two laboratory hybrids made from speckled dace females are further removed from the Umatilla dace in the Boise River, and from the leopard dace in the Fraser River, by their additional node on the tree which has comparatively high bootstrap support. They are thus in somewhat closer association with their maternal parent population from the Kettle River.

The only sample from the Yakima River for which any ITS data could be obtained was speckled dace and these continue to be very different from the laboratory hybrids and again also from the rest of the samples of speckled dace (Chapter 4).

Discussion

Systematics

The genetic analyses of these laboratory crosses were not set up to specifically examine the species status of Umatilla or leopard and speckled dace. Nonetheless, there is still some good incidental support for their species distinctions and for species status of Umatilla dace (Brust et al. 1998). Each of the three dace species retain their integrity in both the cytochrome-b mitochondrial DNA (mtDNA) and on the partial internal transcribed spacer (ITS) sequence data (Figs. 19 and 21).

The addition of the laboratory hybrids did not affect the overall differentiation or general patterns of relationships for these three dace species (Brust et al. 1998, Rieseberg and Linder 1999). The laboratory dace crosses of course cluster with their specific female parent populations for the mtDNA data due to well established maternal inheritance of mtDNA. The two laboratory hybrid crosses are strongly associated, yet also distinct, in the partial ITS data.

Most Umatilla dace are not associated with these laboratory hybrid crosses in the partial ITS data. The laboratory hybrid crosses also remain more closely related to their general parental species in the mtDNA and ITS data, although they are also still associated with the expected specific Umatilla dace groups in mtDNA.

Using the morphometric and meristic data, the laboratory hybrids look more like Umatilla dace than leopard or speckled dace (Chapter 6). This overall evidence suggests at the very least that 'wild' Umatilla dace in natural systems are not simply first generation (F1) hybrids of leopard and speckled dace, and probably not early generation ones either (Aubert and Solignac 1990, Brust et al. 1998, Rieseberg et al. 1996). There is no indication that the uniqueness of Umatilla dace as species is broken down through (further) hybridization (DeMarais et al. 1992, Dowling et al. 1989). This is consistent with the key criterion within many species concepts, but particularly within the biological species concept, that a species must retain its genetic integrity where it co-occurs and could possibly interbreed with other related species (Mayr 1963, 1969, Nelson and Hart 1999).

Evolution

In retrospect, first generation hybrids might not be expected to provide a realistic assessment of the evolution of Umatilla dace through hybridization of leopard and speckled dace (Rieseberg et al. 1996, Turner 1971). This evolution is afterall hypothesized here to have originally taken place around the beginning of end of the last Pleistocene glaciation in western North America, which translates to roughly 15,000 years ago. Nonetheless, there is once more and perhaps even unanticipated support here for this speciation hypothesis. The two laboratory hybrid crosses both provide further good evidence for multiple origins involving the hybridization of these latter two dace species from both mtDNA and nuclear DNA sequence data (Arnold 1997, Brust et al. 1998).

In the mtDNA data, the closest association of any Umatilla dace to the laboratory hybrid cross made with female leopard dace is to those from the Similkameen River. This is also the case for the partial ITS data where the laboratory hybrid cross using the leopard dace female is again more closely associated with Umatilla dace from the Similkameen River than is the opposite hybrid cross. Similarly, the Umatilla dace populations most like the laboratory hybrid cross made with a female speckled dace are all the other Umatilla dace outside of those from the Similkameen and Yakima rivers. The two laboratory hybrid combinations are disparately clustered and more closely associated with either the overall groupings of speckled or leopard dace (DeMarais et al. 1992, Dowling et al. 1989, Tegelström and Gelter 1990). This is a natural result of maternal inheritance of mtDNA, but this pattern also matches those for Umatilla dace. The recognized groups of Umatilla dace are always more closely related to either speckled or leopard dace, even within the unique Yakima River. Umatilla dace are more intermediate to

leopard and speckled dace than the laboratory hybrids, but the laboratory hybrids are only first generation (Brust et al. 1998, Rieseberg et al. 1996, Rieseberg and Linder 1999).

In the ITS data, all three non-hybrid dace in the original analyses that had readable DNA sequence, but with some ambiguity due to variation consisting of occasional nucleotide substitutions, were Umatilla dace (Fig. 21 in Chapter 4; appendix D). The only other fish that show this pattern of readable DNA sequence with ambiguous variation are both of the two possible laboratory hybrid crosses of speckled and leopard dace. This pattern is not seen in the parental populations of the two laboratory hybrid crosses, and these parental dace populations in fact have sufficiently good sequence to be analyzed as part of the complete ITS data set. These particular DNA sequence ambiguities are not found in any speckled or leopard dace.

This is strongly indicative of an association with hybridization for these readable DNA sequence length variations and for Umatilla dace (Hillis and Dixon 1991). The only possible source of that hybridization for Umatilla dace as studied here would be hybrids of leopard and speckled dace. The lack of a specific relationship to Umatilla dace based on the actual partial ITS sequence data as opposed to its ambiguous sequence variations could be interpreted as an argument against this. However, it could also just again be a byproduct of the hybrids only being first generation and of the ITS region being nuclear DNA (Buth et al. 1991, Forbes and Allendorf 1991, Normark and Lanteri 1998). A general relationship of both laboratory hybrid cross types to specific expected Umatilla dace groups does still exist in the mtDNA data (Brust et al. 1998).

Chapter EIGHT

Tests - Reproduction and Viability

Materials and Methods

The laboratory crosses and procedures are fully presented and detailed in the 'Materials and Methods' section of Chapter 6. In brief and pertinent review, three separate laboratory crosses each of speckled dace (*Rhinichthys osculus*), Umatilla dace (*R. o. umatilla*), and leopard dace (*R. falcatus*) were made using allopatric populations from the Columbia and Fraser river drainages in Canada. Three separate laboratory crosses of each of the two possible hybrid combinations of speckled and leopard dace were also made from the same allopatric populations. These two hybrid combinations were speckled dace females × leopard dace males and leopard dace females × speckled dace males. Where two allopatric sites were used for the crosses involving any one dace species, the DNA sequence data was identical for those locations (Chapter 4).

It was further important to determine if the laboratory crosses, and particularly the two hybrid combinations of speckled and leopard dace, were themselves reproductively fertile and viable. To do this, two subsequent laboratory crosses were made from separate initial laboratory crosses of each of the three species and of the two hybrid combinations of speckled and leopard dace. There were limitations to the remaining number of laboratory reared dace because of when this experiment was conceived and of the reproductive condition of the dace at that time. It was decided to be more important to undertake a replicate cross of each of the five laboratory cross types, and thus no inter-hybrid or parental back-crosses were made.

Each of the subsequent laboratory crosses undertaken was made 'dry' with a separate male and female since the egg number and milt output was never large, and the eggs are very adhesive and easily damaged if subdivision was attempted. The eggs for each cross were separately incubated in gently aerated 500 ml glass jars at a constant temperature of 18°C. This temperature was the same that the adult dace from the initial laboratory crosses used for these additional crosses were kept.

After hatching, the fish were briefly kept in their rearing jars and fed live brine shrimp nauplii and an infusoria culture. Once they were swimming freely and demonstrably eating well and properly, their crosses were accepted as viable. The crosses were then terminated with the dace humanely euthanized and preserved as fully described and detailed in the 'Materials and Methods' section of Chapter 2. Ten offspring were randomly selected from each of these subsequent laboratory crosses and examined under a dissecting microscope for any overt malformations or signs of disease or stress.

Results

The egg densities were very similar at the beginning and end of rearing since very few eggs died during fertilization or none died during development. There was no bias detected towards which of the dace species had some very minor mortality during fertilization, and any deaths that did occur at that time were more likely due to my handling and technique than anything inherent to the crosses themselves. In particular, there was no differential mortality for the subsequent hybrid crosses.

As with the original laboratory crosses (Chapter 6), the hatching of their subsequent crosses here occurred after roughly the same time over a two day period. The hours to hatching also again ranged from about 140 to 170, with this translating to degree-days of 105 to 122.

After hatching, all the offspring of the subsequent crosses began behaving and feeding normally. This is probably more appropriately stated as these dace acted and fed just like those in the original laboratory crosses made from the 'wild' fish taken from natural streams. The initial growth and related timing of feeding ability for ground Tetramin tropical fish food was also the same in both the initial and subsequent crosses.

At this stage, these subsequent laboratory crosses were deemed viable and the experiment was terminated. Upon close inspection, none of the offspring examined from these crosses showed any signs of abnormalities, disease, or stress.

Discussion

Systematics

All three dace were viable, developed normally, and hatched in the same time frame as their original crosses made from 'wild' fish collected in natural conditions (Brust et al. 1998). Their subsequent feeding and behaviour were also normal. In particular, Umatilla dace showed no differential problems (Dowling and Moore 1985). There is no indication that Umatilla dace are not a distinct species capable of maintaining their evolutionary lineage without reproduction involving leopard or speckled dace. Umatilla dace thus support primary tenets of both the biological and evolutionary species concepts (Mayr 1963, 1969, Cracraft 1989; for fish specifics see Nelson and Hart 1999). Leopard and speckled dace show the same distinctions.

Evolution

Both possible laboratory hybrid crosses of speckled and leopard dace were viable, developed normally, and hatched in the same time frame as those laboratory crosses of speckled, leopard, and Umatilla dace. If Umatilla dace are derived from such hybrid reproduction there is no evidence that their species would initially suffer any differential mortality or could not maintain their evolutionary distinction or lineage (Arnold 1997, Stauffer et al. 1979,

Tauber and Tauber 1989). This is critical to and supportive of the hypothesis that Umatilla dace evolved from hybridizations of leopard and speckled dace (Chapters 1 and 11).

Chapter NINE

Tests - Ecology - Water Flow Data

Materials and Methods

Flow Tolerance Data - Laboratory

A complete description and discussion of the details, methodology, and protocol for the data from, and for the making and rearing of, the crosses is available in the 'Materials and Methods' section of Chapter 6. The protocol for the laboratory flow experiments was identical here. The crosses used for the laboratory flow tests in this Chapter 9 were again identical to Chapter 6. These once more were speckled, Umatilla, and leopard dace, and in this Chapter 9 now also include both possible hybrid combinations of speckled × leopard dace. These two hybrid combinations were speckled dace females × leopard dace males and leopard dace females × speckled dace males.

As a brief summary reminder, controlled tests of water velocity tolerances of each of the three dace species and their two hybrid combinations were undertaken in a closed system recirculating experimental flow tube (Fig. 24 in Chapter 5). This was done to assess any differences between individuals of these species in their ability to hold their benthic positions under regular increased water velocities that reflected the natural water flow data regime collected in the 'wild'. It was also undertaken to compare the laboratory hybrids to their parental forms and particularly to Umatilla dace. The type of flow preference data collected in the 'wild' could not be readily evaluated with this apparatus. Depth measurements were also not taken since they would not be valid. 'Wild' measurements on natural hybrids of leopard and speckled dace were not possible since they did not likely or definitively exist. No evidence of such early generation hybrids was detected in the genetic data (Chapters 4 and 7).

The laboratory data were all collected on mature dace reared from crosses of allopatric parentals of each species. These laboratory dace were kept under identical and no water flow conditions, and thus had no prior exposure or predilection to water velocity. Their body lengths were similar and ranged from about 50 to 70 mm. The parentals came from the same allopatric sites in Canada that the flow measurements were made in the 'wild'. The laboratory data were taken on 50 individuals each of all three dace species and both hybrid combinations. This resulted in a total of 250 flow measurements.

Since a length measurement was easily collected on the laboratory fish, their flow tolerances could be adjusted for any effects of body size. This was probably not critical for such laboratory fish since most were of similar size due to their common rearing conditions and

maturity status. Proper standardization to size is still desirable if possible though as it makes full comparisons more realistic. Such standardization also did not matter for purposes of comparison to 'wild' data collected under natural stream conditions because that field data was for flow preferences and not tolerances. Only the overall flow patterns and trends are compared between the 'wild' and laboratory.

A standard methodology for size correction is conversion of the laboratory water flow data to 'critical holding velocity' (CHV). CHV is calculated, in terms of body lengths per second based on the fish sizes, as follows:

$CHV = Vi + (t_i / t_{ii} \times V_{ii})$, where: V_i = highest water velocity tolerance;

V_{ii} = water velocity increments;

t_i = time (mins.) each fish held at highest water velocity;

t_{ii} = time period at each velocity increment (15 mins).

Water temperature could be fully controlled in the flow tube and was again set to 18°C. This was the same water temperature that the dace were being maintained at in the laboratory. This temperature was largely selected because it was that at which the dace in the laboratory came into their best reproductive condition and it reflected those temperatures found under natural conditions. This was felt to represent the temperature at which much natural spawning might take place and at which the most critical habitat segregation of the three dace in terms of the ecology and evolution of a species would occur. Water velocity is a reasonable surrogate for overall habitat in this case. The dace were also most active at 18°C and probably would have the best opportunity to exhibit realistic maximum flow tolerances.

Two sets of flow measurements were collected. Water flow at 60 % mean depth was taken because it is a common standard in fisheries work, and is often used to establish legal and regulatory flow conditions. Water flow at the bottom was collected since dace are a benthic fish and that is where all sampled individuals were found under natural conditions. The benthic measurement was determined to be more biologically realistic (Chapter 5). Total depth of each sample location was also measured.

The water flows at the bottom and at 60% mean depth are visually compared using linear least-squares regression. This is to establish that a valid difference exists between the flow measurements taken at these two depths in the laboratory experimental flow tube, and also to compare them to the results from the measurements taken under natural conditions on 'wild' dace specimens.

Flow Preference Data - 'Wild'

The methodology, protocol, and data for collection and analyses of the water velocity measurements taken for each of the three dace species in the 'wild' from allopatric sites in Canada are fully presented and detailed in the 'Materials and Methods' and 'Results' sections of

Chapter 5. The data are from 60 speckled dace (*Rhinichthys osculus*) from four allopatric sites, 57 Umatilla dace (*R. o. umatilla*) from five allopatric sites, and 56 leopard dace (*R. falcatus*) from four allopatric sites (sites listed in appendix A, and shown on map Fig. 16 in Chapter 3). Each allopatric field site consisted of at least ten flow measurements taken at precise locations where each specimen of each species was detected. The DNA sequence data for the multiple allopatric flow sites for any one species indicated that the dace from them were identical or very similar (Chapter 4).

Results

Flow Tolerance Data - Laboratory

The comparison of the laboratory results for just the crosses of the three pure dace species are already presented in Chapter 5 (Figs. 25 and 26). The data are fully compatible with those generated for the two laboratory hybrid crosses in this Chapter 9. Their comparison to the flow preference data for the two laboratory hybrid crosses is thus legitimate and is done and presented directly (Fig. 37).

The two hybrid crosses have very similar patterns of flow tolerance, which are the same on a statistically significant level (Figs. 37A-B). In comparison to their parental species at both depths, the two hybrid crosses are closer to and overlap more with leopard dace than speckled dace. The flow tolerances for both hybrid crosses are nonetheless statistically significantly different from those for all three dace species. The median and data quartile summaries of the hybrid flow tolerances are slightly higher for the hybrid cross of leopard dace females × speckled dace males. The range for this hybrid cross is also somewhat reduced, largely at the lower flow end. With respect to their parentals, this would place the laboratory cross involving leopard dace females as slightly more similar to leopard dace than is the speckled dace female cross. These latter differences in central tendency statistics demonstrating a slight maternal effect for just the two laboratory hybrids crosses are trends, but not statistically significant.

In comparison to all three dace species, the overall placements, patterns, and ranges for the flow tolerance of the two hybrid crosses are clearly closest to and most like that of Umatilla dace (Figs. 37A-B). Of the two laboratory hybrid crosses, the water flow tolerances for the cross made with leopard dace females more strongly resembles that for Umatilla dace. Umatilla dace still have a higher median and slightly higher range of flow tolerance than either laboratory hybrid cross.

Of the three dace species, speckled dace have the lowest and only statistically significantly different level of water flow tolerance (Figs. 37A-B; also see Figs. 26A-B in Chapter 5). The differences in flow tolerance between leopard and Umatilla dace are reduced and not

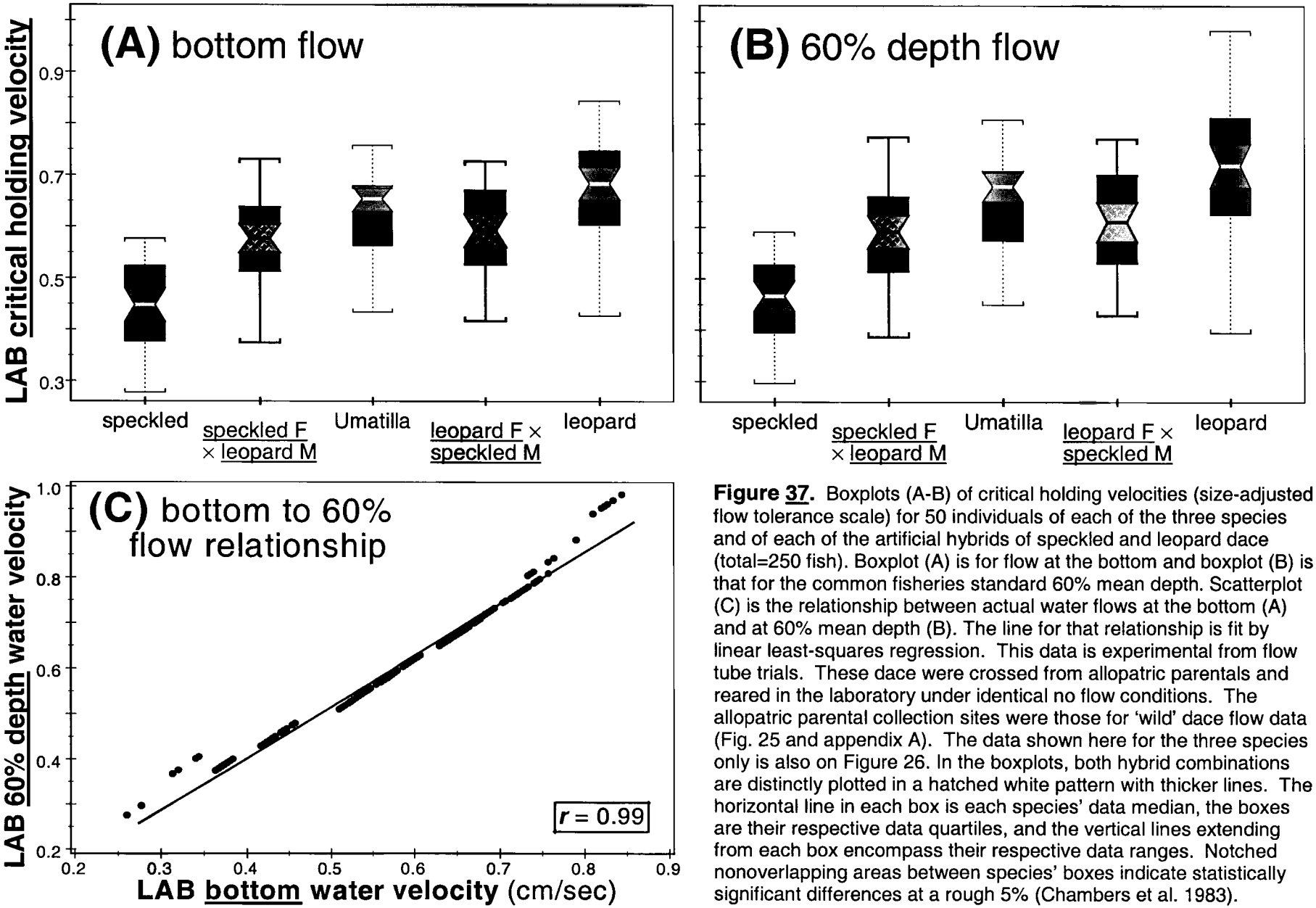


Figure 37. Boxplots (A-B) of critical holding velocities (size-adjusted flow tolerance scale) for 50 individuals of each of the three species and of each of the artificial hybrids of speckled and leopard dace (total=250 fish). Boxplot (A) is for flow at the bottom and boxplot (B) is that for the common fisheries standard 60% mean depth. Scatterplot (C) is the relationship between actual water flows at the bottom (A) and at 60% mean depth (B). The line for that relationship is fit by linear least-squares regression. This data is experimental from flow tube trials. These dace were crossed from allopatric parentals and reared in the laboratory under identical no flow conditions. The allopatric parental collection sites were those for 'wild' dace flow data (Fig. 25 and appendix A). The data shown here for the three species only is also on Figure 26. In the boxplots, both hybrid combinations are distinctly plotted in a hatched white pattern with thicker lines. The horizontal line in each box is each species' data median, the boxes are their respective data quartiles, and the vertical lines extending from each box encompass their respective data ranges. Notched nonoverlapping areas between species' boxes indicate statistically significant differences at a rough 5% (Chambers et al. 1983).

statistically significant. Leopard dace have a slightly higher median value and a larger range of flow tolerances than Umatilla dace. The tendencies and trends of the laboratory flow tolerance data for Umatilla dace are nonetheless still clearly intermediate to speckled and leopard dace.

All the aforementioned flow tolerance relationships for the two laboratory hybrids and the three dace species measured at the bottom and at 60% mean depth are overall and specifically very similar. The relationship between water velocity at the bottom and at 60% mean in the flow tube is very strong (Fig. 37C, r = 0.99; also see Fig. 26C in Chapter 5), and much more so than that found under natural stream conditions (Fig. 25C in Chapter 5, r = 0.73). This suggests that, unlike in the 'wild', there are no real or statistically significant differences in water flow at the bottom and at 60% mean depth in the artificial environment of a completely round and narrow experimental flow tube.

As with the results for the laboratory crosses of the three dace species (Chapter 5), there is only a very small, yet consistent, interspecific difference of about 0.05 cm/sec in the water flow tolerances of the three dace species and of the two laboratory hybrids in the flow tube at water velocity a both depths (Fig. 37). Natural streams have higher water velocities at 60% mean depth too (Figs. 25A-C in Chapter 5), but their flow differences between bottom and 60% mean depth are much more substantial and statistically significant (Fig. 25C in Chapter 5). These differences between the flow tube and natural measurements must also at least partly be the result of the flow tube being of limited diameter and being simply somewhat less realistic (Fig. 24 in Chapter 5).

Flow Preference Data - 'Wild'

The comparison of the natural stream flow data collected in the 'wild' to that for the laboratory crosses of only the three dace species were already presented in Chapter 5 (Figs. 25 and 26). These data are further discussed here only in comparison to the two hybrid crosses and also particularly to Umatilla dace. Comparisons of 'wild' and laboratory data can only be of overall water flow patterns and trends because they necessarily, differentially, and respectively represent flow preferences and flow tolerances. These overall patterns and trends of laboratory flow tolerances for the three dace species (Figs. 37A-B) match the flow preference data taken for them in the 'wild' (Figs. 25 and 26 in Chapter 5).

Discussion

Systematics

As was already demonstrated in more detail (Chapter 5), the ecologies of the three dace species are distinctive based on water flow preferences taken under natural conditions (also see Hughes and Peden 1989, Peden 1991, Peden and Hughes 1988) and on water flow

tolerances derived through laboratory experimentation. Specific habitat segregation and reproductive isolation are important and key components of many species recognition protocols (Blouws 1998), but especially of the biological species concept (Brust et al. 1998, Dowling et al. 1989, Greenfield and Greenfield 1972, Mayr 1963, 1969, Nelson and Hart 1999). Umatilla dace have and maintain their own ecology and evolution distinct from those for leopard and speckled dace based on these water flow criteria. These tests of their distinctiveness as a species are also again done using allopatric populations where some breakdown of species' boundaries might be at least theoretically expected.

Many of the morphometric / meristic distinctions and their allometries for Umatilla dace, and for leopard and speckled dace, are strongly correlated to their water flow preferences and tolerances (for drawings and photographs see Figs. 14-15 in Chapter 2, Figs. 31-34 in Chapter 6, and in Peden and Hughes 1988). This is even the case for the slight maternal effect (Cathey et al. 1998, Crespin et al. 1999) in flow tolerance seen here for the two laboratory hybrid crosses which is mirrored by a similar maternal effect on their overall PC2 shape vector scores (Fig. 29 in Chapter 6). The same or similar morphological specializations have been determined in other studies on other benthic fish species (Baltz et al. 1982, Bartnik 1972, Garner 1997, Lauder 1990, Mills 1991, Schaefer 1991).

Evolution

Umatilla dace have natural water flow preferences and experimental laboratory water flow tolerances that are intermediate to those of leopard and speckled dace. This is a very likely expectation for a species that is hypothesized to have evolved through hybridizations of speckled and leopard dace (Arnold 1992, 1997, Brust et al. 1998, Dowling et al. 1989, Greenfield and Greenfield 1972, Stauffer et al. 1979). Laboratory hybrids made from crosses of both possible combinations of leopard and speckled dace are also intermediate to those parental dace species. The two laboratory hybrid crosses are most similar to Umatilla dace, and not to leopard and speckled dace. Furthermore, the only dace of the three species that is not different on a statistically significant level from the two laboratory hybrid crosses is Umatilla dace.

The two laboratory hybrid crosses most closely resemble Umatilla dace based on morphometric and meristic data as well (Chapter 6; for drawings and photographs see Figs. 14-15 in Chapter 2, Figs. 31-34 in Chapter 6, and in Peden and Hughes 1988). Umatilla dace and the two laboratory hybrids are also intermediate to speckled and leopard dace for the majority of these univariate / bivariate measures and always in the case of their combined multivariate analyses.

Many of these morphological differences can be tightly correlated to water flow conditions and they make intuitive sense (Garner 1997, Mullen and Burton 1998, Persson 1991, Schaefer

1991). Variable characteristics like caudal peduncle depth, tail falcation / indentation, dorsal and anal fin length, and pelvic fin lengths and stays have fairly obvious relationships with swimming and holding capacity in fish. Umatilla dace and the two laboratory hybrid crosses both also show a mild trend towards an intermediate mouth width and barbel size which might again be significant in terms of their species differentiation through diet (Figs. 2 and 3 in Chapter 2; Lammens and Hoogenboezem 1991, Persson 1991, Sibbing 1991, Turner and Grosse 1980, Tyler 1993).

A possible theoretical expectation was that both laboratory hybrids might be expected to have broader water flow tolerances (Stone 2000). The laboratory hybrid crosses are first generation (F1) and no strong selective pressures would have been placed on them during rearing under identical no flow conditions and satiation feeding opportunities. There is no genetic evidence that Umatilla dace are early generation hybrids (Chapters 4 and 7), and they could be assumed to have had a long co-evolutionary history with their specific environments (Turner 1971). The laboratory hybrids were not found to have any broader water flow tolerances or morphometrics and meristics (Chapter 6). This possible theoretical expectation was not met. This result may very likely be due to the Umatilla dace used for water flow experimental testing also being reared under the reduced selection environment of the same no flow and feeding conditions.

The two laboratory hybrid crosses are both more similar to leopard than speckled dace in their water flow tolerance. The range of water flow preference and tolerance is higher for leopard and Umatilla dace than for speckled dace. This is likely because fish that can tolerate a higher water flow regime can presumably live under slower water conditions if necessary or desired as well. The opposite situation where a species has evolved to a slower water regime probably does not provide that fish with the ability to exist in faster water (also see data in Bartnik 1970a, Gee and Northcote 1963, Gibbons and Gee 1972, Gryska et al. 1998, Johannes 1958, Mullen and Burton 1998). In the data here, the faster water species of leopard and Umatilla dace showed no central tendencies to choose to live in slower water even though all the dace species were sampled or reared from allopatric situations and parentals. If the flow tolerance data is reanalyzed so that the boxplots display statistical outliers and do not encompass the full data range, then those outliers are only found at the lower water velocities for leopard and Umatilla dace (Haas unpubl. data).

Water flow preferences and tolerances may not only be associated with the maintenance and evolution of Umatilla dace, but could also be part of the mechanism of its original formation (Balon 1992, Hubbs 1955, Krupka and Holcik 1976). Speciation of Umatilla dace is hypothesized to have occurred during the breakdown of natural flow environments during the last Pleistocene glaciation (Chapter 1). That all three dace species have particular water flow

preferences and tolerances supports this possible mechanism, as particularly does the intermediate specializations of Umatilla and both types of laboratory hybrids of leopard and speckled dace (Arnold 1992, 1997, Brust et al. 1998, Dowling et al. 1989, Gorman 1992, Greenfield and Greenfield 1972, Tauber and Tauber 1989).

The Umatilla dace used for all the water flow preference and tolerance measurements came from the Similkameen River. These specific Umatilla dace are most closely related to leopard dace on sequence data from cytochrome-b mitochondrial DNA and from a region of the internal transcribed spacer (Figs. 19-20 and Table 1 in Chapter 4; appendices C and D). The two laboratory hybrid crosses are also more like leopard dace in both their water flow tolerances here and in these same DNA sequence data (Figs 32-33 in Chapter 7; appendices C and D). The laboratory hybrid crosses involving a leopard dace female are more closely related on the basis of this DNA data and exhibit slightly higher median and overall water flow tolerance than the laboratory hybrid crosses made with a speckled dace female (Crespin et al. 1999). None of these flow differences are statistically significant.

These intriguing results could still be considered as lending some support to the more specific and localized hypothesis that the evolution of Umatilla dace in this population from the Similkameen River are more derived from leopard than speckled dace (Chapter 11). The flow tolerance data could again be thought of as providing a possible mechanism for the origin and evolution of Umatilla dace, but also then for this particular population and not only for the species as a whole. It must be noted that the shorter 28s D3B genomic DNA sequence based on far fewer fish and populations does not support this relationship of Umatilla dace from the Similkameen River to leopard dace, but rather places them with speckled dace (Fig. 23 in Chapter 4). As well, no D3B data were collected on the laboratory crosses.

Fisheries and Habitat

The additional water flow tolerance data collected on the two possible laboratory hybrids of leopard and speckled dace supports the original contention made just on the data from the three dace species (Figs. 25C and 26C in Chapter 5) that bottom flow is probably a much better estimate of such habitat use by dace. The standard fisheries and habitat water flow measurement of 60 % mean depth can nonetheless still accurately determine and distinguish flow tolerances for these dace species and laboratory hybrids. It could thus still be considered for the legal and regulatory criteria that it is generally used to establish. However, the 60 % mean depth measurement is slightly higher for all this flow data collected, and probably as a natural consequence of taking it away from the benthic surface. A true representation of water flow tolerance for benthic fish should involve measurements taken at the bottom of a stream. Consideration of which water flow measurements to take and use for fisheries and habitat work

is worthwhile, especially if they are on benthic species and if their objectives involve biological representation.

PART IV – GENERAL DATA

Chapter TEN

General Data - Fish Hybrids and Hybridization

Materials and Methods

The data here are based on the substantial summaries and catalogues of publications on fish hybrids and hybridization published by Schwartz (1972, 1981). Although a bit dated, the literature coverage up to that time has been generally accepted a reasonably thorough (McPhail, pers. comm.). Schwartz (1972) lists 1945 references, and Schwartz (1981) has 1814 citations. This literature examination is of a total of 3759 citations.

There are undoubtedly some biases in the literature on which hybrids get reported, published, and have additional subsequent research conducted. However, there is no large apparent reason why this bias would favour dace (*Rhinichthys* spp.) or their minnow Family Cyprinidae.

These literature surveys and catalogues were summarized for all citations on native freshwater fish in North America that unequivocally dealt with situations of natural hybridization. These were counted for what taxonomic family membership was involved. Within the Family Cyprinidae, they were further numerated as to whether they dealt with dace or with the other minnows in that taxonomic group, and as to whether the hybridization had occurred west or east of the Continental Divide (Rocky Mountain range). A similar analysis was also been undertaken by Hubbs (1955; also see Sorensen 1978).

Dace are a member of the minnow Family Cyprinidae, and this analysis deliberately involved all their five recognized species (Lee et al. 1980, Robins et al. 1992) and Umatilla dace (*R. o. umatilla*). This was done in order to provide a more general and logistically simpler assessment and to do so across North America. This summary was then not just for speckled (*R. osculus*), leopard (*R. falcatus*) and Umatilla (*R. o. umatilla*) dace, but also for blacknose (*R. atratulus* (Hermann)), longnose (*R. cataractae*) and Umpqua (*R. evermanni* Snyder) dace.

The assessments of natural hybridization were based on information in the provided titles and / or on my professional judgement. Individual readings of each publication in the total summary were probably warranted, but that task was well beyond the simple scope of, and timeframe for, the general assessment desired here. This limitation of my assessment protocol is openly acknowledged, but it is my strong and defendable argument that the overall patterns and trends presented here would not have overly changed as a result of a complete individualized reading of each publication. I had and was aware of many specific papers reviewed by Schwartz (1972, 1981), and my selection process to include or not include that particular reference was not changed from that I would have made based on its title and my

judgement alone. The papers that were selected as pertinent to this assessment were each examined and all corresponded to my categorization and selection of them as appropriate.

Results

The numbers of natural hybrids of native freshwater fish in North America reported and published on are far higher for the minnow Family Cyprinidae than for any other pertinent taxonomic family (Fig. 38) catalogued by Schwartz (1972, 1981). These results are consistent with Hubbs (1955). There are almost four times as many such hybrids reported for the Family Cyprinidae. Most of the dace hybrids reported were for longnose dace, and then for blacknose and speckled dace. This tally probably at least partly reflects the overwhelmingly largest distribution of longnose dace amongst these dace species, and also in comparison to the ranges of virtually if not all other freshwater fish in North America (Lee et al. 1980). Within dace, the next largest ranges after longnose dace are for blacknose and speckled dace.

Within the Family Cyprinidae, the hybrids have a very strong component represented by the dace genus *Rhinichthys*. Dace composed slightly more than one-quarter of these natural native freshwater fish hybrids (Fig. 38A). When the Family Cyprinidae is examined from the geographic perspective of the Continental Divide, 65 % of their hybrids were from drainages on the west side (Fig. 38B).

Discussion

The minnow Family Cyprinidae in general, and dace (*Rhinichthys* spp.) species in particular, show the highest levels of hybridization of any freshwater fish in North America (Fig. 38). The number of genera and species in the Family Cyprinidae is also the highest of any taxonomic family of freshwater fish in North America (Howes 1991, Mayden 1991, Nelson 1984), but their numbers of hybrids are still probably disproportionately higher even when this is accounted for (Hubbs 1955). The minnows are also almost all essentially commercially unimportant and presumably therefore less well studied with fewer resultant literature publications. The dace genus definitely has higher levels of hybridization that are completely out of proportion to its study and its taxonomic representation as a single genus composed of five recognized species as examined here.

These data suggest that hybridization could be an important mechanism in the evolution of many freshwater fish in North America (Hubbs 1940, 1955, Miller 1961, Smith 1992, Stauffer et al. 1979; for Europe see Collares-Pereira 1989, Zardoya and Diadrio 1999), and not just for the three species of dace studied. The phenomenon may nonetheless have particular importance for the Family Cyprinidae and for dace. Hybridization in fishes has long been argued as higher

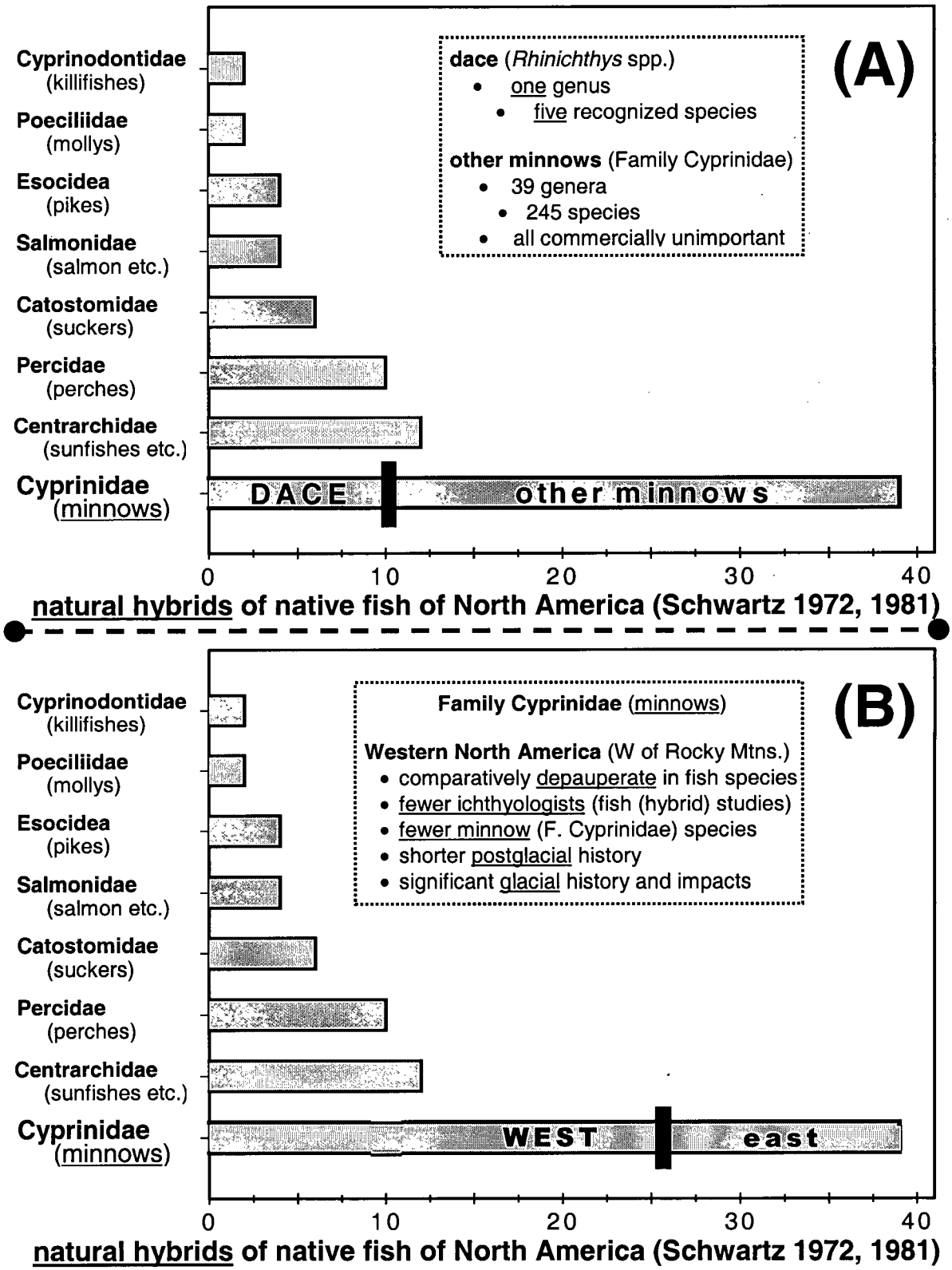


Figure 38. Histogram (A) showing the high level of published hybridization studies for dace within their Family Cyprinidae (minnows) in North America, and also of the Family Cyprinidae itself. Histogram (B) is the same basic figure but instead shows the comparatively higher number of published hybridization studies in the Family Cyprinidae for watersheds west of the Continental Divide within North America. This increased level of hybridization in minnows in western North America is in spite of the first three listed factors that could be thought of as limiting their study and their degree of overall hybridization.

than in other taxonomic groups (Bullini 1985, Hubbs 1955, Sorenson 1978, Smith 1992, Verspoor and Hammar 1991), and more recent genetic research has often confirmed or detected hybridization that was suspected or even completely hidden. In this manner, several fish and other species have been found that morphologically and ecologically behave as a particular distinct species while carrying genetic or physiological evidence attributable only to past hybridization (e.g. Bernatchez et al. 1995, Crawford et al. 1999, Taylor et al. 1999, Wilson and Bernatchez 1998).

Hybridization may not just be the curiosity that it is still generally attributed to, and at least was in the recent past even by most biologists studying the phenomenon (Arnold 1992, 1997, Endler 1989, Stebbins 1959, Straw 1955). This may be especially true for fish, the minnow Family Cyprinidae, and dace (Dowling and DeMarais 1993, Hubbs 1955, Miller 1961, Wiley and Mayden 1985). Hybridization also must move more beyond its general perception as a negative force in evolution (Arnold 1997, Lewontin and Birch 1966, Stone 2000). Hybrids are still usually viewed as detrimental to the integrity of species and often thought of as being restricted to narrow hybrid contact zones between otherwise dominant species (e.g. Barton and Hewitt 1985, Harrison 1993, Hewitt 1989). In conservation biology work and assessments, hybrids are still usually taken to lessen the status of a species or as a sign of its deleterious health. The argument is not that this may not be the case or that hybrids are all good, but more that hybrids are also not necessarily all bad (DeMarais et al. 1992, Dowling and DeMarais 1993).

Limited natural hybridization may provide temporary refuge for a species in populations or even over larger geographic areas (Dowling and Hoeh 1991, DeMarais et al. 1992, Lewontin and Birch 1966). If the overall numbers of a species or one of its populations is decreased to levels insufficient for its immediate survival then there is possibly little alternative for survival but reproduction outside its boundaries (Buth et al. 1991, DeMarais et al. 1992, Dowling et al. 1989). This occurrence is certainly not uncommon or unnatural in fishes or other taxonomic groups (Arnold 1997, Hubbs 1940, 1955, Stauffer et al. 1979), especially toward the edges of a species' range where it may already be more at tolerance maximums and where the often studied hybrid zones usually have been determined. Such hybridization is further influenced by the proportional numbers of other related species present from which viable hybrids can be formed (DeMarais et al. 1992, Hubbs 1955). Hybridization can also be argued to incidentally provide benefits for maintenance and survival (DeMarais et al. 1992, Rieseberg et al. 1996), especially perhaps in the harsher conditions it is often believed to have originated in (Huxel 1999, Potts and Reid 1988, Tauber and Tauber 1989).

The further evidence in the literature is that natural hybrids within the Family Cyprinidae are more abundant west of the Continental Divide in North America (Fig. 38; Hubbs 1955, Miller

1961, Schaefer 1991) and once more within dace. This estimate of hybridization is also again out of proportion to the relative number of recognized minnow and other fish species east and west of the Continental Divide (Avise and Ayala 1976, DeMarais et al. 1992, Dowling and DeMarais 1993, Miller 1961). It does not reflect the number of working biologists and their timeframe of study under which such hybrids are likely to be examined or even simply encountered and recognized.

The main reason though for emphasizing the published hybrid numbers west of the Continental Divide is to highlight the possibility that hybridization is increased there due to the western region's overall stronger and somewhat more recent glacial and postglacial histories and impacts (Dowling and Hoeh 1991, Hubbs 1955, Miller 1961). Hybridization may have been a more common refuge for the maintenance of species or at least significant components of their genome.

The evidence in this overall study has been interpreted to demonstrate that Umatilla dace evolved several related times in a geologically limited time frame through hybridizations of leopard and speckled dace. This hybridization is further argued to have occurred as a result of, and / or in response to, the last Pleistocene glaciation (Dowling and Hoeh 1991, Hubbs 1955, Miller 1961). It also possibly provided immediate benefits for survival, and permitted short and long term successful ecological specialization (Huxel 1999, Kosswig 1963, Potts and Reid 1988, Rice and Salt 1990, Stauffer et al. 1979, Stone 2000). While such hybridization is most likely still not a common occurrence or speciation mechanism, it should probably not be viewed or dismissed solely as an oddity (DeMarais et al. 1992, Tauber and Tauber 1989). Modern situations in reservoirs that are somewhat analogous to this Pleistocene glaciation and speciation scenario have nonetheless been reported to have early generation hybrids (Aspinwall 1968, Aspinwall et al. 1993a/b, Butcher 1980) and even distinct fish species resulting from those hybridizations (Balon 1992, Krupka and Holcik 1976). Any incidence of hybridization and the phenomenon in general should probably receive true consideration in such an overall context before immediate dismissal (Funk 1985, DeMarais et al. 1992, Dowling and DeMarais 1993, Hubbs 1955, Stauffer et al. 1979, Stebbins 1959, Stone 2000, Straw 1955).

PART V –

GENERAL DISCUSSION AND SUMMARY

Chapter ELEVEN

General Discussion and Summary

Biology and Background

Hybridization may not be a major force in speciation and evolution, but it warrants consideration beyond the little it receives now (Arnold 1997, DeMarais et al. 1992, Stone 2000). The research emphasis on the differential mortality of hybrids in restricted hybrid zones should incorporate consideration of hybridization beyond this narrow focus (Endler 1989). A similar bias exists in the interpretation of many hybrid situations that are solely involving parthenogenesis or polyploidy. These views have also engendered a particular mechanistic view towards hybrids and their formation which may be a large part of their present consideration as generally inferior (Arnold 1997, DeMarais et al. 1992, Stone 2000). Hybridization has continued to be suggested as a viable and possible sympatric speciation mechanism (Arnold 1997, Dowling and Brown 1989, Hubbs 1955, Stauffer et al. 1979, Stone 2000). The notion that hybridization itself can generate new bisexual animal and other species warrants more discussion (Arnold 1997, Hewitt 1990, Scudder 1974, Stone 2000, White 1959).

The situations where such speciation and evolution through hybridization might be established and found have many common characteristics (Arnold 1997, Stone 2000). They would also seem to need to pass through a reasonably well known sequence of expectations in order to become independent and established species. The initial hybridizations are usually in conjunction with some range expansion or natural or anthropogenic event that disrupts natural isolating mechanisms. The hybrids that result should have increased variability and often capacity to draw on which might preferentially enhance their survival under these conditions. The hybrids tend to be intermediate in most of their characteristics (Arnold 1997, Hubbs 1955, Stebbins 1959, 1971), both morphometric / genetic and ecological, but also with some unique non-intermediate variables supporting an independent evolution as well (Bloom 1976, Jordan 1991, Stone 2000).

The hybrids must then be able to establish ecological specialization and reproductive isolation in sympatric conditions (Gillespie 1976, Hoekstra et al. 1985, Levene 1953, Maynard Smith 1966, Tauber and Tauber 1989, Taylor 1976), with the latter often seen as at least a possible theoretical byproduct of selection for those initial habitat preferences (Diehl and Bush 1989, Garcia-Dorado 1986, Rausher 1984, Rice 1984, 1987, Rice and Salt 1990, Udovic 1980). This can occur through occupation of new habitats or through spatial partitioning of underexploited ones (Arnold 1997, Rosenzweig 1978, Schilthuizen 2000), and at even with weak habitat preferences and moderate fitness differences as demonstrated theoretically (Diehl

and Bush 1989, Felsenstein 1981, Futuyma and Mayer 1980, Futuyma and Moreno 1988, Grant and Grant 1989, Seger 1985) and with actual data (Jaenike and Holt 1991, Jones 1980, Jones and Probert 1980, Jordan 1991, Parsons 1983, Plante et al. 1989, Rice and Salt 1990, Rosenzweig 1991, Tauber and Tauber 1977b, Turner and Grosse 1980). Habitat specialization can be further accomplished or enhanced by learning or conditioning (Diehl and Bush 1989, Futuyma 1989, Grant 1986, Immelman 1975, Maynard Smith 1970), social selection (Larson et al. 1984, Lens et al. 2000, West-Eberhard 1983) and sexual selection (Blouws 1998, Kaneshiro 1990).

This evolution through hybridization scenario is perhaps most possible in fish in other members of the minnow Family Cyprinidae and for those in western North America (DeMarais et al. 1992, Dowling et al 1989, Smith 1992). The minnows have been recognized for their widespread interspecific and intergeneric hybridization (Chapter 10; DeMarais et al. 1992, Hubbs 1955). These cyprinids are genetically similar and hybridization is not as deleterious under such conditions. This lack of genetic differentiation also likely increases the importance of ecological separation in maintaining cyprinid species (DeMarais et al. 1992, Howell and Villa 1976, Otte 1989, Patton and Smith 1989).

Western North America has always been more depauperate in freshwater fish species (Smith 1981) than eastern North America and it was strongly affected by Pleistocene glaciation (McPhail and Lindsey 1986, Miller 1961). Natural hybridization occurs more in depauperate regions (DeMarais et al. 1992, Hubbs 1955, Smith 1992) and in areas affected by glaciation (DeMarais et al. 1992, Dowling and Brown 1989, Dowling and Hoeh 1991, Kat 1985, Remington 1968, Smith 1992, Spence 1990). Hybrids may also be well suited to survive glaciation and to better disperse (Potts and Reid 1988, 1990, Schemske and Morgan 1990) into the new and empty habitats present after glacial retreat (Hewitt 1989, 1996, Stebbins 1971).
A hybrid's differences relating to habitat specialization would also have existed before deglaciation so they could have been more likely to simply diverge if these differences facilitated coexistence (Otte 1989). In fact, morphological divergence is usually thought to precede reproductive isolation in fish (Dobzhansky 1951, Smith and Todd 1984). Initial hybridization in the dace during glaciation may also have been substantial and the likelihood that hybrids continued to mate true could thus increase (Stebbins 1959, Mossakowski et al. 1990).

This disproportionate level of hybridization in a region historically presenting environmental conditions favouring it, suggests some of its cyprinid species may have sympatrically evolved through hybridization events (Chapter 10; DeMarais et al. 1992, Hubbs 1955, Miller 1961). The specific case of the evolution of Umatilla dace (*Rhinichthys osculus umatilla*) through hybridization hypothesized and argued for in this study fits these overall criteria. Umatilla dace

are a distinct species with a distinct ecology that permits them to remain differentiated and reproductively isolated from leopard and speckled dace. The breakdown of the important characteristics of those ecological parameters occurred during the last Pleistocene glaciation, and both permitted their initial and immediate subsequent evolution. Some aspects of this and other hypotheses for the evolution of a species through hybridization may never be absolutely proven, largely because one cannot yet go back in time to do so. However, this study has tried to obtain and present sufficient good support for the plausibility of the hypothesis and presents it as the most parsimonious explanation (Arnold 1997, Stone 2000).

Specific Hypothesis - Umatilla Dace

Three basic criteria for this hypothesis were stated in the 'General Introduction' (Chapter 1) to this thesis. These were as follows, and each has been well addressed by the data collected and tests conducted for this study. They will be discussed in turn below.

1. Umatilla dace are a distinct biological and evolutionary species from leopard and speckled dace. Umatilla dace exist distinctly in allopatry and sympatry with these other two dace species.
2. Umatilla dace originally evolved from hybridizations of leopard and speckled dace. Mechanisms for the original hybridization event and the subsequent differentiation and maintenance of Umatilla dace are required.
3. This original evolution was made possible by, and occurred during and immediately after, the last Pleistocene glaciation. The hybridizations of leopard and speckled leading to the formation of Umatilla dace were in particular intimately connected to the large glacial lakes formed during that period.

Systematics

Umatilla dace are a distinct species from leopard and speckled dace and presumably from all other dace and fish then as well. They are differentiated from leopard and speckled dace throughout their large mutual ranges in the interior portion of the Columbia River basin by many data types from morphometrics / meristics (Chapters 2 and 6), molecular genetics (Chapters 4 and 7), and ecology (Chapters 5 and 9). These differences for Umatilla dace are at least as large as those seen and designating other accepted species in the Family Cyprinidae (DeMarais et al. 1992, Howes 1991, Schaefer 1991, Simons and Mayden 1999, Stauffer et al. 1979). Artificial laboratory crosses of these three dace species reared under identical conditions at and for the same time period produced specimens that continued to only resemble their parental types collected under natural conditions in the field (Brust et al. 1998, Haas and McPhail 1991). Umatilla dace have specific discrete habitat preferences based on water flow regimes, and these preferences existed even in the artificial laboratory crosses where they were

reared under no flow conditions. These ecological specializations to water flow would seem to have a basis in the inheritance of a specific adapted body form and shape (Cherry et al. 1982, Larson 1989). Umatilla dace appear reproductively isolated, and this again seems to be based on their ecological specializations (Tauber and Tauber 1989, Turner and Grosse 1980).

Umatilla dace have been speculated to just be hybrids of leopard and speckled dace, but there is no good evidence in this study supporting recent hybridization between these three dace species (Stauffer et al. 1979). First generation artificial laboratory hybrid crosses of leopard and speckled dace do very much resemble Umatilla dace in morphometrics and meristics, but there remain differences, particularly in the molecular genetic data, that indicate that this hybridization is ancient and not early generation (Brust et al. 1998, Dowling and Hoeh 1991, Goodfriend and Gould 1996, Rieseberg and Linder 1999). Umatilla dace also exist as different 'forms' or 'groups' within their range, but their within-species variability is not higher than their between-species variability. Their within-species 'forms' or 'groups' do not imply at all that Umatilla dace are not a distinct entity and species (DeMarais et al. 1992, Dowling et al. 1989, Martin and Simon 1990, Mukai et al. 1997). Umatilla dace definitively have and retain a particular distinguishable phenotype, and this is good evidence of convergent evolution from multiple past hybridizations (Arnold 1992, 1997, Rieseberg and Linder 1999). These details will be discussed in full detail in the following section on the 'Evolution' of Umatilla dace.

Umatilla dace also exist in allopatry, parapatry, and all possible combinations of strict sympatry with respect to leopard and speckled dace. Umatilla dace are also abundant and more often dominant in their distribution at least in the USA (DeMarais et al. 1992, Dowling et al. 1989, Greenfield and Greenfield 1972, Hubbs 1955, Martin and Simon 1990). Furthermore, Umatilla dace and the other two dace species all show a reduction in their overall morphometric variability when they occur in strict sympatry with each other (Jordan 1991). This is additional and critical support for species status under species' concepts like the biological (Mayr 1963, 1969, Nelson and Hart 1999) where maintenance of a distinct genome through reproductive isolation is the main tenet.

The molecular genetic data supporting the uniqueness and distinction of Umatilla dace are based both mitochondrial DNA sequences and two types of nuclear DNA sequences. There is overall congruency between these DNA sequence data sets (Arnold 1997, DeMarais et al. 1992). Longnose dace (*R. cataractae*) are distinguished from Umatilla dace and leopard and speckled dace by the nuclear DNA sequence data collected in this study as well.

Nomenclature - Umatilla dace (*Rhinichthys umatilla* (Gilbert and Evermann))

Umatilla dace are presently recognized only as a subspecies of speckled dace by the American Fisheries Society (*R. osculus umatilla*; Robins et al. 1991), whose judgement on such nomenclature and species status is generally accepted as definitive in North America. The

work of Peden and Hughes / Hughes and Peden(1988, 1989) was not judged sufficiently convincing to warrant species status for Umatilla dace. Canada seems to have gone ahead and unilaterally given it species status, probably due to the work of Peden and Hughes (1988) mostly taking place here and because of the likely real rarity of Umatilla dace in Canada and resultant conservation concerns (Haas 1998, Hughes and Peden 1989, Peden 1991, Peden and Hughes 1984). Umatilla dace in Canada are only found in British Columbia (BC), where they are recognized as a species (*R. umatilla*) by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC - see Peden and Hughes 1988) and in BC by the provincial government Conservation Data Centre (CDC - see Cannings and Ptolemy 1998, Haas 1998).

The actual nomenclatures for the three dace species has a lengthy complicated history. Speckled and leopard dace are already long accepted as species by the American Fisheries Society (Robins et al. 1991), and a review of the information on their nomenclatures is deferred. The timeframe of their present specific nomenclature is usually attributed to Hubbs and Miller (1948) for speckled dace and Carl and Clemens (1948) for leopard dace. This assessment of the nomenclature for Umatilla dace is limited to a literature and published data review since type specimens were understandably not available for outside loan and finances did not permit their examination in a distant museum collection (Lee et al. 1980).

The original description of Umatilla dace (Gilbert and Evermann 1894) does seemingly match that summarized by the data here (also see Hughes and Peden 1989, Peden and Hughes 1988). Umatilla dace were somewhat accepted as a species (e.g. Schultz 1936) until seemingly about the 1950s. Since that time, they have been variously attributed subspecies status with speckled or leopard dace (e.g. Carl et al. 1977, McPhail and Lindsey 1986). The original description of Umatilla dace placed them in the genus *Agosia*, but that was also the placement of the other two and still more dace species at that time. Subsequent work and opinion has now put all these and other presumed related dace species in the genus *Rhinichthys* in the minnow Family Cyprinidae. There thus seems to be no reason to question their original common name and species level nomenclatural designation of Umatilla, and their present generic group as *Rhinichthys*. This would also be in accordance with the conventions and priorities of nomenclature accepted by the American Fisheries Society (Robins et al. 1991). It is the recommendation of this study that Umatilla dace being designated as a distinct species and that its name be Umatilla dace *Rhinichthys umatilla* (Gilbert and Evermann).

Evolution

A most parsimonious interpretation of this study's data on the evolution of Umatilla dace is that it occurred through multiple past hybridizations of leopard and speckled dace during and immediately after the Wisconsinan glaciation (Arnold 1997, DeMarais et al. 1992, Kat 1985, Rieseberg and Linder 1999, Smith 1992, Wiley and Mayden 1985). The events which led to

their original hybridizations and contributed to the species success of Umatilla dace were the large glacial lakes formed as a result of ice movement and damming during the last Pleistocene glacial epoch (Allen et al. 1986, Bretz 1919, Bunker 1982, Malde 1965, 1968, Waitt and Atwater 1989). The mechanism that broke down and could have easily permitted their ongoing hybridizations at that time is their specialization to particular water flow regimes (Aspinwall et al. 1993a/b, Balon 1992, Hubbs 1955, Stauffer et al. 1979). The present distribution of, and variability in, Umatilla dace argues strongly for their evolution during such a past time period and within such a physical and geographic association (Arnold 1997, Hewitt 1996, Martin and Simon 1990, Mukai et al. 1997, Rieseberg and Linder 1999).

Umatilla dace are intermediate to leopard and speckled dace in almost all their studied characteristics. They have intermediate morphometrics / meristics (Chapters 2 and 6), distribution (Chapter 3), ecology (Chapters 5 and 9), and to some degree in nuclear DNA sequence (Chapters 4 and 7) for a region of the internal transcribed space (ITS). Umatilla dace were even considered as possible hybrids of leopard and speckled in their original description (Gilbert and Evermann 1894), and within Canada (Carl et al. 1977, McPhail and Lindsey 1986). Their distribution is not only intermediate, but it is largely within the overlapping ranges of speckled and leopard dace (Barraclough and Vogler 2000, Funk 1985, Hubbs et al. 1974, Mayden 1987). Umatilla dace have the highest levels of genetic diversity of these three dace species (Arnold 1997, DeMarais et al. 1992, Stone 2000). This is all very indicative of evolution through past hybridizations of leopard and speckled dace (Rieseberg and Linder 1999).

In spite of their differentiation and distinction as a species, Umatilla dace populations show a relationship to either speckled or leopard dace (Cathey et al. 1998, Crespin et al. 1999). This relationship is seen, and its interpretations are reinforced, by the two laboratory hybrid dace crossed from leopard and speckled dace (Brust et al. 1998). These laboratory hybrid dace also demonstrate the same closer association with speckled or leopard dace, but clarify that it seems to be a maternal effect (Tegelström and Gelter 1990). This is supported by the mitochondrial DNA sequence data as well.

Umatilla dace as studied here are composed of three principal groups based largely on both mitochondrial and nuclear DNA sequence data, with some discordancies between them (Chapters 4 and 7). The specific congruencies and discrepancies in the DNA data seen here have both been argued to be very good evidence for historical hybridization (Arnold 1997, DeMarais et al. 1992). There could have been more groups immediately after Wisconsinan glaciation or presently if more data had been examined (Dowling and Hoeh 1991, Martin and Simon 1990). Umatilla dace are nonetheless still overall very and most similar as a single species, and seem to have converged on a specific and consistent phenotype and ecology (Good 1989, Larson 1984, 1989, McPhail 1992, McPhail and Lindsey 1986). The presence of

these groups largely in Umatilla dace is further evidence of multiple past hybrid origins (Arnold 1997, DeMarais et al. 1992, Dowling et al. 1989, Kat 1985, Martin and Simon 1990, Rieseberg and Linder 1999, Smith 1992).

The first type of Umatilla dace are found in the Similkameen River, and their population here was more closely related genetically and in ecological water flow preferences to leopard dace. They are the second most distinct Umatilla dace based on the molecular genetic data, outside of the unique Yakima River. These Umatilla dace are from a geographically and glacially distinct northcentral portion of the Columbia River basin (Crandell 1965, Porter et al. 1983, Waitt and Thorson 1983). This region would have required the farthest postglacial recolonization, and its watershed in Canada is above a possible barrier found just below the border between Canada and the USA. Enloe dam now sits on this possible barrier and forms a complete one. Of the three dace species, only Umatilla dace here are found above the barrier. However, this drainage formed part of one of the two Wisconsinan glacial connections between the Fraser and Columbia rivers (Mathews 1944, Fulton 1967, 1969), and leopard dace are found well into Canada and much farther upstream in the Fraser River. Leopard dace have shown the farthest postglacial recolonization and highest water flow preferences and tolerances, which could also be related to the presence and this grouping of Umatilla dace. Outside of Umatilla dace, the other predominant dace species in the Similkameen River below the present Enloe dam site and in the surrounding Fraser River watershed is leopard dace (also see Peden 1991). Overall, Umatilla and leopard dace sympatry is amongst the least common found.

In spite of having the largest distribution in regions recently glaciated during the last Pleistocene glacial epoch, leopard dace have the lowest within-species variation. This level of variability in terms of dispersal and peripheral populations largely matches accepted theory and the relationship of the Umatilla dace in the Similkameen River to leopard dace (Arnold 1997, Barraclough and Vogler 2000, Lewontin and Birch 1966, Martin and Simon 1990, Mukai et al. 1997). This theoretical basis is mostly from hybrid zone studies and indicates that terrestrial organisms in glaciated regions usually have more genetically uniform populations than in unglaciated regions if the species are low dispersers (Barraclough and Vogler 2000, Bellemin et al. 1978, Highton and Webster 1976, Sage and Wolf 1986, Schwaegerle and Schaal 1979), whereas the opposite is true for high dispersers (Hewitt 1989, Wheeler and Guries 1982). Glaciated distant regions may be colonized by only a few more specialized animals and could undergo some type of bottleneck (Barraclough and Vogler 2000, Carson and Templeton 1984, Mayr 1963, Slatkin 1987). Present-day populations then show low genetic diversity (Baker and Moeed 1987, Janson 1987, Martin and Simon 1990, Stone 2000), with the most peripheral populations even further reduced (Lande 1979, Patton and Smith 1989). The most distant

populations from glacial refugia are also thought to have had the largest series of preceding founding events (Barraclough and Vogler 2000, Cwynar and MacDonald 1987).

The second and most common overall group of Umatilla dace is found in the rest of the Columbia and Snake rivers. Speckled dace predominate throughout the Columbia River system and in these regions (also see Lee et al. 1980), and their co-occurrence with Umatilla dace is the most common sympatric pairing. Speckled dace are not directly found with the Umatilla dace in this second group in Canada. These Umatilla dace in Canada are in the lower Columbia and associated Okanagan river basin, and are not found with any of the other three dace species. However, speckled or other Umatilla dace dominate downstream in the Columbia River system. Speckled dace in Canada are only found in the Kettle and Granby river systems, which are also most geographically proximal to this second group of Umatilla dace. The postglacial recolonization of these Umatilla and speckled dace in Canada would have been easier and over a shorter distance, which are in compliance with the reduced water flow preferences and swimming abilities of speckled dace. The genetic variability in these speckled dace is in accordance with their low dispersal, but the Umatilla dace still show differentiation in Canada which may yet again reflect their hybrid origins (Arnold 1997, Barraclough and Vogler 2000, Stone 2000).

There is good evidence of subdivision within this overall second group of Umatilla dace, but all its subgroups are still more related to speckled than leopard dace. The subgroups are also somewhat structured around glaciation. The two noteworthy subgroups of Umatilla dace are from the more central portion of the Columbia River system, or are from more southern areas like the Snake and Grande Ronde river which have somewhat distinct glacial histories. The Snake River was most postglacially impacted by Glacial Lake Bonneville (Malde 1965, 1968, Morrison 1965, Wheeler and Cook 1954) whereas the mainstem Columbia regions were more affected by the greater impacts from Glacial Lake Missoula (Allen et al. 1986, Bretz 1919). Glacial Lake Missoula did still affect parts of this southern region of the Columbia River. The upper portion of the Snake River is also completely isolated above Shoshone Falls, and shows a faunal break and distinction at this point (Hubbs and Miller 1948). The Grande Ronde River drains the Blue Mountain region of Oregon, which had its own distinct glaciation, and has a fish fauna composed of some disjunct species. Strong biogeographic morphometric variability in speckled dace has even been noted near this latter region (Bisson and Bond 1971).

This subdivision is at least partly based on the southern subgroup of Umatilla dace also showing some evidence of older or more complete hybridization (Martin and Simon 1990, Mukai et al. 1997, Rieseberg and Linder 1999). The Umatilla dace here are the only ones that did not overall have legibility problems with their ITS sequences. The readability difficulties with ITS came from them having substantial length variation, which was strongly associated with

hybridization here and in other studies. The southern subgroup of the second main group of Umatilla dace are still clearly of hybrid origin, but it may have occurred somewhat more distantly in the past or has subsequently evolved in a 'cleaner' manner. These Umatilla dace also appear to occur more regularly in strict sympatry with speckled dace than other groups of Umatilla dace.

The third Umatilla dace are found in an unique system in Washington State, the Yakima River. These Umatilla dace are again more closely related to speckled than leopard dace, but in this case the relationship to leopard dace is much more distant. These are the most distinct Umatilla dace based on the molecular genetic data. The Yakima River is a tributary in the northcentral region of the Columbia River. This region was not fully glaciated and not as directly impacted by Glacial Lake Missoula (Crandell 1965, Porter et al. 1983, Waitt and Thorson 1983). The area also had distinct, but smaller, glacial lakes associated with it (Mathews 1944, Fulton 1967, 1969). Such phenomena may at least partly explain the distinctiveness of this system (Arnold 1997, Barraclough and Vogler 2000, Martin and Simon 1990).

All three of these dace species are found only in respective allopatry in the drainages in Canada that they postglacially recolonized (Haas 1998, Johnson et al. 1994, R.L.&L. 1991, 1995, Rosenfeld 1996; but see Hughes and Peden 1989, Peden 1991, Peden and Hughes 1988). Umatilla dace are also indeed rare in most of their distribution in Canada, whereas they are abundant and often the dominant dace species where they occur in the USA. Umatilla dace in the Similkameen River also appear to have a very sporadic distribution that seems related to what greatly reduced proportion of the river's substrate is not concreted by siltation (Haas unpubl. data). Umatilla dace warrant continued conservation consideration in Canada (Cannings and Ptolemy 1998, Haas 1998, Hughes and Peden 1989, Peden and Hughes 1988). As well, leopard dace are locally common and abundant in the Fraser River but appear to very rare in the Columbia River system. This is not the present perception among most fisheries biologists and should be considered from a conservation perspective (Haas 1998; also see Buth et al. 1991, Dowling and DeMarais 1993, Johnston 1999, Winfield and Townsend 1991). This is even true in Canada where leopard dace may be locally abundant, but these locations are sporadic, distant, and consequently more susceptible to individual perturbations.

Suggestions for Possible Further Research

There are several further research possibilities that would fill in minor gaps or add to the data collected in this study. Such additional work would largely be towards further examination of the acceptance or rejection of the hypothesis that Umatilla dace evolved through past

hybridizations of leopard and speckled dace during the last Pleistocene glacial period. These include:

(1) Confirmation and examination of 'leopard dace' from the most interior portions of the Columbia and Snake river basins (Chapters 3 and 4). The data collected and literature reviewed here suggest leopard dace should be present at some level, but also that they may now be found only in greatly reduced numbers in many locations where they previously might have been more abundant.

(2) Additional morphometric and meristic data collection and analysis from specific areas in the mutual distribution of the three dace species in the Columbia River drainage to yet more thoroughly investigate them across their complete ranges (Chapters 2 and 3).

(3) Extend the analysis of the allometric and growth patterns of the three dace species to include their specimens culled at specific size intervals from the laboratory crosses (Chapters 2 and 6). This data collection was done to provide further corroboration of these relationships seen with the cross-sectional data from field and museum specimens (Cheverud et al. 1983, Creighton and Strauss 1986, Strauss and Fuiman 1985). It would be based on a true growth series, and might permit more detailed insights into the respective ontogenies and their relationships in the three dace species (Alberch 1980, Bookstein et al. 1985, Goodfriend and Gould 1996, Gould 1971, Strauss 1987).

(4) Further analyses of the proportional abundance and distributions of the three dace species to determine if there is any further evidence of species displacement (Huxel 1999) or of anthropogenic impacts on their distributions and abundances (also see Hughes and Peden 1989, Peden and Hughes 1988). It would also be of interest to know how this relates to the past hybridization and characteristics of Umatilla dace (Chapter 3).

(5) Further molecular genetic work, and again maybe particularly on the Yakima River if more leopard dace could be collected. Aside from the Yakima River, there is a possible need to undertake more ITS sequencing and particularly on samples for which only mitochondrial DNA data presently exists. A few more speckled dace could also be done, especially from the upper Columbia River sites for which mitochondrial DNA sequence data exists. Again, this could be confounded by the difficulties in working with the ITS data in regard to its high levels of length and site variability. Some additional and specific strict sympatric combinations and sites warrant further analysis in mitochondrial and nuclear DNA too Chapter 4).

(6) Creation and rearing of artificial laboratory crosses between speckled and leopard dace in the Yakima River (Chapters 6, 8, and 9). The Yakima River was the only site of sympatry for all three dace species, and the only overall population in which leopard dace were not seemingly related at all to their two other co-occurring dace species. Any work as

suggested here must remember that only a single leopard dace specimen has been collected from the Yakima River in spite of regular intensive surveys by qualified fisheries personnel.

(7) Water flow preference data should be collected at some sympatric sites for two or all of the three dace species (Chapters 5 and 9). It would be useful to know how their habitat selection is influenced by their co-occurrence with one of their closely related species.

(8) Some additional effort to collect dace in modern man-made reservoirs could provide some insights into hybridization between the dace species (Aspinwall 1993a/b, Balon 1992, Butcher 1980, Izyumov et al. 1998, Krupka and Holcik 1976,). The conditions in the reservoirs could be viewed as similar to those in the glacial lakes, and in particular their impact on water flow (Chapters 3, 5, and 9). However, other variables such as water temperature and the sustained input of non-native fish species into these reservoirs could seriously confound any results (also see Peden and Hughes 1984). This study had no success in obtaining specimens from reservoirs in spite of substantial early efforts. Other studies have collected dace from reservoirs and noted some very minor increase in their possible hybrids (Hughes and Peden 1989, Peden 1991, Peden and Hughes 1988), but the data is based on very small fish and suspect identifications.

(9) A similar investigation into other species or groups (e.g. Dauble and Buschbom 1981), particularly those within or associated with the minnow Family Cyprinidae, could be enlightening (Chapter 10). These might best be done in the Columbia River basin (Geist et al. 1996, McIntosh et al. 2000), or at least in heavily glacially impacted regions of western North America (DeMarais et al. 1992, Hubbs 1955, Smith 1992).

(10) There would be some interest in ethological work to try to determine the level and type of dace species' interactions, particularly during reproduction (also see Kaya 1991). This was qualitatively examined in the course of this study, but not in any good or rigorous experimental design (Blouws 1998). Two males from different dace species were sometimes placed briefly in an aquarium together with one or more females from one or more of the dace species. This occurred during their regular examinations for reproductive condition for making the laboratory crosses (Chapters 6 and 8). The two males did not show any obvious predilection towards any particular female, but this may partly have been because they were seemingly completely occupied in strong rivalry displays in which their natural spawning colouration was also greatly intensified.

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Appendix A

Study Sample Sites

Legend - Museum Abbreviations:

- OSU – Oregon State University;
- UA – University of Alberta;
- UBC – University of British Columbia (all study collections are listed as UBC);
- UW – University of Washington.

Legend - Associated Information:

- **sympatric-_/_** - 12 sites with strict sympatry, and their respective dominant species:
  - if no species is dominant, then no corresponding letter for it is in bold font.
  - symS/L - speckled and leopard dace, with speckled dace dominant (one site);
  - symL/S - leopard and speckled dace, with leopard dace dominant (one site);
  - symS/U - speckled and Umatilla dace, with speckled dace dominant (two sites);
  - symU/S - Umatilla and speckled dace, with Umatilla dace dominant (five sites);
  - symL/U - leopard and Umatilla dace, with leopard dace dominant (one site);
  - symU/L - Umatilla and leopard dace, with Umatilla dace dominant (one site);
  - symS/U/L(3) - all 3 dace species, with Umatilla dominant (one site).
    - note: no other site of strict sympatry for all 3 species was found.
- **flow** – thirteen allopatric sites where parental fish for flow experiments on lab crosses were collected, or where field measurements for flow were taken;
- **genetics** – 23 sites with genetic data analyzed. More were collected but not yet done.
- **lab-cross** – five allopatric sites where parental fish for laboratory crosses were collected;
- **nonCF** – 23 allopatric sites not in the Columbia and Fraser river watersheds;
  - composed remainder of 'total 500 fish' sample category.
- **original150** – nine allopatric sites used for the original species discrimination based on 150 dace (50 from each species). Composed 'original 150 fish' sample category;
- **poor** – specimen(s) from three sites were in poor condition, perhaps affecting completely accurate identification (particularly when combined with being *small* specimens too);
- **small** – specimen(s) from 17 sites were all at least ~≤ 30 mm and often much smaller, perhaps affecting completely accurate identification (particularly when combined with being *poor* specimens too). Small fish are harder to measure and identify;
- **'unusual'** – while still classified as one dace species, specimen(s) from eight sites all had an unusual or distinctive appearance, characteristic(s) and / or morphometric(s). This could sometimes be related to specimens also being 'poor' and / or 'small'.

speckled dace (*Rhinichthys osculus*)

British Columbia (Canada)

1. Granby River (UBC – 3 samples) flow (2 sites), genetics, lab-cross, original150;
2. Kettle R. - above barrier at Cascade (UBC – 10 samples) flow (2 sites), genetics, lab-cross, original150;
3. Mission Creek (UBC) poor, very 'unusual' (hybrid?) – single specimen, and no other ever handed in for identification.

Idaho (USA)

4. Boise River [sympatric # 60 on Fig. 16] (UBC and UW) symU/S, genetics;
5. Squaw Creek [sympatric component # 63] (OSU) symS/U, small.

Nevada (USA)

6. Beaverdam Creek (UBC) nonCF, 'unusual'.

Oregon (USA)

7. Bear Canyon Creek (OSU) nonCF;
8. Dorena Lake - unnamed inlet creek (UBC);
9. Emigrant Creek (OSU) nonCF;
10. Grande Ronde River [sympatric # 64 on Fig. 16] (UBC) symU/S, genetics;
11. McKay Creek (OSU) small;
12. Oak Creek (UW) small;
13. Ollala Creek (UBC) nonCF;
14. Umatilla River (UBC and UW) genetics;
15. unnamed creek, Calapooya (UBC) nonCF;
16. Smith River (UBC) nonCF;
17. Willamette River [sympatric # 95 on Fig. 16] (OSU, UBC and UW) symL/S, genetics;
18. Williamson River (OSU) nonCF, 'unusual'.

Washington State (USA)

19. Beaver Creek (UBC and UW) nonCF;
20. Chehalis River (UBC and UW – 3 samples) nonCF;
21. Conner Creek (UBC and UW – 2 samples) genetics, nonCF;
22. Covington Creek (UW) nonCF;
23. Cowlitz River [sympatric component # 97] (UBC) symS/L, 'unusual' speckled dace;

24. Deschutes River (UW) nonCF;
25. Humptulips River (UBC) nonCF;
26. Kettle River - below barrier at Cascade BC [sympatric # 52 on Fig. 16] (UBC) symU/S;
27. Lacamas Creek (UBC and UW – 2 samples) 'unusual';
28. Little Indian Creek (UBC) genetics;
29. Little Klickitat River [sympatric # 75 on Fig. 16] (UBC) symU/S, small;
30. Mill Creek (UW) nonCF;
31. Palouse River (UBC) genetics;
32. Salmon Creek (UW);
33. Satus Creek [other sympatric component # 76] (UBC) symS/U;
34. Scatter Creek (UW) nonCF;
35. Shea Creek [sympatric # 77 on Fig. 16] (UW – 3 samples) symU/S;
36. Toboton Creek (UW) nonCF;
37. unnamed creek - between Bonney and Rock lakes (UW) nonCF;
38. unnamed creek, Centralia (UW) nonCF;
39. unnamed creek, Elma (UW) nonCF;
40. unnamed creek, Malone (UW) nonCF;
41. unnamed drainage ditch, Kent (UW) nonCF;
42. unnamed slough, Hoquiam (UW) non-CF;
43. Willipa River (UW) nonCF;
44. Yakima River [sympatric # 80 on Fig. 16] [other sympatric component # 100] (UBC and UW – 2 samples) symS/U/L(3), genetics.

Wyoming (USA)

45. Salt River (UW) nonCF.

Umatilla dace (*Rhinichthys umatilla*)

British Columbia (Canada)

46. Bear Creek (UA) original150;
47. Beaver Creek (UA) original150;
48. Blueberry Creek (UA) small;
49. Champion Creek (UA);

50. Columbia River (UA – 7 samples and UBC – 5 samples) flows (2 sites), genetics, original150;

51. Jordan Creek (UA) small;

52. Kettle River - below barrier at Cascade BC [sympatric component # 26] (UBC) symU/S;

53. Kootenay River (UA – 2 samples and UBC – 6 samples) flow, genetics, original150;

54. Lower Arrow Lake (UBC – 2 samples) poor, small (mostly);

55. Okanagan Lake (UBC – 2 samples) poor, small;

56. Otter Creek (UBC – 2 samples) 'unusual' (no subsequent collection attempts were successful);

57. Pend d'Oreille River (UBC) small, 'unusual' (but small, and 'river' now a reservoir);

58. Similkameen River - upstream of Enloe dam / former 'barrier' (?)
(UBC – 7 samples) flow, genetics, lab-cross, small (in Wolfe Creek area samples);

59. Slocan River (UBC – 2 samples) flow, genetics.

Idaho (USA)

60. Boise River [sympatric component # 4] (UBC) symU/S, genetics;

61. Sand Hollow Creek (UW);

62. Snake River (UBC) genetics;

63. Squaw Creek [sympatric # 5 on Fig. 16] (OSU) symS/U, small.

Oregon (USA)

64. Grande Ronde River [sympatric component # 10] (UBC) symU/S, genetics;

65. Pine Creek (UBC);

66. Big Sheep Creek (OSU) small;

67. Crooked River (OSU) small;

68. John Day River (OSU) small;

69. Meacham Creek (OSU);

70. Owyhee River (OSU) 'unusual';

71. Powder River (OSU) small;

72. Wallowa River (OSU) small.

Washington State (USA)

73. Columbia River (UBC and UW);

74. Entiat River (UW);

75. Little Klickitat River [sympatric component # 29] (UBC) symU/S, small;

76. Satus Creek [sympatric # 33 on Fig. 16] (UBC) symS/U;

77. Shea Creek [sympatric component # 35] (UW – 3 samples) symU/S;

78. Similkameen R. - downstream of Enloe dam / former 'barrier' (?) [sympatric component # 98] (UBC) symU/L;

79. Wenatchee River [sympatric # 99 on Fig. 16] (UBC and UW) symL/U;

80. Yakima River [sympatric components # 44 and 100] (UBC and UW) symS/U/L(3), genetics.

Leopard Dace (*Rhinichthys falcatus*)

British Columbia (Canada)

81. Euchiniko River (UBC);

82. Fraser River – above canyon (UBC – 4 samples) genetics, original150;

83. Fraser River – below canyon (UBC – 10 samples) flow (2 sites), genetics, lab-cross, original150;

84. Mara Lake (UA);

85. Middle River (UBC);

86. Nazko River (UBC – 2 samples);

87. Nicola / Coldwater rivers (UBC – 10 samples) flow (2 sites), genetics, lab-cross, original150;

88. North Thompson River (UBC – 2 samples);

89. Quesnel River (UBC);

90. Salmon River (UBC);

91. Shuswap Lake (UBC);

92. Stuart Lake (UBC).

Oregon (USA)

93. Long Tom River (OSU);

94. unnamed sand / gravel ponds, Corvallis (OSU);

95. Willamette River [sympatric component # 17] (OSU, UBC and UW) symL/S, genetics.

Washington State (USA)

96. Cle Elem River (UW) small;

97. Cowlitz River [sympatric # 23 on Fig. 16] (UW) symS/L;

98. Similkameen River [sympatric #78 Fig. 16] - downstream of Enloe dam / former 'barrier' (?) (UBC) symU/L;

99. Wenatchee River [sympatric component # 79] (UBC and UW) symL/U;

100. Yakima River [sympatric # 80 on Fig. 16] [other sympatric component # 44] (UBC and UW) symS/U/L(3), genetics.

Appendix B

Morphometrics, Measurements, and Meristics

(A) Morphometric Measurements - Multivariate Analyses

- 42 morphometric characters for chapter 2 principal components analyses (PCAs):
  - (B) lateral truss network (measurements 1 to 28);
  - (D) other measurements (measurements 1 to 14).
  - chapter 2 has minimum group sizes of 50, and minimum total sample of 150 dace.
- 42 morphometric characters for chapter 2 allometry coefficients:
  - (B) lateral truss network (measurements 1 to 28);
  - (C) dorsal truss network (measurements 1 to 14);
- 39 morphometric characters for chapter 2 allopatry – strict sympatry analysis:
  - (B) lateral truss network (measurements 1 to 28);
  - (D) other measurements (measurements 1 to 11).
    - length measure (D-13) not used as its allometry would be inappropriate;
    - snout overhang (D-12) and barbel length (D-14) not used as their discriminatory power could have biased these descriptive statistics.
- 27 morphometric characters used for chapter 6 PCAs and linear discriminant function analysis:
  - (B) lateral truss network (measurements 1 to 24);
  - (D) other measurements (measurements 12 to 14).
  - chapter 6 has minimum group sizes of 30, and minimum total sample of 90 dace;
    - this reason necessitated the character set reduction from that for chapter 2;
      - sample, and preferably group, size must not exceed the number of variables.

(B) Lateral Truss Network

- see landmark point placements and their definitions in Figure 1(A) in Chapter 2;
- all 26 measurements are total straight-line distances (Hubbs and Lagler 1964);
- noted when equivalent to traditional fish measurements (Hubbs and Lagler 1964).

1. landmark points 1 to 2:
2. landmark points 1 to 3;
   - ≈ upper jaw (premaxillary and maxillary) length (Hubbs and Lagler 1964).
3. landmark points 2 to 3;
4. landmark points 2 to 4;

5. landmark points 2 to 5;
6. landmark points 3 to 4;
7. landmark points 3 to 5;
8. landmark points 4 to 5;
   - ≈ head depth (Hubbs and Lagler 1964).
9. landmark points 4 to 6;
10. landmark points 4 to 7;
11. landmark points 5 to 6;
    - post orbital length (Hubbs and Lagler 1964).
12. landmark points 5 to 7;
13. landmark points 6 to 7;
    - ≈ body depth (Hubbs and Lagler 1964).
14. landmark points 6 to 8;
15. landmark points 6 to 9;
16. landmark points 7 to 8;
17. landmark points 7 to 9;
18. landmark points 8 to 9;
19. landmark points 8 to 10;
20. landmark points 8 to 11;
21. landmark points 9 to 10;
    - dorsal fin base length (Hubbs and Lagler 1964).
22. landmark points 9 to 11;
23. landmark points 10 to 11;
24. landmark points 10 to 12;
25. landmark points 10 to 13;
26. landmark points 11 to 12;
27. landmark points 11 to 13;
28. landmark points 12 to 13;
    - caudal peduncle depth (Hubbs and Lagler 1964).
29. landmark points 1 to 14 (fork length);
30. landmark points 1 to 15.
    - total length (Hubbs and Lagler 1964).

(C) Dorsal Truss Network

- see landmark point placement and their definitions in Figure 1(B) in Chapter 2;
- all 14 measurements are total straight-line distances (Hubbs and Lagler 1964);

- noted when equivalent to traditional fish measurements (Hubbs and Lagler 1964).

1. landmark points A to B;
   - = *mean* (landmark points A to B and landmark points A to C);
2. landmark points B to C;
3. landmark points B to D;
   - = *mean* (landmark points B to D and landmark points C to E);
4. landmark points B to E;
   - = *mean* (landmark points B to E and landmark points C to D);
5. landmark points D to E;
   - (bony) interorbital width (Hubbs and Lagler 1964).
6. landmark points D to F;
   - = *mean* (landmark points D to F and landmark points E to G).
7. landmark points D to G;
   - = *mean* (landmark points D to G and landmark points E to F).
8. landmark points F to G;
   - head width (Hubbs and Lagler 1964);
9. landmark points F to H;
   - = *mean* (landmark points F to H and landmark points G to I).
10. landmark points F to I;
    - = *mean* (landmark points F to I and landmark points G to H).
11. landmark points H to I;
12. landmark points H to J;
    - = *mean* (landmark points H to J and landmark points I to K).
13. landmark points H to K;
    - = *mean* (landmark points H to K and landmark points I to J).
14. landmark points J to K.

(D) Other (non-truss) Measurements

- see fish pictures in Figures 1, 2, 14 and 15 in Chapter 2 for these body parts;
- all 14 measurements are total straight-line distances (Hubbs and Lagler 1964);
- noted when equivalent to traditional fish measurements (Hubbs and Lagler 1964).

1. pectoral fin length:
   - origin of pectoral fin to posterior tip of pectoral fin;
   - Hubbs and Lagler 1964.
2. pectoral fin base length:

- origin to insertion of pectoral fin;
    - Hubbs and Lagler 1964.

3. pelvic fin length:
    - origin of pelvic fin to posterior tip of pelvic fin;
        - Hubbs and Lagler 1964.
4. pelvic fin base length:
    - origin to insertion of pelvic fin;
        - Hubbs and Lagler 1964.
5. anterior anal fin length:
    - origin of anal fin to ventral tip of anterior edge (first ray) of anal fin;
        - anal fin height (Hubbs and Lagler 1964).
6. posterior anal fin length:
    - insertion of anal fin to ventral tip of posterior edge (last ray) of anal fin.
7. anal fin base length:
    - origin to insertion of anal fin;
        - Hubbs and Lagler 1964.
8. anterior dorsal fin length:
    - origin of dorsal fin to dorsal tip of anterior edge (first ray) of dorsal fin;
        - dorsal fin height (Hubbs and Lagler 1964).
9. posterior dorsal fin length:
    - insertion of dorsal fin to dorsal tip of posterior edge (last ray) of dorsal fin.
10. 'eye' size:
    - anterior to posterior edges of orbit (eye socket);
        - eye, orbit, and their horizontal lengths are very similar in dace (*Rhinichthys* spp.);
            - orbit (and eye) length (Hubbs and Lagler 1964).
11. mouth width:
    - transverse width of closed mouth (left to right corners of lower (mandible) and upper (premaxillary and maxillary) jaws);
        - gape width (Hubbs and Lagler 1964).
12. snout overhang:
    - distance from (anterior) tip of snout to anterior tip of lower jaw (mandible).
13. standard length:
    - body length from (anterior) tip of snout to posterior end of hypural plate.
        - Hubbs and Lagler 1964.
14. barbel length:

- joining / contact point of barbel with body (almost always hidden) to its most distal point from body (also sometime hidden);
    - *note* - accurate and valid measurement as taken and verified here, but could be very 'personal' and probably has low inter-user repeatability. The barbel score index (Figs. 2 and 3 in Chapter 2) was created for species discrimination instead;
        - barbel score index recommended for species discrimination
            - see barbel score index in section (D) meristics in this appendix B (below).

(E) 'Meristics'

- see fish pictures in Figures 1, 2, 14 and 15 in Chapter 2 for these body parts;
- noted when equivalent to traditional fish measurements (Hubbs and Lagler 1964).

1. barbel score index:
    - score =1 (absent or inconspicuous barbel);
    - score = 2 (short, usually visible, and generally pointing more terminal);
    - score = 3 (long, conspicuous, and generally pointing more subterminal).
        - developed from barbel length categorization (Figs. 2 and 3 in Chapter 2).
2. pelvic fin stay score index:
    - score =1 (weak or absent pelvic fin stays);
    - score = 2 (moderate and / or inconspicuous pelvic fin stays);
    - score = 3 (strong and / or conspicuous pelvic fin stays).
        - developed from a pelvic fin area categorization (Figs. 2 and 3 in Chapter 2).
3. dorsal fin ray number:
    - total principal rays in dorsal fin;
        - Hubbs and Lagler 1964.
4. lateral line scales:
    - scales in lateral line from its beginning at shoulder girdle to end of hypural plate;
        - Hubbs and Lagler 1964.
        - *note* –consistent good discrimination not found; low measurement repeatability, especially amongst users; very hard to accurately collect, particularly on small fish; lateral line scale pores present in fish at about 20 mm, but did not seem to fully develop until about 30 mm (Hubbs et al. 1974).
            - This meristic was thus evaluated but not used (but see Peden and Hughes 1988, 1989).
        - *note* – Hubbs et al. (1974) discuss some dace identification by lateral line scale *morphology*, but not useful to our field oriented identification objectives.

Appendix C

DNA Sequence for ITS *COMPLETE* data (316 bp)

Legend

Key to DNA Sequence Symbols

. = identical
- = insertion / deletion
K = G or T
M = A or C
R = A or G
S = G or C
W = A or T
Y = C or T

Key to Sample Site Symbols

#*n* = 4 specific dace from same sample that are different fish in ITS and mtDNA sequence data.
- *n* is the particular number given that specific dace throughout the thesis.

♦ (in superscript) = only 6 specific dace common to both the partial ITS and mtDNA sequence data sets.
(*no.*) = number of dace analyzed from that particular sample site locality

Other Related Figures

- distribution map of complete and partial ITS DNA groups summarized in Figure 22 (Chapter 4).
  - group names kept constant to those on map in this Figure 22 (Chapter 4).
- tree for complete ITS DNA groups, excluding lab hybrids, presented in Figure 21 (Chapter 4).

Data - Analyzed

- DNA sequences for mtDNA begins on the next page:

S1. speckled **dace** *Group 1*
Willamette R., OR #3 (1) GGTCTCCGGG CCTCGGCAGT CCCAGGAACG AAAAACCAAA TGCCTCGGGT CCGCCCCGGC GGACCTGAAC CCCCCTTCGG
Boise R., ID (1 . . . . . .
Kettle R., BC (1) . . . . . .
S2(U5). speckled **dace** *Group 2* (and Umatilla dace Group 5 in ITS partial data; no ITS complete data available)
Yakima R., WA (1) . . . . . .
U1. Umatilla **dace** *Group 1* – see leopard dace group 1 below (names kept constant to ITS partial data groupings on map Fig. 22 in Chapter 4)
U2. Umatilla **dace** *Group 2*
Boise R., ID (1) . . . . . .
U3. Umatilla **dace** *Group 3 [A and B]*
Grande Ronde R., OR *[A]* #3 (1) . . . . . .
Snake R., ID *[B]* #2 (1) . . . . . .
U4. Umatilla **dace** *Group 4 [A and B]*
lower Columbia R., BC *[A]* (1) . . . . G . . . . . .
Kootenay R., BC *[B]* (1)♦ . . . . . .
(U1)**L1.** leopard dace *Group 1* (and Umatilla dace Group 1 in ITS partial data; no ITS complete data available)
Willamette R., OR (1)♦ . . . . . .
L2. leopard dace *Group 2*
lower Fraser R., BC (1)♦ . . . . . .

S1. speckled **dace** *Group 1*
Willamette R., OR #3 (1) TGAAAAACGA GTGGCGAGAG TTGCAACCCC GACGGGTACC CGTAGGCTCC GGACACCCCG GCCC--T-TC ACGGG----G
Boise R., ID (1) . . . . . .
Kettle R., BC (1) . . . . . .
S2(U5). speckled **dace** *Group 2* (and Umatilla dace Group 5 in ITS partial data; no ITS complete data available)
Yakima R., WA (1) . . . . . T . . . . . CC.-. . G. . . . GG--.
U1. Umatilla **dace** *Group 1* – see leopard dace group 1 below (names kept constant to ITS partial data groupings on map Fig. 22 in Chapter 4)
U2. Umatilla **dace** *Group 2*
Boise R., ID (1) . . . . . .
U3. Umatilla **dace** *Group 3 [A and B]*
Grande Ronde R., OR *[A]* #3 (1) . . . . . .
Snake R., ID *[B]* #2 (1) . . . . . .
U4. Umatilla **dace** *Group 4 [A and B]*
lower Columbia R., BC *[A]* (1) . . . . . .
Kootenay R., BC *[B]* (1)♦ . . . . . .
(U1)**L1.** leopard dace *Group 1* (and Umatilla dace Group 1 in ITS partial data; no ITS complete data available)
Willamette R., OR (1)♦ . . . . . .
L2. leopard dace *Group 2*
lower Fraser R., BC (1)♦ . . . . . .

Appendix C *continued* – DNA Sequence for ITS *COMPLETE* data (306 bp)

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S1. speckled dace Group 1
  Willamette R., OR #3 (1)  TC-GGGGGAG AAGGGGACCG TGGGTTTAAA GACCTCCTCC CCATCCCTCG ACCGGGGGGG AGGAGGCGCC CGTCCGGGTC
  Boise R., ID (1)          ..-..... ..... ..... ..... ..... ..... ..... .....-..
  Kettle R., BC (1)         ..-..... ..... ..... ..... ..... ..... ..... .....
S2(U5). speckled dace Group 2 (and Umatilla dace Group 5 in ITS partial data; no ITS complete data available)
      Yakima R., WA (1)     ..A..... ..... ..... ..... ..... ..... ..... .....
U1. Umatilla dace Group 1 – see leopard dace group 1 below (names kept constant to ITS partial data groupings on map Fig. 22 in Chapter 4)
U2. Umatilla dace Group 2
      Boise R., ID (1)      ..G...-... ..... ..... ..... ..... ..... ..-..... .....
U3. Umatilla dace Group 3 [A and B]
  Grande Ronde R., OR [A] #3 (1)  CGG..--... ..... ..... ..... ..... ..... ..... .....
  Snake R., ID [B] #2 (1)         CGG..--... ..... ..... ..... ..... ..... ..... .....
U4. Umatilla dace Group 4 [A and B]
    lower Columbia R., BC [A] (1)  ..G..--... ..... ..... ..... ..... ..... ..... .....
    Kootenay R., BC [B] (1)♦       ..G..--... ..... ..... ..... ..... ..... ..... .....
(U1)L1. leopard dace Group 1 (and Umatilla dace Group 1 in ITS partial data; no ITS complete data available)
      Willamette R., OR (1)♦  ..-..... ..... ..... ..... ..... ..... ..... .....
L2. leopard dace Group 2
    lower Fraser R., BC (1)♦  -------... ..... ..... ..... ..... ..... ..... .....
```

```
S1. speckled dace Group 1
  Willamette R., OR #3 (1)  TCGCCACCAC TCTTTTTTTT TCCATTCCCA CGTCTCGGTT TGTGTTGGTC GGCCCCGAAA ACAAAAACAC TCAAGT
  Boise R., ID (1)          .......... ..... -.-..... ..Y..... ..... ..... .G..... .....
  Kettle R., BC (1)         ..... ..... --..... ..... ..... ..... ..... .....
S2(U5). speckled dace Group 2 (and Umatilla dace Group 5 in ITS partial data; no ITS complete data available)
      Yakima R., WA (1)     ..... ..... ..----... ..... ..... ..... ..... .....
U1. Umatilla dace Group 1 – see leopard dace group 1 below (names kept constant to ITS partial data groupings on map Fig. 22 in Chapter 4)
U2. Umatilla dace Group 2
      Boise R., ID (1)      ..... .....- -.--..... -.-....-.- ..... ..... ..... .....
U3. Umatilla dace Group 3 [A and B]
  Grande Ronde R., OR [A] #3 (1)  ..... ..... -..... .....C ..... ..... ..... .....
  Snake R., ID [B] #2 (1)         ..... ....---.. -T-T..... T.C..... ..... ..... ..... .....
U4. Umatilla dace Group 4 [A and B]
    lower Columbia R., BC [A] (1)  ..... ....---.. -T-T..... Y.Y.....Y ..... ..... ..... .....
    Kootenay R., BC [B] (1)♦       ..... ....---.Y -Y-W..... Y.Y.....Y ..... ..... ..... .....
(U1)L1. leopard dace Group 1 (and Umatilla dace Group 1 in ITS partial data; no ITS complete data available)
      Willamette R., OR (1)♦  ..... ..... ..... .....C ..... ..... ..... .....
L2. leopard dace Group 2
    lower Fraser R., BC (1)♦  ..... ....---.. -Y-W..... .....C ..... ..... ..... .....
```

Appendix D
DNA Sequence for ITS *PARTIAL* data (255 bp)

Legend

Key to DNA Sequence Symbols

. = identical | **K** = G or T | **M** = A or C | **R** = A or G
- = insertion / deletion | **S** = G or C | **W** = A or T | **Y** = C or T

Key to Sample Site Symbols

* = 6 specific dace with somewhat ambiguous DNA sites of multiple bases, but no length variation.
#*n* = 4 specific dace from same sample that are different fish in ITS and mtDNA sequence data.
- *n* is the particular number given that specific dace throughout the thesis.

♦ (in superscript) = only 6 specific dace common to both the partial ITS and mtDNA sequence data sets.
(*no.*) = number of dace analyzed from that particular sample site locality

Other Related Figures

- distribution map of partial ITS DNA groups, excluding lab hybrids, shown in Figure 22 (Chapter 4).
  - group names kept constant to those on map in this Figure 22 (Chapter 4).
- tree for partial ITS DNA groups, including lab hybrids, presented in Figure 36 (Chapter 7).
- tree for partial ITS DNA groups, excluding lab hybrids, presented on Figure 21 (Chapter 3).

Data – Not Analyzed

- 5 other dace from 5 samples that had sufficient length variation to render their data unreadable:
  1. Umatilla dace – Grande Ronde R., OR #2 (1)
  2. Umatilla dace – Slocan R., BC (1)
  3. Umatilla dace – Yakima R. at Benton City, WA (1)
  4. leopard dace – Yakima R., WA (1)
  5. longnose dace – Yakima R., WA (1)

Data – Analyzed

H1. ***LAB HYBRIDS*** speckled dace ♀ [Kettle R., BC] × leopard dace ♂ [lower Fraser R., BC] (2)
* lab hybrid S♀×L♂ 1 (1) **GGTCTCCGGG CCTCGGCAGT CCCAGGAACG AAAAACCAAA TGCCTCGGGT**
* lab hybrid S♀×L♂ 2 (1) ....

H2. ***LAB HYBRIDS*** leopard dace ♀ [lower Fraser R., BC] × speckled dace ♂ [Kettle R., BC] (1)
* lab hybrid L♀×S♂ (1) ....

S1. speckled dace *Group 1*
Willamette R., OR #3 (1) ....
Boise R., ID (1) ....
Kettle R., BC (1) ....

U2. Umatilla dace *Group 2*
Boise R., ID (1) ....

U3. Umatilla dace *Group 3*
Grande Ronde R, OR #3 (1) ....
Snake R., ID #2 (1)♦ ....

U4. Umatilla dace *Group 4*
lower Columbia R., BC (1) ....G..... ....
Kootenay R., BC (1)♦ ....

L2. leopard dace *Group 2*
lower Fraser R., BC (1)♦ ....-....... ....

U1L1. Umatilla dace *Group 1*
* Similkameen R., BC (1)♦ ....
leopard dace *Group 1* ....
Willamette R., OR (1)♦ ....

S2U5. speckled dace *Group 2*
Yakima R., WA (1) ....
Umatilla dace *Group 5*
*Grande Ronde R, OR #1 (1)♦ ....
* Snake R, ID #1 (1) ....

H1. *LAB HYBRIDS* speckled dace ♀ [Kettle R., BC] × leopard dace ♂ [lower Fraser R., BC] (2)
* lab hybrid S♀×L♂ 1 (1) **CCGCCCCGGC GGACCTGAAC CCCCCTTCGG TGAAAAACGA GTGGCGAGAG**
* lab hybrid S♀×L♂ 2 (1) .....

H2. *LAB HYBRIDS* leopard dace ♀ [lower Fraser R., BC] × speckled dace ♂ [Kettle R., BC] (1)
* lab hybrid L♀×S♂ (1) .....

S1. speckled dace *Group 1*
Willamette R., OR #3 (1) .....
Boise R., ID (1) .....
Kettle R., BC (1) .....
U2. Umatilla dace *Group 2*
Boise R., ID (1) .....
U3. Umatilla dace *Group 3*
Grande Ronde R, OR #3 (1)
Snake R., ID #2 (1)♦ .....
U4. Umatilla dace *Group 4*
lower Columbia R., BC (1) .....
Kootenay R., BC (1)♦ .....
L2. leopard dace ***Group 2***
lower Fraser R., BC (1)♦ .....
U1L1. Umatilla dace *Group 1*
* Similkameen R., BC (1)♦ .....
leopard dace ***Group 1***
Willamette R., OR (1)♦ .....
S2U5. speckled dace *Group 2*
Yakima R., WA (1) .....
Umatilla dace *Group 5*
*Grande Ronde R, OR #1 (1)♦ .....**Y**..... **K**.....**R**. .**K**...**R**.**R**..
* Snake R, ID #1 (1) .....**YK**. .....**R**. .**W**..**S**.....

H1. *LAB HYBRIDS* speckled dace ♀ [Kettle R., BC] × leopard dace ♂ [lower Fraser R., BC] (2)
* lab hybrid S♀×L♂ 1 (1) **TTGCAACCCC GACGGGTACC CGTAGGCTCC GGACACCCCG GCCC-TT-YM**
* lab hybrid S♀×L♂ 2 (1) .....**--.-T.**

H2. *LAB HYBRIDS* leopard dace ♀ [lower Fraser R., BC] × speckled dace ♂ [Kettle R., BC] (1)
* lab hybrid L♀×S♂ (1) .....**--.-TA**

S1. speckled dace *Group 1*
Willamette R., OR #3 (1) .....**--.-TC**
Boise R., ID (1)
Kettle R., BC (1) .....**--.-TC**
U2. Umatilla dace *Group 2*
Boise R., ID (1) .....**--.-TA**
U3. Umatilla dace *Group 3*
Grande Ronde R, OR #3 (1) .....**--.-TA**
Snake R., ID #2 (1)♦ .....**CC.-TA**
U4. Umatilla dace *Group 4*
lower Columbia R., BC (1) .....**CC.-T.**
Kootenay R., BC (1)♦ .....**CC.-TA**
L2. leopard dace ***Group 2***
lower Fraser R., BC (1)♦ .....**--.-TA**
U1L1. Umatilla dace *Group 1*
* Similkameen R., BC (1)♦ .....**--.-TW**
leopard dace ***Group 1***
Willamette R., OR (1)♦ .....**--.GTA**
S2U5. speckled dace *Group 2*
Yakima R., WA (1) .....**T** .....**CC.-TC**
Umatilla dace *Group 5*
*Grande Ronde R, OR #1 (1)♦ .....**S**.....**M**.. .....**Y**... .....**CY.-TA**
* Snake R, ID #1 (1) .....**S**..... .**S**..... .....**CC.-TA**

H1. *LAB HYBRIDS* speckled dace ♀ [Kettle R., BC] × leopard dace ♂ [lower Fraser R., BC] (2)

* lab hybrid S♀×L♂ 1 (1)	-CGGG----G TC-GGKGGAG AARGGRACCG YSSGTTTAAA GACCTACTTC
* lab hybrid S♀×L♂ 2 (1)	A.....----. ..-..G.... ..G..G.... TGG..... .....M..Y.

H2. *LAB HYBRIDS* leopard dace ♀ [lower Fraser R., BC] × speckled dace ♂ [Kettle R., BC] (1)

* lab hybrid L♀×S♂ (1)	A.....----. ..-..G.... ..G..G.... TGG..... .....C..C.

S1. speckled dace *Group 1*

Willamette R., OR #3 (1)	A.....----. ..-..G.... ..G..G.... TGG..... .....C..C.
Boise R., ID (1)	A.....----. ..-..G.... ..G..G.... TGG..... .....C..C
Kettle R., BC (1)	A.....----. ..-..G.... ..G..G.... TGG..... .....C..C.

U2. Umatilla dace *Group 2*

Boise R., ID (1)	A.....----. ..G..G-... ..G..G.... TGG..... .....C..C.

U3. Umatilla dace *Group 3*

Grande Ronde R, OR #3 (1)	A....---GT CGG..--... ..G..G.... TGG..... .....C..C.
Snake R., ID #2 (1)♦	G....GG-GT CGG..--... ..G..G.... TGG..... .....C..C.

U4. Umatilla dace *Group 4*

lower Columbia R., BC (1)	G....GG--. ..G..--... ..G..G.... TGG..... .....C..C.
Kootenay R., BC (1)♦	G....GG--. ..G..--... ..G..G.... TGG..... .....C..C.

L2. leopard dace *Group 2*

lower Fraser R., BC (1)♦	A.....----- -------... ..G..G.... TGG..... .....C..C.

U1L1. Umatilla dace *Group 1*

* Similkameen R., BC (1)♦	A..S.----. ..-..... ....R..... W..... .....

leopard dace *Group 1*

Willamette R., OR (1)♦	A.....----. ..-..G.... ..G..G.... TGG..... .....C..C.

S2U5. speckled dace *Group 2*

Yakima R., WA (1)	G....GG--. ..A..G.... ..G..G.... TGG..... .....C..C.

Umatilla dace *Group 5*

*Grande Ronde R, OR #1 (1)♦	G....GG--. ..-..G.R.R ..G..G.... TGG..... R..Y.CY.C.
* Snake R, ID #1 (1)	R....GG--. ..-..GR..R ..G..G.... TGK..W.... .....Y..C.

H1. *LAB HYBRIDS* speckled dace ♀ [Kettle R., BC] × leopard dace ♂ [lower Fraser R., BC] (2)

* lab hybrid S♀×L♂ 1 (1)	CYATCCMTCS AYCGGGGGGG RGGRAGGACC CGYCCSGGYC TTTCYMCCMM
* lab hybrid S♀×L♂ 2 (1)	.C....C..G .C..... ....R..GS. ..T.....TY ..KSCA....

H2. *LAB HYBRIDS* leopard dace ♀ [lower Fraser R., BC] × speckled dace ♂ [Kettle R., BC] (1)

* lab hybrid L♀×S♂ (1)	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

S1. speckled dace *Group 1*

Willamette R., OR #3 (1)	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC
Boise R., ID (1)	.C....C..G .C..... A..AG.CG.. ..T..G.-T. .CG.CA..AC
Kettle R., BC (1)	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

U2. Umatilla dace *Group 2*

Boise R., ID (1)	.C....C..G .C..... A.-AG.CG.. ..T..G..T. .CG.CA..AC

U3. Umatilla dace *Group 3*

Grande Ronde R, OR #3 (1)	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC
Snake R., ID #2 (1)♦	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

U4. Umatilla dace *Group 4*

lower Columbia R., BC (1)	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC
Kootenay R., BC (1)♦	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

L2. leopard dace *Group 2*

lower Fraser R., BC (1)♦	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

U1L1. Umatilla dace *Group 1*

* Similkameen R., BC (1)♦	.... A..AR.SR.S ..T..G.... .CG..A..AA

leopard dace *Group 1*

Willamette R., OR (1)♦	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

S2U5. speckled dace *Group 2*

Yakima R., WA (1)	.C....C..G .C..... A..AG.CG.. ..T..G..T. .CG.CA..AC

Umatilla dace *Group 5*

*Grande Ronde R, OR #1 (1)♦	.CW...Y..R .C..... A..AG.CS.. ..T..G..TY .CG.CA..AY
* Snake R, ID #1 (1)	.C....C..G .C..... A..AG.CG.. .S...G..T. .CS.CA..AC

H1. ***LAB HYBRIDS*** speckled dace ♀ [Kettle R., BC] × leopard dace ♂ [lower Fraser R., BC] (2)
* lab hybrid S♀×L♂ 1 (1) **TCTTT**
* lab hybrid S♀×L♂ 2 (1) .....

H2. ***LAB HYBRIDS*** leopard dace ♀ [lower Fraser R., BC] × speckled dace ♂ [Kettle R., BC] (1)
* lab hybrid L♀×S♂ (1) .....

S1. speckled dace *Group 1*
Willamette R., OR #3 (1) .....
Boise R., ID (1) .....
Kettle R., BC (1) .....

U2. Umatilla dace *Group 2*
Boise R., ID (1) .....

U3. Umatilla dace *Group 3*
Grande Ronde R, OR #3 (1) .....
Snake R., ID #2 (1)♦ .....

U4. Umatilla dace *Group 4*
lower Columbia R., BC (1) .....
Kootenay R., BC (1)♦ .....

L2. leopard dace *Group 2*
lower Fraser R., BC (1)♦ .....

U1L1. Umatilla dace *Group 1*
* Similkameen R., BC (1)♦ .....

leopard dace *Group 1*
Willamette R., OR (1)♦ .....

S2U5. speckled dace *Group 2*
Yakima R., WA (1) .....

Umatilla dace *Group 5*
*Grande Ronde R, OR #1 (1)♦ .....
* Snake R, ID #1 (1) .....