THE EVOLUTION OF BIRD POLLINATION IN MACARONESIAN *LOTUS* SECTION *RHYNCHOLOTUS* (LEGUMINOSAE)

by

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ABSTRACT

In order to understand the evolutionary transition from bee pollination (melittophily) to bird pollination (ornithophily), I studied a group of *Lotus* from Macaronesia. First, I provided a combined phylogenetic framework using nuclear and plastid genes, where I showed that the morphological features adapted to opportunistic passerine birds in Tenerife and La Palma are derived and evolved recently within the last 1.2 Ma in four species. I also identified *Lotus sessilifolius* as the most likely closely related species with melittophily.

I showed that *L. sessilifolius* and the clade where this syndrome evolved had a pre-adaptation to produce a color change to red flowers (and the associated anthocyanidin pigment, cyanidin) as a possible strategy to increase bee foraging efficiency. The transition from yellow to red flowers in this group required only a redirection in the flux of pigment production and a modification in the proportions of flavonols and anthocyanidins, especially within the cyanidin branch.

I also found that petal micromorphology is highly modified between the two syndromes. I found that ornithophilous flowers lack the typical papillose conical cells, which are distributed in the exposed sides of the petals in bee-pollinated flowers. This reduction of conical cells is associated with an early down-regulation of a dorsal identity gene in legumes, *LjCYC2*. Bird-pollinated flowers also have a higher proportion of tabular rugose cells in all three types of petals in comparison with the bee-pollinated species. The increase of this epidermal type is associated with an up-regulation of *LjCYC3*, a lateral petal identity gene, during early stages of flower development in the dorsal and lateral petals. *Lotus sessilifolius* also seems to have this early expression in comparison with other bee-pollinated species.

All this evidence suggests that the transition from bee to bird pollination in this group required only heterochronic modifications of the genes involved in flower color and petal micromorphology. It seems that ornithophily in *Lotus* evolved within a group which has at least two pre-adaptations, production of pigments required for red colors and an increase in the amount of tabular rugose cells, which likely facilitated the evolution of phenotypes associated with this pollination syndrome in the Canary Islands.

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DEDICATION

Dedicated with all my heart to you,

SANDRA and EMILIO

“Regarding the small size of these islands the sheer amount of endemic species is really remarkable. Furthermore, every mountain is crowned by a young crater and the borders of each lava flow are still clearly recognisable. We have to conclude that not long ago, the ocean was reigning out here. It seems to me, that here in space as well as in time, the secret of all secrets, that is the appearance of new creatures on earth is readily perceptible.”

CHARLES DARWIN, 1845

CO-AUTHORSHIP STATEMENT

Chapter 2 has been published: Cronk, Q. and Ojeda, I. (2008) Bird-pollinated flowers in an evolutionary and molecular context. *Journal of Experimental Botany* 59: 715-727. The opportunity of this work came as an invitation to Q.C.B. Cronk. The idea came after discussions with Q.C.B. Cronk. The manuscript was written in equal proportion by both authors.

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1. Introduction

1. 1 Bird pollination

Bird pollination or ornithophily has evolved in several plants groups in various regions in the world, especially in the tropics. This coevolutionary adaptation involves the association of several distinctive traits which form the so called "pollination syndromes" (Proctor et al., 1996). The genetic mechanisms by which these floral adaptations have been modified during pollinator shifts have been addressed in a few plant groups. However, the majority of these plant groups have evolved under the selective pressure of hummingbirds in North America (e.g., *Mimulus, Aquilegia, Penstemon*) (Grant and Grant, 1968).

North American bird-pollinated flowers represent a small proportion of the great diversity in floral morphology adapted to birds. Additionally, these bird-pollinated flowers enter into contact with hummingbirds relatively recently, and therefore these model systems do not represent the range of adaptations displayed in ornithophilous plants. This lack of additional groups, in part, motivated the research described in this thesis.

In this thesis, I studied how the flower features adapted to bird pollination (opportunistic passerine nectar-feeders) evolved in a group of four species of *Lotus* (Leguminosae) in the Canary Islands (Macaronesia).

I start my thesis (Chapter 2) with a review of what it is currently known about this pollination syndrome. In this chapter, I give a general overview of the bird pollination syndrome. I also summarize the major traits that have been associated with this syndrome (for instance red flowers, nectar composition, and flower shape), with the current knowledge that is known about the candidate genes that control some of these traits. I also discuss some of the models systems used to dissect the genetic factors responsible for these floral traits. I should clarify that there are other plant models not mentioned in this chapter, notably are the cases of *Aquilegia* (Fang et al., 2010; Kramer, 2009; Kramer and Hodges, 2010) and *Petunia* (Gübitz et al., 2009). All these

plant models will provide in the near future fruitful insights about pollinator transitions and the genetic basis of some of these floral traits.

1.2 Macaronesian *Lotus*

In this thesis, I propose that this group can provide some insight about transitions from be to bird pollination. This group has four species (the "rhyncholotus group") adapted to opportunistic passerine nectar-feeders in the Canary Islands. Although there is no evidence to support the efficiency of opportunistic passerine nectar feeder birds in this plant group, there are additional data, such as nectar composition (Dupont et al., 2004), visitation observation, and other floral traits, that strongly suggest that this group is bird-pollinated (Olesen, 1985; Ollerton et al., 2009; Sletzer, 2005; Valido et al., 2004). The group has a contrasting floral morphology in comparison with its closely relatives. Additionally, the group seems to have evolved recently and crosses between species with contrasting syndrome are likely possible. Therefore, this group represents another model system to address questions in pollination shifts, from a region not represented in previous plant models.

Finally, there are a number of genomic and genetic resources from four model systems in legumes (*Pisum sativum*, *Glycine max*, *Medicago trunculata* and *Lotus japonicus*) which are useful in studying the genetic basis of some of these traits. Of particular interest is *L. japonicus* which has extensive EST libraries and a whole genome sequence (Sato et al., 2008), tools that are easily transferred to Macaronesian *Lotus*.

In particular, there has been notable advance in the understanding of the genes that determine petal identity in legumes and its association with particular petal micromorphology (Feng et al., 2006b). However, outside the two models species where these features have been

characterized (*L. japonicus* and *Pisum sativum*) (Feng et al., 2006b; Wang et al., 2008); there was a general lack of the epidermal types in Leguminosae before this thesis.

In Chapter 3, I provide a survey of the different epidermal types in a representative group of legumes. This analysis provides a general overview of the major epidermal types within legumes and how some of these traits have evolved within the family. Especially important is the finding that the highly specialized papilionoid flower, with three petal types, is also highly specialized at the micromorphological level, where specific petal surfaces are associated with a particular petal type. This analysis also indicates that this specialization has been lost in some groups in the papilionoids.

1. 3 Molecular phylogenetics and DNA barcoding of Macaronesian *Lotus*

Macaronesian *Lotus* comprises a group of about 40 species distributed in five volcanic archipelagos and mainland Africa and Europe. The group is high in species diversity and endemics in Morocco and the Canary Islands. Despite the large species numbers within this group, there are relative few phylogenetic analyses within the group and there is a lack of resolution due to low levels of variability of the ITS region and a lack of complete representation of the species.

In Chapter 4, I provide a phylogenetic framework of the group where I included the most comprehensive sampling within this group. I used a combined analyses of six gene regions (plastid and nuclear) in three different data sets. I identified one species, *Lotus sessilifolius*, as the closest relative of the four bird-pollinated species. I also determined that the group evolved relatively recently (within the last 2 Ma) and this radiation is associated with recent volcanic activity in Tenerife and the emergence of La Palma. My analyses suggest that this syndrome is

derived in Macaronesian *Lotus* and may have evolved under the selective pressure of these passerine nectar-feeders ("de novo opportunistic hypothesis") (Valido et al., 2004).

In Chapter 5, I addressed whether six gene regions (ITS, *matK, rpoB, rbcL, rpoC1* and *trnH-psbA*) have a practical application as barcodes in this group. This legume group represents a particular challenge for barcodes, as some of these clades diverged relatively recently. My analyses suggest that these barcodes have low levels of resolution in comparison with other plants groups that have been analyzed at a floristic level (González et al., 2009; Kress et al., 2009).

I was able to identify only 18% at species when I used the recommended barcode combination (*rbcL+matK*) (CBOL, 2009). When all six regions were combined I identified 52% of the species included and only four (out 10 species) considered endangered within this group.

1.4 Flower colour and petal micromorphology modifications during the transition from bee to bird pollination in Macaronesian *Lotus*

In this thesis, I particularly studied the modification of two floral traits: petal micromorphology and flower colour.

In Chapter 6, I addressed the modifications in petal micromorphology between the two pollination syndromes. The transition from bee to bird pollination in this group required the modification of the three types of petals (dorsal, lateral and ventral) within the flower. During the transition the role of each petal is modified within the flower and this modification occurred at the micromorphological level as well. Bee-pollinated species have papillose conical cells on the dorsal and lateral petals, which enhance petal perception and aid grip when pollinators land on the lateral petals. In contrast, all four bird-pollinated species completely lack papillose conical cells and this epidermal type is restricted to only a highly localized area of the lateral petal. This

shift in epidermal surface suggests that papillose conical cells may have been lost as an anti-bee strategy and it is only maintained where it enhances bird attraction. I found an association between the micromorphological modifications with the expression patterns of two petal identity genes. These analyses suggest that the micromorphological modifications in the petals may have evolved due to heterochronic changes of expression patterns of two petal identity genes (*LjCYC2* and *LjCYC3*) between the two pollination syndromes.

Bird-pollinated flowers have red/orange flowers while the majority of the bee-pollinated species have yellow flowers. In Chapter 7, I explain how red flowers might have evolved in this group. I found that 58% of the bee-pollinated species have the capacity to modify flower color after anthesis. This ability may have evolved in this group and other plant groups as a strategy to increase foraging efficiency (Jones and Cruzan, 1999). I found that this ability has evolved at least three times in Macaronesian *Lotus* and the rhyncholotus group evolved within a clade where all species have the ability to modify from yellow to red flowers.

I found that pre-change yellow flowers differ in spectral reflectance to the post-change red flowers and insect pollinators can distinguish between the two colours. I also show that the pigments found in bird-pollinated flowers are already present in the most closely related species, and the transition required only the re-direction of delphinidin and flavonols towards the cyanidin branch of the anthocyanidin pathway. I suggest that this transition evolved as a heterochronic transition of pigment production and as an anti-bee, rather than a pro-bird strategy.

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2. Bird-pollinated flowers in an evolutionary and molecular context

2.1 Pollination syndromes

The concept of pollination syndromes, where specific floral traits are associated with particular pollination mechanisms, dates back to the work of the Neapolitan botanist Federico Delpino (1833-1905). The attraction and utilization of a specific group of animals for pollination, for instance, is associated with specific characteristics of flower morphology, colour, nectar, odour, and orientation (Faegri and van der Pijl, 1966; Fenster et al., 2004; Proctor and Yeo, 1973). However, pollination systems are often more complex than floral morphology would at first sight suggest, and this has led to criticisms of the pollination syndrome concept, mainly based on evidence that flowers attract a broader spectrum of visitors than expected (Waser et al., 1996). Nevertheless, there is ample evidence supporting a strong association between certain floral traits and functional groups of pollinators that exert similar selective pressures (Fenster et al., 2004).

One well-recognized syndrome of floral traits is that associated with bird pollination (ornithophily). Ornithophilous flowers (Fig. 2.1) are very often red with copious dilute nectar. Furthermore they lack characters associated with other pollination syndromes, such as scent.

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2.2 The syndrome of ornithophily

2.2.1 The birds

Many birds will casually visit flowers in search of food, often primarily to seek insects concealed in inflorescences although they will take nectar if it is available. Flower visiting of some sort has been reported in as many as fifty families of birds (Proctor and Yeo, 1973; Proctor et al., 1996). However, three families of birds have evolved as major groups of flower specialists. These are the hummingbirds (Trochilidae), the sunbirds (Nectariniidae) and the honey-eaters (Meliphagidae) (Fig. 2.2).

Hummingbirds are exclusive to the New World ranging from Southern South America to Alaska with the highest diversity in the northern Andes (Grant and Grant, 1968). As hummingbirds visit flowers by hovering there is no need for a perch and in consequence some hummingbird flowers are long and pendulous. Hummingbirds have beaks that are highly specialized for nectar feeding, even though insects form a normal part of their diet, necessitating a remarkable flexibility of the jaw (Yanega and Rubega, 2004). They have undergone a major evolutionary radiation in South America and a secondary radiation in North America (Mayr, 1964). Fossil evidence from Europe, however, suggests that the early evolution of this group was not exclusive to the New World (Mayr, 2004).

Sunbirds and spiderhunters (Nectariniidae) are the major group of pollinating birds in Africa and Asia. The honeyeaters (Meliphagidae) are very important pollinators of styphelioid Ericaceae (Epacridaceae), Myrtaceae and Proteaceae in Australia and they extend north to Wallace's line and east to New Zealand and Hawaii.

Other important groups include the American orioles (Icteridae) in North and South America, the honeycreepers (Thraupidae) in tropical America and the Hawaiian honeycreepers (Fringillidae, subfamily Drepanidinae) in the Hawaiian Islands. In Africa the White-eyes

(Zosteropidae) are another important group, as are the South African sugar-birds (Promeropidae).

Bird pollination is particularly common in relatively aseasonal tropical and subtropical regions as flowers and nectar are available year-round to support nectarivorous birds. It tends to be absent or rare in regions in which vegetation has a long dormant period. North America is an exception as here hummingbirds migrate north during the summer. The migration is particularly remarkable on the NW coast where hummingbirds migrate as far as Alaska. Bird pollination is almost entirely absent in Europe and in Asia north of the Himalayas. In Europe, although there are reports that some passeriform birds occasionally feed on nectar (Kay, 1985; Merino and Nogueras, 2003; Proctor et al., 1996; Schwilch et al., 2001), there is only one report of a bird-pollinated native plant, *Anagyris foetida* L. (Leguminosae). In Spain this is apparently pollinated by three warblers, *Phylloscopus collybita* Vieillot, *Sylvia atricapilla* L. and *S. melanocephala* Gmelin (Ortega-Olivencia et al., 2005).

2.2.2 Ecology of bird pollination

Some attributes of birds, such as long flight distances and high visual acuity, make them excellent pollinators, especially valuable during inclement weather conditions when other pollinators, such as bees, are inactive. Birds may therefore be important supplemental pollinators in environments where insects have low population densities, such as high altitudes ecosystems (Van der Pijl and Dodson, 1966), dry environments (Stiles, 1978), isolated islands where insect colonization has been poor (Dupont et al., 2004; Micheneau et al., 2006) and for plants flowering during winter months when insects are few (Kunitake et al., 2004).

However, birds are large and require more energy than insects. For this reason plants with bird-pollinated flowers tend to put more energy into nectar production and often produce larger

flowers to accommodate their avian pollinators. Bird-pollinated plants may also deploy more resources in floral structures that protect against thieves of their abundant nectar (Stiles, 1978). Environments with low plant productivity may be limiting for nectar production and for this syndrome in general. The tropical forest understorey, with limited photosynthetic rates, has relatively few bird-pollinated plants (Stiles, 1978) and the same appears to hold true for cold, hyper-arid and nutrient poor environments. In constrast, the syndrome is particularly common in tropical and subtropical shrublands, open woodland and riverine communities.

2.2.3 Perching vs hovering

The behaviour of those birds associated with ornithophily can be broadly divided into two types, hovering and perching. The hovering behaviour is found mainly in hummingbirds, but is also present in some families of passeriforms. In hovering, birds collect nectar without landing on the plant, which may therefore have hanging or pendant flowers. On the other hand, perching birds land on stems, leaf stalks, adjacent branches, flower buds, which must provide an adequate perch. Low herbaceous plants may be pollinated by birds that perch on the ground, and they usually orient their flowers vertically erect. Examples include *Lotus berthelotii* Masf. and its relatives in the Canary Islands (Olesen, 1985), and *Gastrolobium praemorsum* (Meisn.) G. Chandler & Crisp in the southwest of Western Australia (Keighery, 1982).

Among birds, flower foraging by perching is more widespread and involves fewer specialist adaptations than foraging while hovering. Flower visiting has evolved in several families of perching passeriforms in the New World and the Old World. Unlike hummingbirds, passeriforms tend to forage and travel in groups and can be effective in cross-pollinating even large trees (Stiles, 1981). In the New World, pollination by perching passeriforms appears to have evolved recently, usually, although not always, involving plant species in genera already

adapted to hummingbird pollination (Cruden and Toledo, 1977; Toledo, 1975).

2.2.4 Hermit vs non-hermit hummingbirds

Bird behaviour is important in determining the nature of bird-plant interaction. The vast majority of bird-pollinated species in the Neotropics, are herbs, shrubs, small trees and epiphytes, and rarely large canopy trees. Solitary behaviour and territoriality in hummingbirds do not allow the levels of pollinator saturation or cross-pollination necessary for the effective pollination of large canopy trees (Stiles, 1975), which therefore tend to be bee-pollinated. Hummingbirds may be divided into two subgroups, hermits and non-hermits, and this division has important implications for pollination.

The majority of hermits have long, curved bills and a tendency to forage on flowers with long, curved corollas. They inhabit the understorey of the tropical forest and decrease in abundance and diversity at higher elevations and in dry habitats. Hermits are non-territorial with a traplining method of foraging. Traplining involves visiting many plants sequentially for short visits, flying from plant to plant, often over some distance, and is highly effective in promoting cross pollination (Snow and Snow, 1972; Stiles, 1975, 1981).

Non-hermits have short straight bills and a tendency to hold territories and thus non-hermit pollination behaviour favours self-pollination. Non-hermits are widely distributed but have their greatest diversity in the tropical highlands (Snow and Snow, 1972; Stiles, 1975, 1981). The North American hummingbird fauna comprises a fairly homogenous assemblage of non-hermit hummingbirds and probably for this reason North American ornithophilous plants are fairly uniform in floral form compared to those of South America (Grant, 1966; Stiles, 1981). Interestingly, the existence of these two types of hummingbird has had a strong influence on patterns of floral diversity in the genus *Heliconia* (Heliconiaceae) (Stiles, 1975, 1981). *Heliconia*

species that are pollinated by hermit hummingbirds usually have long, curved corollas, and grow in the forest understorey where light is limiting. They grow in small, scattered clumps and produce a few flowers over a long flowering period. Daily nectar production is low with usually moderate to high nectar concentration (Stiles, 1975), and hermit hummingbirds trapline between clumps. By contrast, non-hermit associated *Heliconia* species grow in large clumps with highly synchronous flowering, producing many flowers during a definite flowering period. These *Heliconia* species usually have short straight corollas and are usually found in highly productive environments of forest gaps or along rivers with abundant light (Stiles, 1975). They produce a large quantity of relatively dilute nectar. Territorial non-hermit hummingbirds will defend these clumps because of their abundant nectar reward, which is beneficial for pollination intensity but not for cross-pollination between clumps.

2.2.5 Major groups of angiosperms with bird pollination

Bird pollination is widespread in the flowering plants and appears to have evolved many times. It is present in some 65 flowering plant families and in most of these it probably represents a separate origin, usually from a bee-pollinated precursor. However, bird pollination is notably absent in some of the largest families of flowering plants. In Asteraceae, for example, only the South American genus *Mutisia* (Buzato et al., 2000; Proctor et al., 1996) and *Dendroseris litoralis* from Juan Fernandez Islands (Bernardello et al., 2001) are bird pollinated. On the other hand, there are some large clades in which bird pollination is particularly common, such as in the monocot order Zingiberales. Families of this order are Cannaceae, Costaceae, Heliconiaceae, Lowiaceae, Marantaceae, Musaceae, Strelitziaceae, Zingiberaceae, and bird pollination occurs in all of them. Genera such as *Canna* (Cannaceae), *Strelitzia* (Strelitziaceae), *Heliconia* (Heliconiaceae) and *Costus* (Costaceae) are well known for their showy bird-

pollinated species. In the Costaceae, for instance, ornithophily associated with hummingbirds evolved several times in the neotropics from bee-pollinated ancestral species (Specht, 2006).

Ornithophily has also evolved several times, mainly from a bee-pollinated ancestor, in many families of the eudicots. A common pattern within these groups is the parallel evolution of the traits associated with ornithophily among non-closely related groups of plants. For example, within Gesneriaceae many *Columnea* species have similar morphological traits (trailing epiphytes with fairly large and showy red flowers) to another group from the Old World, *Aeschynanthus*. *Columnea* is distributed in Central and South America and is pollinated by hummingbirds, whereas *Aeschynanthus* is distributed in the Palaeotropics.

The multiple origin of bird pollination in the flowering plants raises the question of what pre-adaptations promote this evolutionary transition. Two features, commonly associated with bee pollination, appear to be permissive of a transition to bird pollination. One is floral zygomorphy (monosymmetry or asymmetry) and the other is the possession of a floral tube. Bird pollination is common in many families with strongly zygomorphic flowers (e.g. Scrophulariaceae, Heliconiaceae, Gesneriaceae and Leguminosae). It is generally considered to be an adaptation to bee pollination and allows precise placement of pollen on the body of the bee. It seems that these adaptations may be important for bird-pollination also.

The same applies to the character of perianth segments being connate into a tubular corolla (or the presence of a tubular hypanthium in *Passiflora* and *Fuchsia*). Tubular flower originally evolved for insect pollination but are equally suitable for the probing foraging of bird's beak. Hummingbird flowers in North America are predominantly sympetalous dicots and it has been suggested that this corolla condition, with its tubular shape, is another pre-adaptation to hummingbird pollination (Grant and Grant, 1968).

2.3 The floral phenotype

2.3.1 Types of floral adaptation

Floral adaptations to bird pollination fall into four broad types (1) attraction mechanisms, (2) exclusion mechanisms, (3) protection mechanisms, and (4) pollination mechanisms (Grant and Grant, 1968). Attraction mechanisms are those such as copious nectar and vivid floral display that attract birds to flowers. The floral display may be just red or orange, or a combination of contrasting colours, including orange, yellow, green and blue ("parrot colours"). *Strelitzia reginae* W. Aiton, for instance, presents a striking display of orange and blue.

Exclusion mechanisms are those features that help to deter illegitimate flower visitors that might otherwise interfere with pollination and rob nectar. Red colour, long and narrow floral tubes and the absence of insect landing platforms are the most obvious of these and are further discussed below. Pendent flowers, as in *Aquilegia formosa* Fisch. ex DC., are difficult for insects to work but easy for hummingbirds. Recurving petals, as in *Ipomopsis aggregata* (Pursh) V. Grant, or the short and recurved lower lip of *Mimulus cardinalis* Dougl. ex Benth., serve to deny insects a landing platform and similarly make the flowers difficult to work except by hummingbirds. In *Trichostema lanatum* Benth., the long-exserted stamens are recurved to block entrance to the tube.

Protection mechanisms are also important, as birds are large and potentially destructive pollinators. A common form of protection is provided by mechanical strengthening of the flower by the formation of sclerenchyma or collenchyma tissue in various floral parts. The ovary and ovules, often situated near to the nectaries are particularly vulnerable to the probing of bird's beaks. Protection of ovules takes many forms. There may be separation of ovary and nectary, either by the sheathing of the ovary by a staminal tube, or by a stalked or inferior ovary. Alternatively there may be a groove formed by the corolla to guide birds' beaks to the nectary

without causing damage, or ridges of the corolla to provide direct protection to the ovary. In addition, the style may be protected in a groove formed from rides of the upper petals, as in *Justicia californica* (Benth.) D. Gibson. In *Penstemon* it is the nectaries rather than the ovary that shift. In bird-pollinated species of this genus, nectaries are displaced upwards from the base of the ovary to the outer bases of the upper pair of stamens.

Pollination mechanisms are those that enhance the precise deposition of pollen on bird and stigma. These include both spatial and temporal relations of the reproductive organs to the position of pollinating birds. The long exserted stamens of bird-pollinated species of *Aquilegia* (*e.g. A. formosa*), *Fuchsia* and *Ribes* (*e.g. R. speciosum* Pursh) dust birds' heads or even backs, with pollen. In the radially symmetrical *Ipomopsis aggregata* the ring of five stamens place pollen all around the base of the birds' beaks. Zygomorphic flowers, on the other hand, tend to place pollen on the top of the beak or on the top of the bird's head.

2.3.2 Why red?

Explanations for the remarkably consistent association of bird pollination with red or reddish flowers take two forms, either avoidance of bees (and other insect pollinators), or attraction of birds. It seems that red colour is not necessary to attract birds. There are examples known where birds are effective pollinators of species with orange, yellow and white flowers (and less frequently reddish violet and blue flowers) (Micheneau et al., 2006; Ortega-Olivencia et al., 2005; Proctor and Yeo, 1973). This has led to the suggestion that avoidance of bees (which cannot see red) is more important than attraction of birds (Proctor and Yeo, 1973; Proctor et al., 1996). Birds perceive colour over wavelengths ranging between 300 and 660 nm, whereas bee vision is in the range 300 to 550 nm. In the neotropical forests bird-pollinated flowers have been shown to have typical median reflectance greater that 585 nm, outside the visual range of bees

(Altshuler, 2003). However it should be noted that bees can perceive (and do visit) some flowers seen as red by humans, if they have at least some reflectance in the shorter wavelengths as well (Chittka and Waser, 1997).

However, as well as its invisibility to bees, the fact that red is very readily detectable by birds is also likely to be significant. For instance the visual prominence of red may be important for migratory hummingbirds, which can easily detect red flowers when entering a new habitat and associate it with reward (Grant, 1966). Indeed, the association of red flowers and bird pollination may be explained via optimal foraging theory and the relative efficiency of bees and birds in detecting red (Rodriguez-Gironés and Santamaría, 2004), with red flowers acting as a signal of high caloric reward (Raven, 1972). With the association between high energetic rewards and the colour red already well established, there may be strong selective pressure for other bird-pollinated flowers to adopt this "common advertising strategy". However, there is a curious twist in *Fuchsia excorticata* L.f. of New Zealand, in which a developmental change, from green to red flower colour, signals lack of nectar reward in post-reproductive flowers (Delph and Lively, 1989). Birds therefore avoid the red flowers in favour of green.

2.3.3 Flower colour and its perception

Flowers that appear red to humans are of three types (Chittka and Waser, 1997). Some species have an additional peak that stimulates the blue receptor of bees; for example, the red flowers of *Dianthus carthusianorum* L. are perceived as blue-bee, as this species has a reflectance peak that stimulates the blue receptor of bees. The red flowers of field poppies (*Papaver rhoeas* L.) have a reflectance peak below 400 nm and are perceived by bees as ultraviolet. However, typical red hummingbird flowers, such as *Ipomopsis aggregata* and *Justicia rizzinii* Wassh., will be perceived as green by bees (Chittka et al., 1994; Chittka and

Waser, 1997). These flowers only stimulate the green receptor of bees, and there is no additional peak of reflectance in other wavelengths. This type of flower, with a peak only in the red, and with no blue or UV reflection, is more difficult to detect than flowers of other colours (Chittka et al., 1994; Chittka and Waser, 1997).

On the other hand, birds are tetrachromatic and they have an additional UV receptor, compared to humans making colour perception in birds more complex than in mammals (Bowmaker, 1977). Further, they have oil droplets that can act as filters that increase the complexity of colour perception. Birds can see in theory twice the number of colours compared with trichromats (Ödeen and Håstad, 2003). It seems that birds do not have an innate preference for red colour, although most of them have their greatest spectral sensitivity and hue discrimination towards the long wavelength end of the spectrum (Stiles, 1981). Experiments with hummingbirds have shown that they learn to associate a range of colours with rewards and that this behaviour can be modified (Proctor et al., 1996).

Floral pigments (anthocyanidins and flavonols) have the major impact on the wavelength of light reflected from flowers. There are three major types of anthocyanidin pigments: pelargonidin (generally red), cyanidin (typically magenta or blue depending on pH) and delphinidin (generally blue). Bird-pollinated flowers are much more likely to contain pelargonidin and much less likely to contain delphinidin than flowers generally (Scogin, 1988). A general predominance of pelargonidin in tropical floras (Beale et al., 1941) has been attributed to the tropical distribution of hummingbird pollination (Harborne, 1976). A difference in pigment composition has been reported in comparisons between perching-bird and hummingbird-visited flowers (Scogin, 1988), with a greater prevalence of cyanidin, as opposed to pelargonidin, in perching-bird flowers.

2.3.4 Nectar

Bird-pollinated flowers generally produce a large quantity of dilute nectar as the main pollinator reward. Nectar characteristics, such as (1) volume, (2) sugar concentration and viscosity, (3) sugar composition, and (4) amino acid composition, are extremely important in determining the success of plant-pollinator interactions (Baker and Baker, 1983a; Proctor et al., 1996) and nectars of bird-pollinated plants tend to be recognizable as such. The volume of nectar in flowers is generally correlated with the size of the flower (Baker, 1978). However, size for size, bird-pollinated plants tend to secrete larger quantities of nectar relative to bee-pollinated species. The same is true in bat-pollinated species. Some bat-pollinated *Bombax* species, for example, can produce between 200-300 µl of nectar daily (Baker, 1978).

Nectar concentration is a characteristic often inversely linked to nectar volume. This holds true in bird-pollinated flowers, which produce relatively dilute nectars but in large quantities. Mean sugar concentrations in nectars of bird-pollinated flowers range between 20 and 26% (Pike and Waser, 1981; Proctor et al., 1996; Stiles and Freeman, 1993) with extremes between 10 and 34% (Baker, 1975). The sugar concentration of nectar determines its viscosity, which is an important physical property that is thought to affect the ease of uptake of nectar by birds. Large quantities of nectar at low concentrations have therefore been explained on the basis of constraints of its uptake associated with viscosity (Baker, 1975), and nectar viscosity tends to remains constant even under different environmental conditions (Proctor et al., 1996). However, dilute nectar with low sugar concentration is less optimal for bees, and so this trait may be more anti-bee than pro-bird (Bolten and Feinsinger, 1978). Nectar with sugar concentration below 18% is not beneficial to honeybees because of the high energetic cost of evaporating water in order to produce honey (Percival, 1965).

Nectar sugar composition is also important. Nectar is mainly composed of fruit sugars such as glucose and fructose (hexoses), and/or the disaccharide sucrose. Based on composition, the following types of nectar have been distinguished: (1) sucrose-dominant nectar, (2) sucrose-rich, (3) hexose-rich, and (4) hexose-dominant (Baker and Baker, 1983a). Hummingbird pollinated flowers generally have sucrose-dominant nectar, whereas flowers pollinated by passerine perching birds tend to have hexose-dominant nectar (Baker and Baker, 1983b, 1990; Baker et al., 1998; Elisens and Freeman, 1988; Freeman et al., 1984; Lammers and Freeman, 1986; Perret et al., 2001; Stiles and Freeman, 1993). Bee-pollinated flowers characteristically have sucrose-rich or sucrose-dominant nectar (Baker and Baker, 1983a), so such nectar composition in hummingbird-pollinated flowers that have evolved from bee-pollinated flowers is not surprising. More difficult to explain are the hexose-rich nectars. Passerine birds often feed on fruits as well, and it has been suggested that the "taste" for hexose fruit sugars derived from this (Baker and Baker, 1983b). Also, there is evidence that some Old World birds (such as Sturnidae, which includes some facultative nectarivores) have difficulty digesting sucrose (Schuler, 1977).

Amino acids are another important constituent of nectar. They provide a source of protein-producing substances, perhaps affect the "taste" of the nectar (Baker and Baker, 1983a) or stabilize the sugars in the nectar, i.e. to avoid crystallization (Baker and Baker, 1983a). Surveys have reported between 2 to 24 amino acids in nectar from different species (Baker, 1978). Bird-pollinated flowers contain low concentration of amino acids, which is mainly because birds have additional sources of amino acids (Baker and Baker, 1986). One exception to this trend has been studied in *Erythrina* (Leguminosae), which is pollinated by territorial orioles and tanagers, whose uptake of amino acids mainly depend on this plant (Baker and Baker, 1982).

2.3.5 Corolla morphology in bird-pollinated flowers

Flower morphology associated with ornithophily may be divided into five major groups (Proctor et al., 1996). In the brush-flower group, the flowers are arranged in clusters, usually in spheres or cylinders, with protruding stamens, and pollen placement is generalized. The Australian flora is particularly notable for its brush flowers, for instance in Proteaceae and in *Acacia* species (Lara and Ornelas, 2001). Another common flower trait is the hanging bell, producing copious nectar. Examples of this are found in *Canarina* (Campanulaceae) (Dupont et al., 2004; Valido et al., 2004), *Fritillaria* (Liliaceae) (Burquez, 1989; Peters et al., 1995) and *Cadia* (Fabaceae) (Citerne et al., 2006).

However, many ornithophilous flowers have long sympetalous corolla tubes. Such corolla tubes differ from those that are bee pollinated by being long and narrow (the shape of a bird's beak rather than a bee's body) and the inconspicuous size and position of the corolla lobes. Rather than forming a landing platform, as in bee-pollinated flowers, the lower corolla lobes are often reflexed under the flower so preventing alighting by insects. This has been termed the "dogfish" flower-type (Proctor and Yeo, 1973) from a fancied resemblance to the backwards-sloping snout of elasmobranch fish. The long, narrow tubular corolla is important in deterring bees from accessing nectar. However, this trait may be circumvented by nectar robbing (Lara and Ornelas, 2001) if the base of the corolla tube is not protected by a robust calyx. The corolla tube may also be downcurved (arcuate) in a similar way to many bird beaks. There is also a benefit to being downwardly directed in that insect access is made more difficult. However, most North American hummingbirds have relatively short straight beaks and, probably in consequence, most North American ornithophilous flowers have rather similar short straight and narrow corolla tubes.

Differences between corolla tubes of bee and bird-pollinated species are well illustrated in the genus *Streptocarpus* (Gesneriaceae) (Harrison et al., 1999; Hughes et al., 2007b). Between the bee-pollinated *S. rexii* Lindl. and the bird-pollinated *S. dunnii* Mast. there are five major floral differences associated with pollination syndrome. *S. dunnii* has small flowers that are massed into large inflorescences, while in *S. rexii* the floral display is provided mainly by large individual flowers. *Streptocarpus dunnii* has a cylindrical corolla tube suitable for probing by a bird's beak, whereas *S. rexii* has an open funnel-shaped flower allowing for ingress by relatively large bees. The orientation of the flowers is varied in *S. rexii*, while in *S. dunnii* all the flowers on the inflorescence face the single, large leaf that probably serves as a perching surface for birds. *S. dunnii* has red flowers without nectar-guides, whereas *S. rexii* has pale violet flowers marked with the dark nectar-guides that are often found in bee pollination. Finally, *S. dunnii* has a slightly longer adaxial than abaxial corolla tube length, producing a straight or slightly down-curved flower. This morphology is suitable for an ascending approach by a bird perching on the foliage below. *S. rexii*, on the other hand, has a shorter adaxial than abaxial corolla length, giving a swept-back flower, so providing an insect landing platform.

2.3.6 Other characters associated with bird pollination.

Whereas red flower colour and characteristic nectar are nearly universal in ornithophily, a number of other characters are more minor or associated only with specific examples of ornithophily. These include: (1) floral posture (2) secondary perches (3) protection (4) floral clustering and (5) prominent nectar and (6) absent characters

(1) Floral posture is closely correlated to pollinator behaviour. Nodding flowers without perches such as *Fuchsia* and bird-pollinated *Aquilegia* species are invariably hummingbird pollinated, as hummingbirds have the ability to hover under a downward facing flower and direct

their beaks upward into what is generally a long nectar path. This posture effectively excludes other pollinators by making the flower difficult to access.

Bee flowers, by contrast, are often horizontally oriented, in accord with the generally horizontal approach flight of bees, and have horizontal or near-horizontal lower lips for alighting. Flowers pollinated by perching birds that probe from above for nectar are often upwardly oriented. The genus *Tillandsia* provides examples of both types: short inflorescences with upwardly directed flowers for foraging by birds perching on the stiff leaves, or long arching inflorescences bearing pendulous flowers for hummingbird pollination. The bird-pollinated *Streptocarpus dunnii* has a single leaf, which probably functions as a perch for foraging birds and the flowers are unidirectionally oriented and down-curved towards the single leaf. Related bee-pollinated *Streptocarpus* species have flowers oriented in all directions. The South African *Phygelius capensis* E. Mey. ex Benth. has flowers that are somewhat resupinate, turning back towards the stems from where they may be easily probed by perching birds. In contrast insect-pollinated and hummingbird pollinated flowers tend to face outwards towards the incoming flight path of the pollinators.

(2) Secondary perches are important for facilitating pollination by birds requiring a perch for floral foraging. The most striking example is provided by the 'rat's tail' babiana (*Babiana ringens* Ker Gawl.) (Anderson et al., 2005). This produces completely sterile robust inflorescence stalks functioning exclusively to provide a perch for foraging by the malachite sunbird (*Nectarinia famosa* (L.)). The stiff inflorescence bract of *Strelitzia* provides a strong perch for birds working the *Strelitzia* flowers with their feet.

(3) Protection. Large vertebrate pollinators can be damaging to all but the most robust of flowers. Perching birds are frequently highly destructive of flowers and may destroy them in the search for insects and nectar. For this reason many flowers have protection in the form of tough

construction of parts. *Strelitzia reginae* is an example of a flower in which the floral parts are rather cartilaginous in texture and robust enough to survive rough foraging by pollinating birds.

(4) Floral clustering. Dense inflorescences are frequently associated with pollination by perching birds in the Old World. Rather than flying from single flower to single flower as bees and hummingbirds do, perching foragers will exploit flower clusters by probing several flowers from the same perch. A good example is provided by the robust, multi-flowered inflorescences of *Banksia*, which are pollinated by honey-eaters and other animals.

(5) Prominent nectar is frequently associated with bird pollination in those flowers wide enough for the nectar to be seen. Birds have great visual acuity and often nectar is presented to birds with striking visual cues. The "nectar globes" often found at the base of hanging bell shaped flowers are an example of this. In *Fritillaria imperialis* L., a plant known since the 18th century to attract birds to feed on the nectar (White, 1789), these take the form of prominent white depressions in the bases of the perianth segments that fill with large drops of nectar. *Nesocodon mauritianus* (I.B.K. Richardson) Thulin has nectar that is prominent for a different reason, it is coloured red with an aurone pigment, a phenomenon which is surprisingly common and generally associated with bird or reptile pollination (Hansen et al., 2006; Olesen et al., 1998).

(6) Characters conspicuous by their absence. These include scent, night blooming and nectar guides. In contrast to bats, which visit flowers that often have a rancid or mousey smell, many birds appear to have a poor sense of smell and bird-pollinated flowers are usually odourless. Not all birds have poor olfaction and exceptions include vultures, tube-nosed Procellaridae (storm petrels and albatrosses) and the nocturnal kiwis (*Apteryx* spp), all of which rely heavily on olfaction for foraging. However, nectar-feeding birds do not belong to this type and appear to forage by sight, and strictly diurnally. Nectar guides (markings under visible or

ultraviolet light that guide insects to floral rewards) are generally absent in bird-pollinated flowers. This is unsurprising as while the insect compound eye is optically crude, bird vision is excellent (with good depth perception) rendering foraging within flowers easy.

2.4 The molecular basis for the evolution of ornithophily

The bird-pollination syndrome appears to have evolved independently many times in a wide variety of plant families and genera (Stiles, 1981; Thomson et al., 2000). The syndrome may be initiated with birds experimentally foraging for insects or other sources of food into the flowers (Proctor and Yeo, 1973; Proctor et al., 1996), followed by selection of mutations that increase flower visitation and effectiveness of pollen transfer by birds. The molecular basis for these mutations has been addressed using model systems, in which a shift from one syndrome to another is examined in closely related species with contrasting flower morphology.

2.4.1 Some model systems

Ipomoea. The genus *Ipomoea* (morning glories) has tubular flowers in a wide variety of colours associated with different pollinators. The ancestral colour in this group is blue/purple and together with other traits (broad floral tube, moderate nectar production, inserted stigma and non-versatile anthers) this indicates an adaptation to bee pollination (McDonald, 1991). In one clade, however, there has been a shift to red flowers and hummingbird pollination, encompassing some six species (including *I. quamoclit* L.). Generally, the anthocyanin cyanidin, and its derivatives, produces purple flowers whereas pelargonidin and its derivatives result in red flowers. The gene flavonoid-3'-hydroxylase (*F3'H*) is important in controlling pigment production, and the flower colour shift in the *I. quamoclit* lineage has been shown to be due to a down-regulation of the *F3'H* enzyme (Zufall and Rausher, 2003, 2004). *F3'H* is directly responsible for the

hydroxylation of anthocyanidin precursors at the 3' position that is required for the production of cyanidin rather than pelargonidin. Furthermore the enzyme dihydroflavonol 4-reductase (*DFR*), with a role downstream of *F3'H*, seems to have lost its substrate affinity in this species. Although the major change in this case is a shift in flower colour, other flower traits, such as stigma position, flower tube width and the amount of nectar produced, also distinguish the two pollination syndromes.

Mimulus. In this genus two closely related species, *M. lewisii* Pursh and *M. cardinalis* Douglas ex Benth., display a great differences in floral characteristics. The former is pollinated mainly by bumblebees and has pink flowers with a wide corolla, nectar guides, and a modest amount of nectar; the latter is associated with hummingbird pollination and has red flowers with reflexed corolla lobes, a narrow corolla tube, and copious production of nectar (Ramsey et al., 2003). A major QTL for flower colour is associated with the allele *YELLOW UPPER* (*YUP*) that controls yellow pigment concentration (Bradshaw et al., 1998; Bradshaw et al., 1995). In *M. lewisii* the dominant allele *YUP* prevents carotenoid deposition, thus petals have only their pink anthocyanin pigments; whereas in *M. cardinalis,* the recessive allele *yup* allows carotenoid deposition and flowers with red colouration. Flower colour is known to have a very marked effect on pollinator visitation (Schemske and Bradshaw, 1999), and when the allele of *M. cardinalis yup* allele is introgressed into the *M. lewisii* background, hummingbird visitation increases dramatically, whereas bee visitation is considerably lowered (Bradshaw and Schemske, 2003). This suggests that an adaptive divergence in pollination syndrome can be initiated by a major change in flower colour alone. No gene has yet been cloned as responsible for *YUP* and its molecular identity still remains unknown.

Lotus. Lotus is a large genus in the Fabaceae, containing about 130 species divided into 14 sections. *Lotus* flowers are zygomorphic and typical of the papilionoid legumes. There are

five petals: one dorsal (the standard) usually large and conspicuous, two lateral petals (wings) and two ventral petals (keel petals) that enclose the stamens and the ovary. Pollination takes place when a bee lands and depresses the wings and the keel, forcing out a string of pollen from the stamens located beneath the keel and placing it in the underside of the visitor. Other floral features of *Lotus* that are associated with bee pollination are the horizontal position, yellow colour, sucrose-dominant nectar composition and scent production.

Bird pollination occurs in four species of *Lotus* from the Canary Islands (Olesen, 1985; Valido et al., 2004). In this group (also known as *Rhyncholotus*) the flowers are red-orange, in a vertical position, with hexose-dominant nectar and no scent production (Dupont et al., 2004). However, the most striking differences are the size and shape of the petals (Fig. 2.3). In the bird-pollinated species the flowers are about twice the size of those of typical bee-pollinated species and the dorsal petal is bent backwards while the ventral petals point up. The floral mechanism is effective in depositing pollen either on the top of the head or on the throat of a foraging bird (Fig. 2. 3). To date, two bird species, the Canarian chiffchaff *Phylloscupus canariensis* and the Blue tit *Parus caeruleus* have been reported feeding on some species of this group (Ollerton et al., 2009; Stelzer, 2005).

This system is a promising one in which to study the role of petal identity in transitions between pollination systems. The genes responsible for dorsal petal identity have been identified in *Lotus japonicus* (Regel) K. Larsen. These are the legume *CYCLOIDEA*- like genes (Citerne et al., 2003; Cronk, 2006b; Feng et al., 2006a), which have already been shown to be involved in the shift to bird-pollination in the papilionoid legume *Cadia* (Citerne et al., 2006).

Figure 2.1 Forms of bird-pollinated flowers. (A) *Strelitzia reginae*. (B) *Erythrina suberosa*, (C) *Babiana ringens* with sterile inflorescences for perching birds (arrow). (D) *Cadia purpurea*, a member of the genistioids with radial symmetry and nectar globes (arrow). (E) *Ipomopsis aggregata*. (F) *Phygelius capensis*. (G) *Psittacanthus* sp. (H) *Fritillaria suberosa* with nectar globes (arrow).

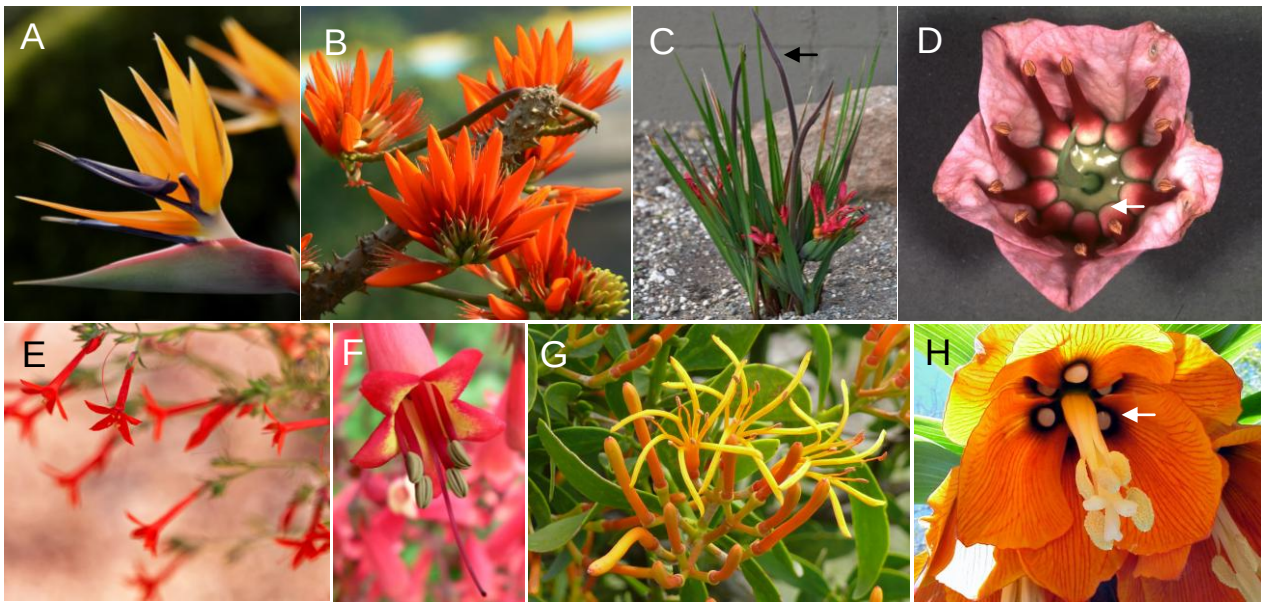


Figure 2.2 Approximate world distributions of the three main families of flower visiting birds: hummingbirds (Trochilidae), sunbirds (Nectariniidae), and honey-eaters (Meliphagidae).

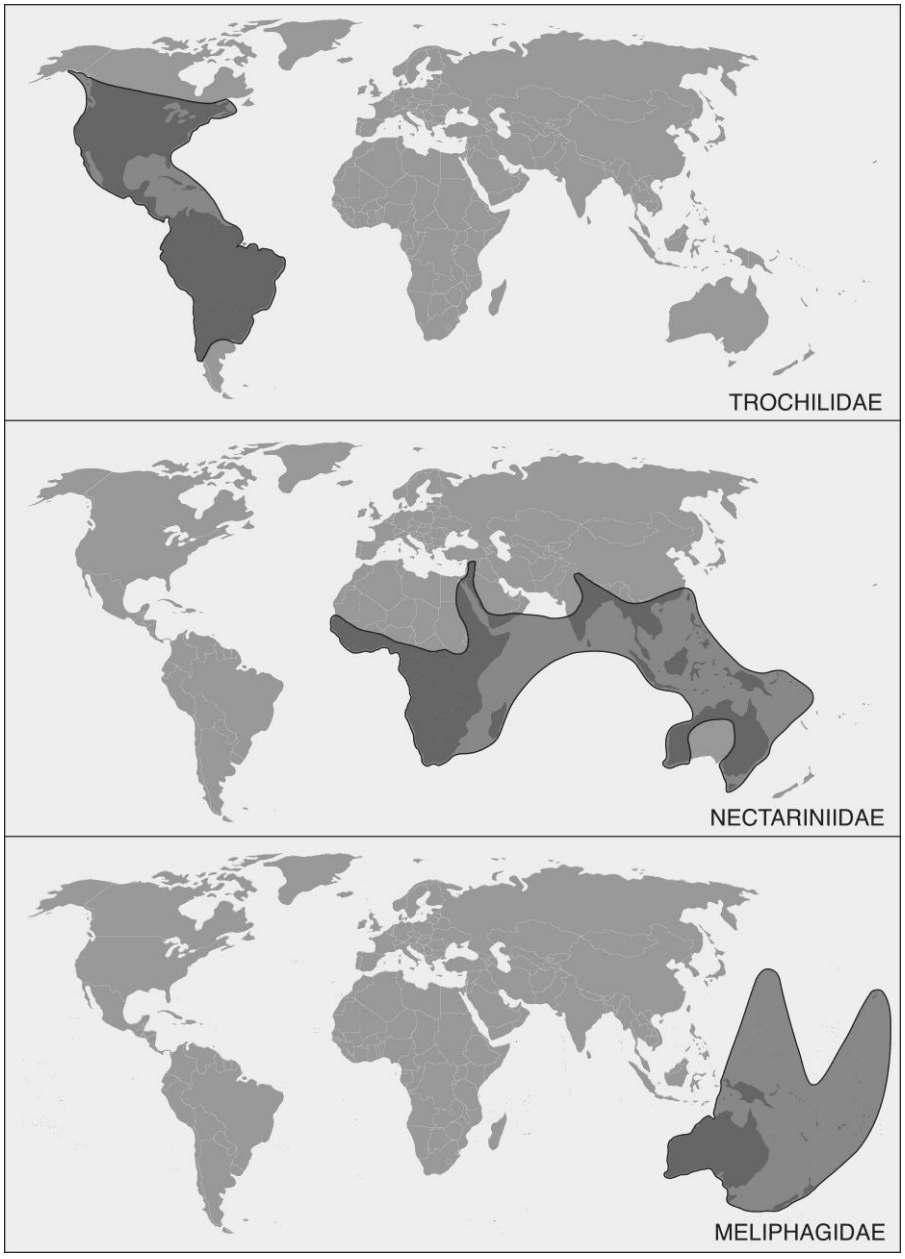
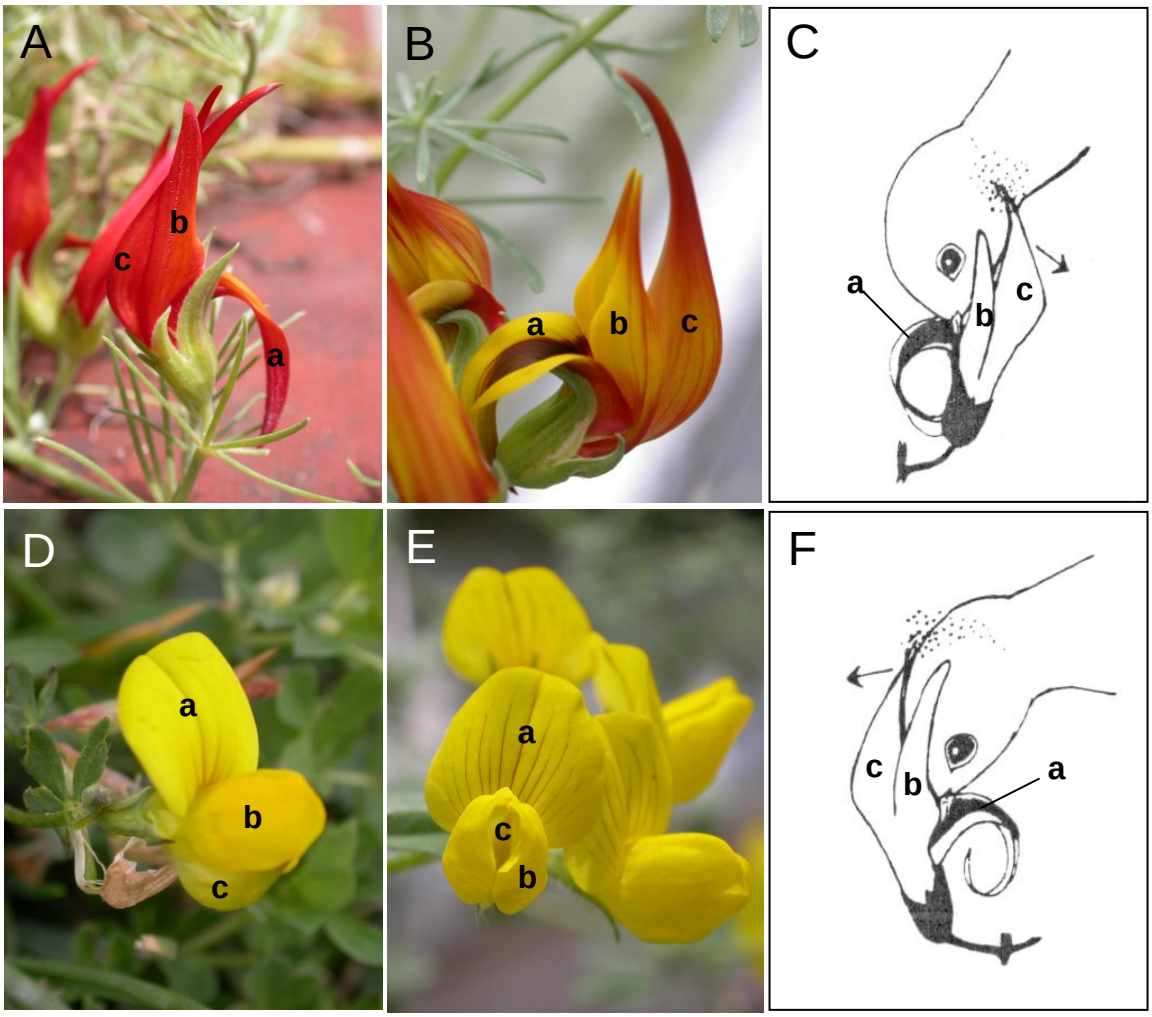


Figure 2.3 Flowers of *Lotus* species. Bird-pollinated species of (A) *Lotus berthelotii* Masf. and (B) *L. maculatus* Breitfeld, two members of the subgenus *Pedrosia* s.l. (D) The model legume *L. japonicus* GIFU B-129 and (E) *L. arenarius* Brot. a closely related species of the bird-pollinated species within the subgenus *Pedrosia* s.l. (C, F) Diagrammatic representation of the hypothetical mechanism by which a bird seeks nectar in flowers of *L. berthelotii*. Arrows indicate the direction in which the dorsal petal is pushed. a, Dorsal petal; b, lateral petal; and c, ventral petal. (C, F) Modified from Olesen (1985).



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3 Evolution of petal epidermal micromorphology in Leguminosae and its use as a marker of petal identity[1]

3.1 Introduction

The petal epidermal surface is important in pollination, as it influences the way in which pollinators perceive and interact with the flower. There is evidence that petal epidermal type and its surface affect colour depth (Dyer et al., 2007; Gorton and Vogelmann, 1996; Kay, 1988; Kay et al., 1981), iridescence (Whitney et al., 2009b), scent production (Kolossova et al., 2001), temperature (Comba et al., 2000; Dyer et al., 2006) and provides tactile cues (Comba et al., 2000; Kevan and Lane, 1985b; Stirton, 1981; Whitney et al., 2009b).

Previous surveys have classified and analyzed the distribution of the epidermal surface of petals within the angiosperms (Barthlott and Ehler, 1977; Kay et al., 1981). Extensive and detailed analyses have been provided for a few groups, such as Asteraceae (Baagøe, 1977, 1980; Hansen, 1991) and Leguminosae (Stirton, 1981), in which the characteristics of these epidermal types have been used for taxonomic and phylogenetic analyses.

Among the epidermal types described in these studies, papillose conical cells are frequently reported. Between 60 to 80 % of the angiosperm species analyzed have at least one petal with this epidermal type on the adaxial surface (or upper side, towards the floral axis) of the petals (Christensen and Hansen, 1998; Kay et al., 1981). The frequency of this cell type, coupled with other evidence, suggests that it has an adaptive value (Glover and Martin, 2002).

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Several recent studies have elucidated the genetic basis of the papillose conical cell type. The *MIXTA* gene, a MYB transcription factor, is required for the development of papillose conical cells in *Antirrhinum majus* L. (Noda et al., 1994). Further evidence suggests that this gene is sufficient to produce papillose conical cells in other groups within the asterid clade (Solanaceae and Scrophulariaceae). The developmental program leading to this epidermal pattern is therefore quite conserved, at least in these groups (Glover and Martin, 2002). However, it seems that a different developmental pathway may operate in the Brassicaceae (rosid clade) (Glover et al., 1998; Payne et al., 1998).

Petal identity, whether dorsal (adaxial), lateral or ventral (abaxial) is set at an early developmental stage by the expression of identity genes that induce transcription of other genes responsible for the shape, size, colour and epidermal type characteristics of individual petals (Cronk, 2006a). A change of petal identity may therefore change features of the epidermal surface. The functions of two petal identity genes have so far been described in the Leguminosae (Feng et al., 2006b). One of these genes, *Lotus japonicus CYCLOIDEA 2* (*LjCyc2*) is a transcription factor in the TCP gene family (named after its first three characterised members, TB1 in maize, CYC in snapdragon and PCFs in rice) with a dorsalizing action. This gene is associated with dorsal petal identity and hence papillose conical cell formation, as papillose conical cells are characteristic of standard petals in *L. japonicus*. The second gene, *LjCYC3* is a lateralizing factor. This gene is responsible for the lateral petal identity and hence the formation of tabular rugose cells with a jigsaw puzzle-shape that are characteristic of wing petals. The molecular basis of jigsaw puzzle shape cells has recently been elucidated (Fu et al., 2005).

These results suggest that each petal in the papilionoid legume flower has a distinct molecular identity, that may be marked by epidermal type. In transgenic plants that overexpress *LjCYC2* all petals have papillose conical cells, indicating that all petals have been converted to a

dorsal identity. Furthermore, natural evolution in legume flowers may occur by shifts in petal identity (Citerne et al., 2006).

Many legume species have a specialized flower morphology that promotes pollinator specificity (Fig. 3.1). Papilionoid legumes generally have three kinds of petals: a dorsal petal, called a "standard", two lateral petals or "wings", and two ventral petals forming the "keel". The distribution of the epidermal types within this family has not been analyzed in detail. A few studies have included some legume species (Christensen and Hansen, 1998; Kay et al., 1981), but in the first case only the dorsal petal was analyzed (C.I. Christensen, University of Copenhagen, Denmark, pers. comm.). A more extensive survey (Stirton, 1981) focused only on the papilionoids and was restricted to lateral petals. Another study has reported the epidermal type of two *Lathyrus* species, but only the dorsal and lateral petals were analyzed (Hammett et al., 1994). There is therefore a lack of detailed information about the epidermal types within the family.

Knowledge of the molecular developmental basis of petal identity has therefore not been coupled with a systematic analysis of the epidermal types and their distribution within the Leguminosae, even though epidermal types could be useful micromorphological markers of petal identity in developmental studies, for instance in the analysis of developmental mutants. In order to address this lack of information, we conducted a survey of the epidermal surface of the various types of petals within this family.

3.1.1 Objectives of the study

The aims of the present study are (1) to characterize the epidermal cell types of petals in the major clades of the Leguminosae, (2) to determine their distribution along four axes of distribution, one within the flower (dorsiventral) and three within each petal (abaxial-adaxial

surfaces, proximo-distal and medio-lateral), and (3) to relate these patterns to our current understanding of the molecular genetic basis of petal identity and flower evolution within the family.

3.2 Material and methods

3.2.1 Taxon sampling

Representative species were chosen from each of the three subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) and all 12 major clades currently recognized in the Leguminosae following recent phylogenetic analyses and taxonomic treatments (Lavin et al., 2005; Lewis et al., 2005; Wojciechowski et al., 2004). In total our sampling included 175 species representing 26 of the 37 tribes of the family, and 89 genera (Table 3.1) (Lewis et al., 2005). For an outgroup comparison four species of the closely related family Polygalaceae were analyzed. Polygalaceae also has zygomorphic flowers, but of a different constitution (Prenner, 2004a; Prenner and Klitgaard, 2008). Comparison with Polygalaceae is problematic as in this family the two adaxial petaloid organs are sepals. The lateral petals are reduced and the two adaxial petals may be considered to have closest functional equivalence to the lateral petals in legumes. The single abaxial petal forms the keel-like structure. We have therefore chosen to compare functionally equivalent structures (Table 3.2).

Mimosoid legume flowers also have a contrasting morphology (Prenner, 2004b) with an abaxial median petal rather than an adaxial median petal. In this case the dorsal petals are compared to the dorsal petal in other legumes and the ventral petal is compared to the ventral petals in other legumes.

To explore the variation within species several individuals were studied in two taxa (*Lotus japonicus,* four individuals and *Trifolium repens,* three individuals) but subsequent

sampling used only one individual to represent each species as intra-specific variation was found to be negligible. The variation among species within the same genus was explored in 11 genera, in all of which four or more species were analyzed. In *Senna*, 12 species were included representing three of the six recognized sections (*Chamaefistula, Senna, Peiranisia*) (Irwin and Barneby, 1982). These species represent all major clades currently recognized in this genus and included the entire range of flower symmetry within the group (Marazzi and Endress, 2008; Marazzi et al., 2006). Seven species of *Lathyrus* belonging to two of the nine sections of this genus (*Lathyrus* and *Orobus*) were studied (Kenicer et al., 2005). In *Lotus* seven species from three of its 14 sections (*Bonjeana*, *Lotus* and *Pedrosia*) were included (Degtjareva et al., 2006). In *Bauhinia* we analyzed six species with diverse flower morphology. In *Cassia* six species were included with a range of flower morphology and petal differentiation. Six species of *Dalbergia* were analyzed. Within *Erythrina* five species with diverse flower morphology were included. Within *Vicia* five species were sampled representing the two subgenera *Cracca* (sections *Cracca* and *Cassubicae*) and *Vicia* (sections *Vicia* and *Faba*) currently recognized within this genus (Choi et al., 2006a). In *Trifolium*, five species belonging to two of its subgenera, *Chronosemium* and *Trifolium*, and including sections *Trifoliastrum, Involucrarium, Trifolium* and *Vesicastrum*, were analyzed (Ellison et al., 2006). In *Genista* we sampled four species representating the subgenera *Spartocarpus* (section *Spartocarpus*) and *Genista* (section *Spartioides*), together with the species *Ulex europaeus* and *Chamaespartium sagittale*, currently considered to be nested within this group (Pardo et al., 2004). Finally, four species of *Dalea* were included.

3.2.2 Microscopy and cell type classification

Petals of fully open mature flowers were the subject of micromorphological studies using either a Hitachi S-2600N or a JEOL JSM-5900 LV scanning electron microscope (SEM) at an

acceleration voltage of 10-15 Kv. Either high vacuum or low vacuum conditions were used without significant differences on the structures observed. Some species were analyzed using a light microscope (Motic B Series) from preserved flowers in 70% ethanol or from herbarium specimens. In the latter case the flowers were first rehydrated, fixed in FAA, preserved in 70% ethanol and then analyzed. One species, *Lotus japonicus*, was tested under all different conditions to determine treatment effects or artifacts. Entire or partial petals were mounted on SEM stubs with double-sided adhesive tape. The distribution of the epidermal types was recorded using the following four axes using as a reference the centre of the flower (Fig. 3.2,A-F),where differentiation may occur, as listed below:

1) a dorsiventral axis within the flower. In the case of zygomorphic (monosymmetric) flowers (Endress, 2001), the dorsal, one lateral and one ventral petal were analyzed (Fig. 3.2,A, B). In actinomorphic (or polysymmetric) flowers all five petals were analyzed. In some caesalpinioids, with asymmetric flowers, all five petals were also analyzed separately.

2) abaxial-adaxial axis within the petal, i.e. upper and lower surfaces.

3) a proximo-distal axis within each petal, i.e. base to tip (Fig. 3.2, D-F).

4) a medio-lateral axis within the petal, i.e. middle to edge (Fig. 3.2, D-F].

The epidermal types were classified based on cell-shape traits (the primary sculpture) and on the fine relief of the cell wall (or secondary sculpture) (Barthlott, 1981, 1990), using the standard terminology regularly applied in similar studies within angiosperms (Kay et al., 1981). The epidermal types were classified into two main types: papillose and tabular (Table 3.3). These two types were further subdivided based on cell shape and sculpture of the outer surface. The distribution of these epidermal types was then recorded in relation to the four axes described above, either on entire petals (in the case of small flowers) or portions (representing each one of the sections) in the case of species with large flowers.

3.2.3 Reconstruction of evolutionary changes

Character evolution was studied using parsimony (DELTRAN) as implemented in MacClade 4.0 (Maddison and Maddison, 2000) and maximum likelihood using Mesquite (Maddison and Maddison, 2009). No major differences were found using the different methods. Traits were coded as binary characters (absence and presence). The distribution of these traits was mapped on a tree built according to recent phylogenetic analyses of the legumes (Lavin et al., 2005; Wojciechowski et al., 2004). Additional phylogenetic studies of individual genera or tribes were consulted in order to have a fully resolved phylogeny (Choi et al., 2006a; Degtjareva et al., 2006; Ellison et al., 2006; Kenicer et al., 2005; Marazzi et al., 2006; McMahon and Hufford, 2004; Pardo et al., 2004). In a few cases where there was a lack of information on specific groups, the polytomies were resolved randomly using MacClade.

3.3. Results

3.3.1 Epidermal types within Leguminosae

A total of six major epidermal types were recorded; five of which occurred in the papilionoids, three were found in the caesalpinioids and only one type was detected in the mimosoids (Table 3.3) (Fig. 3.3, A-R). Of the major categories only the tabular rugose cells with striations (TRS) had marked variation in cell shape, size and in the fine relief of the cell wall. Therefore, this epidermal type was further subdivided into three minor subtypes (i, ii and iii) described in Table 3.3. Some papilionoid species had only TRS on all three types of petals, but these minor differences (as marked by the TRS subtypes) can potentially be used to distinguish petal types in at least some species (Table 3.3 and Fig. 3.4).

3.3.2 Strong micromorphological variation in petals is exclusive to papilionoid legumes

The three distinct types of petals found in papilionoids generally showed clear micromorphological differences at the epidermal level (Fig. 3.5, A). Character analysis using parsimony and maximum likelihood indicates that this represents a single character state gain (Fig. 3.6). The early divergent *Cladrastis* branch of the papilionoids has only two major epidermal types, one occurring on the dorsal and lateral petals, and the other found on the ventral petal. Within papilionoids two groups, the tribe Loteae and the genistoid clade, commonly have the greatest micromorphological differentiation; their standards, wings, and keels are each characterized by specific epidermal features. Similarly, all of the five epidermal types that occur in papilionoids tend to be associated with a particular petal type (Table 3.4). For instance tabular flat striate cells (TFS) are restricted to keel petals and papillose conical cells (PCS) are generally characteristic of the standard petal. Further details of the association between epidermal types and petal types are given elsewhere (Table 3.2).

An interesting feature of papilionoid legumes is the occasional presence of more than one epidermal type on the same petal. In general the base of each petal has poorly differentiated cells (cells of simple shape, without prominent surface features and characteristics of early developmental stages), while cells in the middle and distal part are more strongly differentiated (Fig. 3.7,A-J). However, in some species two strongly differentiated but quite different epidermal types were observed in the same petal (Fig. 3.7). For example, in *Lathyrus* the dorsal petal has mainly tabular rugose cells (TRS) but has a border with papillose lobular cells (PLS) (Fig. 3.7,K-P).

3.3.4 Loss of micromorphological variation in Indigofera, Amorpheae and IRLC.

Although most papilionoids have high micromorphological variation along their floral dorsiventral axis, three papilionoid groups do not exhibit this pattern and have only one major epidermal type on all petals. The *Indigofera* clade, the Amorpheae and most species of the large Inverted Repeat-lacking clade (IRLC) have TRS covering most of their petals. In these groups, papillose conical cells are absent from the dorsal petal (although there are numerous exceptions, as noted in Table 3.2) (Fig. 3.6) and the area covered by TFS on the ventral petal is commonly reduced to a small region at the tip.

In the Amorpheae, all species analyzed have TRS on the three types of petals, with the exception of *Dalea leporina*, which has TRS on ventral and PCS on dorsal and lateral petals. The lack of micromorphological variation is evident both in species with a typical papilionaceous corolla, with three types of petals, such as in *Psorothamnus arborescens* and *Marina* spp. and in species with two petal types, such as *Apoplanesia paniculata*.

It is noteworthy that although TRS is the dominant epidermal type in all three petals, the ventral petal of these groups may still have a small amount of the TFS epidermal type that characterizes this petal type in other papilionoids. Furthermore, although these groups have the same major epidermal type (TRS) on the dorsal petal and on the wings, in some cases we detected minor differences in cell size and shape that made it possible to distinguish them easily.

This was particularly clear in the IRLC. Most of the species in this clade have lost the diversity of major epidermal types, as they have only tabular rugose cells with striations (TRS); however, in some instances we found striking differences between the TRS cell morphology on different petals within the flower. Variation in several features, such as cell size, shape of the cells (whether elongated or isodiametric), marginal features (such as the waviness of the cell

margin) and features of the surface (such as density of striations), allows clear micromorphological identification of each petal type.

I therefore further subdivided the TRS cell type into three subtypes: TRS subtype i (TRSi) has elongated cells, TRS subtype ii (TRSii) has isodiametric cells. In these two subtypes, the cell wall is usually well delimitated with dense striations. In contrast, TRS subtype iii (TRSiii) has elongated cells that tend to have weak cell wall delimitation and a lower density of striations. TRSi and TRSii were mainly observed on the dorsal and lateral petals, respectively, while TRSiii was found exclusively in the ventral petal (Fig. 3.4).

In some of the IRLC species, e.g. *Pisum sativum* (Fig. 3.4, A-C), this variation allows all three types of petals to be differentiated, or at least for the ventral (keel petal) to be differentiated from the dorsal and lateral petals, as in *Vicia hirsuta* (Fig. 3.4, D-F) or *Trifolium repens* (Fig. 3.4, G-I). However, in some IRLC species, such as *Melilotus* spp. (Fig. 3.4, J-L), there are no obvious differences between the different petals (Table 3.2).

3.3.5 Zygomorphy in caesalpinioids is not associated with strong micromorphological variation

The caesalpinioids show micromorphological differences of major epidermal types between species (Fig. 3.5, B-D and Fig. 3.4, M-O). Despite the variation in petal size and shape within flowers of many of the caesalpinioids surveyed (Fig. 3.2, A-F), the major epidermal type of each petal within a flower is uniform, usually TRS (64 %) while PCS is less common. Character state reconstruction indicates that TRS is the ancestral petal cell type in caesalpinioids and in legumes as a whole (Fig. 3.6).

There is therefore no association of a particular major epidermal type with any specific petal, although we found some very minor variations in cell size and form. Even *Cercis*, a

caesalpinioid with strongly zygomorphic flowers that superficially resemble papilionoid flowers has all its petals as TRS with only minor differences distinguishing them (Fig. 3.4, M-O). We included species representing all the main variation of flower symmetry within the genus *Senna*, which ranges from radial to asymmetric flowers (enantiostyly) (Marazzi and Endress, 2008; Marazzi et al., 2006). All the petals in individual flowers of this genus have the same major epidermal type (either TRS, PCS or PKR), even in species such as *Senna mucronifera* or *Cassia emarginata* (Fig. 3.2, E), where each of the five petals are different in size and shape.

3.3.6 The occurrence of papillose cells in the Leguminosae

Papillose cells (of all types) are particularly characteristic of papilionoids, but also appear to have evolved independently in some caesalpinioids (Fig. 3.6). Papillose cells (PCS and PKR) are mainly found in the dorsal part of the papilionoid flower (especially the standard). However some species have papillose cells on the wings. More rarely, papillose cells are found on the keel. For example, in *Lespedeza thunbergii* (Fig. 3.2, H) and *Desmodium incanum*, the keel petals are mainly covered by TFS, as in the majority of papilionoids. However, the tip of the keel has some cells intermediate between tabular flat cells (TFS) and papillose conical cells (PCS) in the most exposed area.

In the 175 species analyzed in this study only seven species (*Dalbergia brownei*, *Canavalia rosea* and five species of *Erythrina*) had well developed papillose conical cells on the keel. *Dalbergia brownei* and *Canavalia rosea* display PCS only in small patches. The genus *Erythrina* is notable for having papillose cells (PCS and PKR) on all three types of petal.

3.4 Discussion

3.4.1 Functional significance of papillose cell types

Papillose cells are characteristic of many papilionoid lineages and this group of cell types may have evolved in papilionoids due to possible functional advantages. Papillose cells may increase petal brightness and therefore increase pollinator visitation rates (Comba et al., 2000; Dyer et al., 2006; Glover and Martin, 1998).

It is interesting to consider why papillose cells (PCS and PKR) tend to be characteristic of the dorsal and lateral petals in papilionoids (Fig. 3.5, A), while these cell types are virtually absent in the ventral petals of the papilionoid subfamily (Table 3.4). Functionally, this may be because keel petals are in many cases covered by the lateral petals and are not usually prominent in pollinator attraction. This explanation is supported by the fact that species with papillose cells on the keel generally have an exposed keel, which probably does function in pollinator attraction (e.g. *Erythrina*, *Crotalaria* and *Lespedeza*).

3.4.2 Lack of micromorphological variation in the Caesalpinioideae

In contrast to papilionoids, caesalpinioids have relatively little micromorphological variation. Differences between dorsal, lateral and ventral petals do occur but are small and never involve major epidermal types (Fig. 3.5). This implies that a strong connection between the dorsiventral patterning of the flower and the developmental patterning of cell form, that is so evident in papilionoids, never evolved in caesalpinioids. Within the legumes, this connection between symmetry and cell type differentiation is therefore a unique derived character of papilionoids.

This lack of variation in caesalpinioids is independent of floral patterning, from nearly radial, as in *Bauhinia natalensis* and *B. petersiana*, to strongly zygomorphic flowers, as in *Tara*

cacalao or *Cercis* spp. (Fig. 3.2, D). For instance, *Cercis* has highly zygomorphic flowers (Fig. 3.2, A), and still displays much less micromorphological variation (Fig. 3.4, M-O) than a typical papilionoid (Fig. 3.5, A). These results support previous studies suggesting that the floral morphology of this genus, although superficially similar to a papilionoid, is only a weak convergence at the anatomical level to the papilionoid flower (Tucker, 2002).

In most caesalpinioids the adaxial and abaxial surface of the petal have the same major epidermal type. However, this is not the case in *Senna alata* (with all petals having PCS on the abaxial side and PKR on the adaxial one) and *Bauhinia tomentosa* (with PCS on the abaxial side and TRS on the adaxial side of all petals) (Table 3.2). These two species have flowers which do not fully open and it is the abaxial (exposed) (Fig. 3.2, B) surface that has papillose cells, which may enhance brightness and therefore the pollinator attractiveness of the flowers.

Another interesting feature of the caesalpinioids is the presence of trichomes in about 29% (11 species of 39) of the species analyzed and stomata in 15% (6/39). In most species the distribution of trichomes was homogeneous on all the petals within the flower (all petals having trichomes). This feature is shown in *Cassia emarginata* (Fig. 3.5). However, in six species trichomes were localized on a specific petal and hence the distribution of the trichomes could be used as an indicator of petal identity in these species. In Table 3.2 species with trichomes and stomata are denoted by a superscript t and s, respectively. Trichomes and stomata are also occasionally found in papilionoids but more rarely.

3.4.3 Mixing of epidermal types in the same petal surface

A unique feature of papilionoids is the occasional occurrence of more than one major epidermal type within a single petal. Between the two major epidermal types there is a transition zone or morphocline (Baagøe, 1977; Hansen, 1991), which has been reported previously in other

groups of angiosperms (Barthlott and Ehler, 1977; Christensen and Hansen, 1998; Hansen, 1991, 1992). The shifts observed involve changes from TRS to PLS (Fig. 3.7, K-P), TRS to PCS (Fig. 3.7, Q-V) or TRS to PKR in the dorsal and lateral petals, and from TRS to TFS in the ventral petal. The transition zone, with intermediate cell morphology between the two major epidermal types, is always relatively narrow (Fig. 3.7).

If the epidermal cell type is responding to the expression of underlying petal identity genes, as seems to be the case in at least some legumes (Feng et al., 2006b; Wang et al., 2008), these morphoclines may then indicate gene expression boundaries within organs. It might even suggest that such petals are of "mixed identity" and hence developmentally composite organs.

3.4.4 Contrasting loss of micromorphological variation within flowers of the Amorpheae and IRLC

Despite the striking micromorphological variation observed within the papilionoid flower, some species lack this variation. This is especially evident in the Amorpheae. It is interesting to note that the Amorpheae is often characterized by flowers of altered zygomorphy. They vary from zygomorphic (flowers with corollas of three types of petals as in *Psorothamnus*, *Marina* and some *Dalea* species) to subactinomorphic (five petals poorly differentiated into two types as in *Apoplanesia* and *Eysenhardtia*), while some species have only one petal (*Amorpha*) and other species lack petals altogether (*Errazurizia* and *Parryella*) (McMahon, 2005; McMahon and Hufford, 2004, 2005).

The tendency to weak dorsiventrality and subactinomorphy in this group is therefore associated with a lack of micromorphological diversity in the major epidermal types. Other dalbergioids, the group to which Amorpheae belongs, generally have flowers typical of other papilionoids.

The IRLC has also lost diversity of major epidermal types (TRS being the most common type), but in this case it is not accompanied by any loss of dorsiventral patterning as the flowers are highly zygomorphic and some species show differentiation in subtypes of TRS (Fig. 3.4). This indicates that the loss of epidermal diversity in Amorpheae and IRLC may be different in mechanism with different underlying genetic control in each group.

3.4.5 Genetic control of petal micromorphology and petal identity

Most studies of genetic control of petal micromorphology have focused on papillose conical cells. *MIXTA*, a transcription factor of the MYB family, has been associated with the differentiation of PCS in *Anthirrinum majus*, and in some species of Solanaceae (Glover and Martin, 2002; Noda et al., 1994). In *Petunia hybrida* Vilm. an ortholog of *MIXTA*, *mybPh1*, is associated with conical cells (Avila et al., 1993; van Houwelingen et al., 1998). However, there is not yet any evidence that *MIXTA* homologues play a role in PCS differentiation in legumes. An additional network of genes has been associated with the jigsaw puzzle shape of *Arabidopsis* leaf pavement cells. Pavement cell morphogenesis is controlled by two antagonistic pathways, Rho GTPase (*ROP*) and *ROP* effector protein (*RIC*) pairs with opposing action. The countersignaling of these two pathways has been associated with the interdigitation and final shape observed in these epidermal cells, with lobes and indentations. The pair *ROP2*/*RIC4* promotes cell growth on lobes, and the gene pair *ROP2*/*RIC1* restrains outgrowth, hence producing the indentations (Fu et al., 2005; Guimil and Dunand, 2007; Mathur, 2006). There is at present no information as the precise pathway by which petal identity genes promote the differentiation of the epidermal types described in this survey.

Lotus japonicus CYCLOIDEA 2 (*LjCYC2*), a transcription factor of the TCP family, promotes dorsal petal identity, i.e. the expression of this gene activates other gene networks

necessary for dorsal petal traits and it therefore confers specific organ fate on the primordium in which it is expressed. *LjCYC2* expression is therefore necessary for the differentiation of PCS in this petal (Feng et al., 2006b). Overexpression of this gene in transgenic plants promotes a dorsalization of all petals, with the consequent production of PCS cells in all petals.

In *Lotus japonicus, LjCYC2* is exclusively expressed on the adaxial side of the flower (dorsal petal) and thus affects dorsal petal identity (Feng et al., 2006b). It has been demonstrated that in species with a gain of dorsal identity, such as *Cadia purpurea* (G. Piccioli) Aiton, this gene is expressed throughout the flower, and all petals have the same shape, symmetry and identity (Citerne et al., 2006). Studies of the petal micromorphology of *Cadia* and related species would therefore be of great interest.

Another gene, *Lotus japonicus KEELED WING 1* (*KEW1* or *LjCYC3*), is associated with lateral petal identity. *LjCYC3* is required for normal lateral petal development, which includes tabular rugose cells (TRS) with a jigsaw puzzle shape. As in *LjCYC2*, mutations that knockout the activity of *LjCYC3* causes a ventralization of the lateral petal, with a subsequent lack of TRS and the presence of TFS (Feng et al., 2006b).

To date, the role of *LjCYC2* has been only explored in detail in *Lotus japonicus* (Feng et al., 2006b) and *Pisum sativum* (Wang et al., 2008). Orthologues of *LjCYC2* (*PsCYC2*) and of *LjCYC3* (*PsCYC3*) have recently been cloned in *Pisum sativum* (Wang et al., 2008). These two genes are required for normal zygomorphic development. *PsCYC2* is associated with dorsal identity and *PsCYC3* with lateral identity. But unlike *L. japonicus*, all petals have the same major micromorphology (TRS) and the identity of each petal (dorsal, lateral and ventral) is associated with the variation of features within this major epidermal type (Wang et al., 2008). Homologues of *CYCLOIDEA* have been also explored in Genisteae (Citerne et al., 2003; Citerne et al., 2006; Ree et al., 2004).

Therefore, the activation of the *ROP*/*RIC* pathway, essential for jigsaw puzzle cell shape, must be downstream of the *LjCYC3* identity gene. No ventral identity gene has yet been found, and the cause of ventral petal identity (and hence TFS differentiation) in legumes is still unknown. However, in both *Lotus japonicus* and *Pisum sativum*, the double knockout of the dorsal and lateral identity genes causes a ventralization of all the petals, suggesting that ventral identity and TFS are perhaps the default states in these groups.

3.4.6 Evolution of petal micromorphology

I have have shown that in the Caesalpiniaceae different petals within a flower are not strongly distinguished micromorphologically and the most common pattern is TRS/TRS/TRS (in dorsal, lateral and ventral petals respectively). The ancestral state reconstruction analysis suggests that this pattern probably represents the ancestral condition in the Leguminosae (Fig. 3.6). More or less papillose cells have independently evolved at least six times in the Caesalpinioideae and, when they occur, species have either PKR (in all petals) or PCS (in all the petals). Papillose cells appear to be absent in Mimosoideae, and their very small petals all have TRG.

In addition, my study suggests that the strong micromorphological differentiation within the flower is an advanced condition of the family that has evolved only within the papilionoid clade. Dorsiventral differentiation of major epidermal types appears to have evolved at the base of the papilionoid clade (with the less derived papilionoids state being PKR/PKR/TFS), reaching maximum differentiation independently in Loteae (PCS/TRS/TFS) and genistoids (PKR/PCS/TFS). Papillose cell types appear to have evolved many times in legumes but may have evolved only once in papilionoid legumes (at the base of that clade) and this character has apparently been lost at least four times in the subfamily (Fig. 3.6). However, most of the

papilionoid lineages that have lost papillose cells (groups predominantly with TRS/TRS/TRS as a character reversal), still display dorsiventral differentiation between petals by means of different TRS subtypes (e.g. TRSi, TRSii, and TRSiii).

Table 3.1 List of species sampled during this study. Clades are recognized following Wojciechowski et al., 2004; Lavin et al., 2005; Lewis et al., 2005. Tribal classification following Lewis et al., 2005. * = species analyzed from fresh flowers preserved in ethanol using a light microscope, + = species analyzed from flowers re-dehydrated from voucher specimens and preserved in ethanol using a light microscope. Other species were studied using the SEM and fresh material. UBCBG = UBC Botanical Garden, FTBG= Fairchild Tropical Botanical Garden, MBG = Montgomery Botanical Garden, QEG = Queen Elizabeth Garden, Vancouver, JBRCICY = Jardín Botánico regional del CICY, CICY = Centro de Investigación Científica de Yucatán, Mexico. JAO = Jardín de Aclimatación de la Orotova, Spain.

Taxon	Collection site and number	Voucher information
POLYGALACEAE		
Polygala chamaebuxus L.	UBCBG, No. 31175-575-94	*Ojeda 56*
P. diversifolia L.	FTBG, No. 941312A	*Ojeda 111*
P. grandiflora Walter	Wild collected, Big Pine Key, Florida	*Ojeda 121*
P. myrtifolia L.	FTBG, No. 2007-0032A	*Ojeda 116*
LEGUMINOSAE		
CAESALPINOIDEAE		
Cercis crown node		
Tribe Cercidae		
Cercis canadensis L.	UBCBG, No. 03872-0691-2006	*Ojeda 48*
C. yunnanensis Hu & Cheng	UBCBG, No. 27814-568-89	*Ojeda 47*
Bauhinia divaricata L.	MBG, No. S-19-04	*Ojeda 92*
B. natalensis Oliv.	MBG, No. 2006-0207	*Ojeda 95*
B. petersiana Bolle	FTBG, No. 2002-0513A	*Ojeda 127*
B. tarapotensis Benth.	FTBG, No. 981198A	*Ojeda 105*
B. tomentosa L.	MBG, No. S-67-06	*Ojeda 90*
**B. variegata* L.	JAO, No. 348-99	*Ojeda 161*
Tylosema fassoglensis (Kotschy ex Schweinf.) Torre & Hillc.	FTBG, No. 941095B	*Ojeda 112*
Caesalpioid crown node		
Tribe Detarieae		
**Brownea ariza* Benth.	JAO, No. 0270-70	*Ojeda 155*
B. capitella Jacq.	FTBG, No. 821821D	*Ojeda 104*
Tamarindus indica L.	Public street, Coral Gables, Florida	*Ojeda 130*
Umtiza crown node		
Tribe Caesalpineae		
**Gleditsia triacanthos* L.	JAO	*Ojeda 159*
Tribe Cassieae		
Cassia emarginata L.	FTBG, No. 2000-119D	*Ojeda 126*
**C. grandis* (L.F.) Pers.	JAO, No. 14-96	*Ojeda 156*
C. fistula L.	MBG, No. 7687A	*Ojeda 101*
C. javanica L.	MBG, No. 91268A	*Ojeda 87*
**C. javanica* L.	Cultivated, Australia Queesland	*Endress 6411*
C. nodosa Buch.-Ham. ex Roxb.	FTBG, No. 7978A	*Ojeda 128*
C. roxburghii DC.	FTBG, No. F64901A	*Ojeda 113*
Chamaecrista lineata (Sw.) Greene	Wild collected, Big Pine Key, Florida	*Ojeda 122*
Senna alata (L.) Roxb.	FTBG, No. 2006-1082A	*Ojeda 107*
S. corymbosa (Lam.) H.S. Irwin & Barneby	Blodel conservatory QEG	*Ojeda 40*
S. ligustrina (L.) H.S. Irwin & Barneby	FTBG, No. 2006-1082A	*Ojeda 109*
S. mexicana (Jacq.) H.S. Irwin & Barneby	MBG, without number	*Ojeda 131*
**S. mucronifera* (Mart. ex Benth.) H.S. Irwin & Barneby	Wild collected, Paraguay, Caaguazú	*Marazzi et al. BM 019*
**S. nicaraguensis* (Benth.) H.S. Irwin & Barneby	Wild collected, México, Chiapas	*Marazzi et al. BM 185*
**S. pallida* (Vahl) H.S. Irwin & Barneby	Wild collected, México, Oaxaca	*Marazzi et al. BM 178*
S. polyphylla (Jacq.) H.S. Irwin & Barneby	MBG, No. S-111-05	*Ojeda 93*
**S. racemosa* (Mill.) H.S. Irwin & Barneby	JBRCICY	*Ojeda 148*
S. septemtrionalis (Viv.) H.S. Irwin & Barneby	MBG, No. 2002-0775	*Ojeda 96*
**S. siamea* (Lam.) H.S. Irwin & Barneby	Wild collected, Panamá City, Panamá	*Marazzi et al. BM 157*
**S. wislizeni* (A. Gray) H.S. Irwin & Barneby	Wild collected, México, Puebla	*Marazzi et al. BM 169*
Tribe Caesalpineae		
Caesalpinia pulcherrima (L.) Sw.	FTBG, No. 2003-0464A	*Ojeda 114*
**C. gaumeri* Greenm.	JBRCICY	*Ojeda 150*

Taxon	**Collection site and number**	**Voucher information**
Delonix regia (Bojer ex Hook.) Raf.	MBG, No. 2000-1152A	*Ojeda 103*
Peltophorum pterocarpum (DC.) Backer ex K. Heyne	MBG, No. 76178A	*Ojeda 83*
Tara cacalao unpub. combination	FTBG, No. 93585C	*Ojeda 117*
MIMOSOIDEAE		
Mimosoid crown node		
Tribe Acacieae		
Acacia tortuosa (L.) Willd.	FTBG, No. 67330C	*Ojeda 129*
Tribe Ingeae		
Albizia julibrisii Durazz.	Public street, Vancouver	*Ojeda 42*
Calliandra haematocephala Hassk.	Blodel Conservatory QEG	*Ojeda 41*
Inga paterno Harms	MBG, No. 2002-0124A	*Ojeda 100*
Lysiloma sabicu Benth.	MBG, No. 99568A	*Ojeda 86*
Pithecellobium arboreum (L.) Urb.	MBG, No. 2003-1080B	*Ojeda 102*
Tribe Mimoseae		
Leucaena leucocephala (Lam.) de Wit	MBG, without number	*Ojeda 88*
PAPILIONOIDEAE		
Swartzia crown node		
Tribe Swartzieae		
+*Swartzia pittieri* Schery	Voucher specimen, CICY herbarium	G. Carnevali & F. Guanhez 1491
Cladrastis crown node		
[Tribe Sophoreae]		
+*Cladrastis lutea* (Michx.) K. Koch	Voucher specimen, UBC herbarium	*Jenninson 558*
+*C. sinensis* Hemsl.	Voucher specimen, UBC herbarium	*Straley 4284*
Genistioid crown node		
Tribe Crotalarieae		
Crotalaria pallida Aiton	Public garden, Vaca Key, Florida	*Ojeda 119*
Crotalaria sp.	FTBG, No. 2006-0220A	*Ojeda 108*
Tribe Genisteae		
Adenocarpus decorticans Boiss.	UBCBG, No. 32873-620-95	*Ojeda 50*
**Chamaecytisus albus* Rothm.	UBCBG, No. 037228-0474-2004	-
Chamaespartium sagitale (L.) P. Gibbs	UBCBG	*Ojeda 61*
Cytisus nigricans L.	UBCBG, No. 005631-0171	*Ojeda 62*
C. scoparius (L.) Link	UBC campus, Vancouver	*Ojeda 20*
Erinacea anthyllis Link	UBCBG, No. 10584-156-74	*Ojeda 51*
Genista lydia Boiss.	UBCBG, No. 037943-0117-2005	*Ojeda 55*
G. pilosa L.	UBCBG, No. 0112128-0117-2005	*Ojeda 49*
G. radiata DC.	UBCBG, No. 036849-5491-2003	*Ojeda 54*
G. tenera (Jacq.) Kuntze	UBCBG, No. 22652-050-82	*Ojeda 134*
Laburnum x *watereri* cultivar Vossii	UBCBG, No. 023189-0284-1983	*Ojeda 57*
Lupinus littoralis Douglas ex Lindl.	Spanish Bank beach, Vancouver	*Ojeda 15*
L. polyphyllus C.E. Anderson	UBC campus, Vancouver	*Ojeda 30*
**Retama rhodorhizoides* Webb & Berthel.	Wild collected, Tenerife, Spain	*Ojeda 164*
Spartium junceum L.	UBCBG, No. 014629-0275-1977	-
Ulex europaeus L.	UBCBG, without number	*Ojeda 35*
Tribe Sophoreae		
Sophora davidii (Franch.) Skeels	UBCBG, No. 034350-0077-1998	*Ojeda 133*
Sophora tomentosa L.	FTBG, No. 2001-0108B	*Ojeda 110*
Tribe Thermopsideae		
Baptisia australis (L.) R. Br.	UBCBG, No. 032961-0614-1996	*Ojeda 59*
Thermopsis macrophylla Hook. & Arn.	UBCBG, No. 036707-0640-2003	*Ojeda 63*
Dalbergioid crown node		
Tribe Adesmieae		
+*Adesmia atacamensis* Phil.	Voucher specimen, UBC herbarium	*Clarke 16-01*

Taxon	Collection site and number	Voucher information
Tribe Amorpheae		
+*Amorpha georgiana* Wilbur	Voucher specimen, UBC herbarium	*Leonard and Moore 1720*
A. herbacea Walter	MBG, No. 2005-0070	*Ojeda 94*
+*Apoplanesia paniculata* C. Presl	Voucher specimen, CICY herbarium	*J. S. Flores 9885*
+*Dalea carthagenensis* (Jacq.) J.F. Macbr.	Voucher specimen, CICY herbarium	*Ventura E. & Lopez E. 4138*
+*D. eysenhardtioides* Hemsl.	Voucher specimen, CICY herbarium	*D. Tenorio 2114*
+*D. greggii* A. Gray	Voucher specimen, UBC herbarium	*Helmkamp 5619*
+*D. leporina* (Aiton) Bullock	Voucher specimen, CICY herbarium	*J. Martinez 1021*
+*Marina diffusa* (Moric.) Barneby	Voucher specimen, CICY herbarium	*J.I. Calzada 20413*
+*M. ghiesbreghtii* Barneby	Voucher specimen, CICY herbarium	*Mendez Tun A. 7159*
+*Psorothamnus arborescens* (Torr. ex A. Gray) Barneby	Voucher specimen, UBC herbarium	*Sanders 2707*
Tribe Dalbergieae		
+*Andira galeottiana* Standl.	Voucher specimen, CICY herbarium	*Gutierrez B.C. 7740*
+*A. inermis* (W. Wright) Kunth ex DC.	Voucher specimen, CICY herbarium	*Tenorio P. 19415*
+*Aeschynomene americana* L.	Voucher specimen, CICY herbarium	*A. Mendez t. 6786*
+*A. ciliata* Vogel	Voucher specimen, CICY herbarium	*Magaña M.H. & Correl C. 613*
+*A. fascicularis* Schltdl. & Cham.	Voucher specimen, CICY herbarium	*E. Cabrera 15268*
**Arachis hypogaea* L.	Propagated from comercial seeds	-
Brya ebenus (L.) DC.	FTBG, No. 992154A	*Ojeda 125*
+*Dalbergia ecastaphyllum* (L.) Taub.	Voucher specimen, CICY herbarium	*I. Espejel 476*
+*D. brownei* (Jacq.) Schinz	Voucher specimen, CICY herbarium	*C. Chan 6484*
+*D. glabra* (Mill.) Standl.	Voucher specimen, CICY herbarium	*J. Flores & E. Ucan E. 8303*
+*D. glomerata* Hemsl.	Voucher specimen, CICY herbarium	*G. Aguilar M. 413*
+*D. stevensonii* (Standl.)	Voucher specimen, CICY herbarium	*Gutierrz B.C. 6675*
+*D. violacea* (Jacq.) Hoffmanns.	Voucher specimen, UBC herbarium	*Mimura 227*
**Diphysa carthagenensis* Benth. ex Benth. & Oerst.	JBRCICY	*Ojeda 143*
+*Pterocarpus acapulcensis* Rose	Voucher specimen, CICY herbarium	*R. Torres 11961*
+*P. hayesii* Hemsl.	Voucher specimen, CICY herbarium	*C:P. Cowan 4654*
Stylosanthes hamata (L.) Taub.	FTBG, without number	*Ojeda 118*
**Tipuana tipu* (Benth.) Kuntze	JAO, No. 0680-70	-
Mirbelioid crown node		
Tribe Mirbelieae		
+*Daviesia ulicifolia* Andrews	Voucher specimen, UBC herbarium	*Beamish 1314*
+*Gastrolobium floribundum* S. Moore	Voucher specimen, UBC herbarium	*Beamish 418*
+*G. subcordatum* (Benth.) G. Chandler & Crisp	Voucher specimen, UBC herbarium	*Beamish 675*
+*Gompholobium grandiflorum* Andrews	Voucher specimen, UBC herbarium	*Dempster 3075*
+*G. virgatum* Sieber ex DC.	Voucher specimen, UBC herbarium	*Beamish 155*
+*Isotropis cuneifolia* Benth. ex B.D. Jacks.	Voucher specimen, UBC herbarium	*Beamish 239*
+*Mirbelia dilatata* R. Br.	Voucher specimen, UBC herbarium	*Beamish 674*
+*Oxylobium scandens* (Small) Benth.	Voucher specimen, UBC herbarium	*Dempster 3427*
Milletioid crown node		
Tribe Desmodieae		
Desmodium incanum DC.	Wild collected, Coral Gables, Florida	*Ojeda 99*
Lespedeza thunbergii (DC.) Nakai	UBCBG, No. 011143-0013-2002	*Ojeda 136*
Tribe Indigofereae		
Indigofera decora Lindl.	UBCBG, No. 007973-0241-1974	*Ojeda 137*

Taxon	Collection site and number	Voucher information
I. kirilowii Maxim. ex Palibin	UBCBG, No. 33916-598-98	*Ojeda 77*
Tribe Phaseoleae		
**Canavalia rosea* (Sw.) DC.	Wild collected, Yucatán, México	*RD 2229*
Centrosema virginianum (L.) Benth.	MBG, No. 2003-1125	*Ojeda 97*
Clitoria fairchildiana R.A. Howard	MBG, No. 84	*Ojeda 84*
C. ternatea L.	MBG, No. 2005-1338[a]	*Ojeda 91*
**Erythrina carnea* Blanco	JAO, No. 254-99	*Ojeda 162*
**E. crista-galli* L.	JAO, No. 0222-94	*Ojeda 163*
**E. indica* Lam.	JBRCICY	*Ojeda 140*
**Erythrina* sp.	JAO, No. 504-99	*Ojeda 165*
**E.corallodendron* L.	Cultivated at La Palma, Spain	*Ojeda 157*
Galactia striata (Jacq.) Urb.	Wild collected, Big Pine Key, Florida	*Ojeda 120*
Glycine max (L.) Merr.	Propagated from comercial seeds	*Ojeda 76*
**Vigna elegans* (Piper) Maréchal, Mascherpa & Stainier	Wild collected, Dzitya, Yucatán, México	*Ojeda 144*
V. luteola (Jacq.) Benth.	MBG, without number	*Ojeda 89*
Phaseolus coccineus L. var. Scarlet Runner	Propagated from comercial seeds	*Ojeda 36*
P. vulgaris L. var. Blue Lake	Propagated from comercial seeds	*Ojeda 38*
Tribe Psoraleae		
***Bituminaria bituminosa* (L.) C.H. Stirt.	Wild collected, Gran Canaria, Spain	*Ojeda 166*
Tribe Millettieae		
***Piscidia piscipula* (L.) Sarg.	JBRCICY	*Ojeda 139*
Robinioid crown node		
Tribe Loteae		
Anthyllis hermanniae L.	UBCBG, No. 035419-0389-2000	*Ojeda 138*
Coronilla valentina L.	UBCBG, without number	*Ojeda 33*
C. varia L.	UBC campus, Vancouver	*Ojeda 39*
Hosackia chihuahuana S. Watson	From seeds, Pullman No. 18085	*Ojeda 79*
Lotus arenarius Brot.	From seeds, Pullman KBG 5688	*Ojeda 78*
L. burttii Borsos	Cultivated from seeds, Miyasaki University	*Ojeda 72*
L. corniculatus L.	UBC campus, Vancouver	*Ojeda 27*
L. eriosolen (Maire) Mader and Podlech	From seeds, Pullman KBG 5810	*Ojeda 243*
L. filicaulis Durieu	Propagated from seeds	*Ojeda 71*
L. hirsutus L.	UBCBG, No. 032962-0447-1996	*Ojeda 58*
L. japonicus (Regel) K. Larsen Gifu B-129	Propagated from seeds	*Ojeda 70*
**L. japonicus* (Regel) K. Larsen Gifu B-129	Propagated from seeds	*Ojeda 70*
L. japonicus (Regel) K. Larsen MG 20	Propagated from seeds	*Ojeda 69*
Tribe Robineae		
**Coursetia caribea* (Jacq.) Lavin	Wild collected, Dzitya, Yucatán, México	*Ojeda 146*
**Gliricidia maculata* (Kunth) Walp.	JBRCICY	*Ojeda 141*
+G. sepium (Jacq.) Kunth ex Walp.	Voucher specimen, CICY herbarium	*G. Aguilar 629*
Robinia hispida L.	UBC campus, Vancouver	*Ojeda 75*
R. pseudoacacia L.	UBCBG, without number	*Ojeda 63*
Tribe Sesbanieae		
**Sesbania grandiflora* (L.) Pers.	Voucher specimen, CICY herbarium	*M. Narváez 1400*
**S. herbaceae* (Mill.) McVaugh	Voucher specimen, CICY herbarium	*Duran et al 3506*
S. punicea (Cav.) Benth.	FTBG, No. 2005-0522A	*0jeda 124*
IRLC crown node		
Tribe Cicereae		
Cicer arietinum L.	Propagated from seeds	*Ojeda 73*
Tribe Fabeae		
Lathyrus japonicus Willd.	Wild collected, Spanish Bank beach, Vancouver	*Ojeda 16*
L. latifolius Visiani	Wild collected UBC campus, Vancouver	*0jeda 31*
L. odoratus L. cultivar April in Paris	Propagated from comercial seeds	*Ojeda 43*
L. sativus L. cultivar Electric Blue	Propagated from comercial seeds	*Ojeda 80*
L. sylvestris L.	Wild collected, UBC campus, Vancouver	*Ojeda 29*

Taxon	Collection site and number	Voucher information
L. venetus Rouy	UBCBG, No. 036759-0684-2003	*Ojeda 60*
L. vernus (L.) Bernh.	UBCBG, No. 032718-0620-	*Ojeda 52*
Lens culinaris Medik.	Propagated from comercial seeds	*Ojeda 74*
Pisum sativum L.	Propagated from comercial seeds	*Ojeda 44*
Vicia cracca L.	Wild collected, UBC campus, Vancouver	*Ojeda 18*
V. faba L.	Propagated from comercial seeds	*Ojeda 37*
V. hirsuta (L.) Gray	Wild collected, UBC campus, Vancouver	*Ojeda 28*
V. nigricans Hook. & Arn.	Wild collected, UBC campus, Vancouver	*Ojeda 24*
V. sativa L.	Wild collected, UBC campus, Vancouver	*Ojeda 26*
Medicago sativa L.	Wild collected, UBC campus, Vancouver	*Ojeda 34*
M. lupina L.	Wild collected, UBC campus, Vancouver	*Ojeda 25*
Melilotus albus Medik.	Wild collected, UBC campus, Vancouver	*Ojeda 17*
M. officinalis (L.) Pall.	Wild collected, UBC campus, Vancouver	*Ojeda 19*
Ononis spinosa L.	UBCBG, No. 034702-0433-1999	*Ojeda 132*
Trifolium dubium Sibth.	Wild collected, UBC campus, Vancouver	*Ojeda 22*
T. hybridum L.	Wild collected, UBC campus, Vancouver	*Ojeda 23*
T. incarnatum L.	UBCBG, without number	*Ojeda 32*
T. repens L.	Wild collected, UBC campus, Vancouver	*Ojeda 21*
T. wormskioldii Lehm.	Wild collected, UBC campus, Vancouver	*Ojeda 16*
Tribe Galegeae		
Astragalus crassicarpus Nutt.	UBCBG, No. 037261-0653-2004	*Ojeda 135*
Tribe Millettieae		
Wisteria brachybrotis Siebold & Zucc.	UBC campus, Vancouver	*Ojeda 68*
W. floribunda (Willd.) DC. var *.violacea*	UBCBG, No. 022727-0481-1983	*Ojeda 66*
W. floribunda (Willd.) DC. var. *rosea*	UBCBG, No. 037625-0075-2005	*Ojeda 67*
W. sinensis (Sims) Sweet	UBCBG, No. 014050-0223-11976	*Ojeda 65*

Table 3.2 Distribution of the major epidermal types in each of the three types of petal in the Leguminosae. PCS= papillose conical cells, PKR= papillose knobby with rugose sculpture, PLS = papillose lobular cells, TRG= tabular rugose with a granular scupture, TRS= tabular rugose striate, (TRS^{i} TRS^{ii} TRS^{iii} represents minor variations within this epidermal type that allow petal identification within the same species) and TFS= tabular flat longitudinally striate. More than one major epidermal type with more or less equal distribution are separated by a slash. Cell types in bold indicates the side more differentiated in each type of petal. If both sides are bold, then more or less the same level of differentiation is implied. PLS is a rare type that is never characteristic of whole petals and its presence is therefore only noted by the symbol ‡ (it is found in some *Lathyrus* species where it is restricted to the margin of the dorsal petal). s= stomata, t= trichomes, st= trichomes and stomata. *= flowers preserved in ethanol and analyzed using a ligth microscope, + = flowers re-dehydrated from voucher specimens, preserved in ethanol and analyzed using a light microscope. Other species were studied using the SEM and fresh material. -= petals absent.

Taxon	**Petaloid sepal**		**Dorsal petal**		**Ventral petal**	
POLYGALACEAE	**abaxial**	**adaxial**	**abaxial**	**adaxial**	**abaxial**	**adaxial**
Polygala chamaebuxus	TRS	TRS^{t}	PCS	PCS	PCS	PCS
P. diversifolia	TRS	**TRS**	**TRS**	**TRS**	**TFS**	TFS
P. grandiflora	TRS^{s}	**TRS**^{s}	**TRS**	TRS	**TRS**	TRS
P. myrtifolia	PKR	**PKR**	**TFS**	TFS	**TFS**^{t}	TFS^{t}
	Dorsal petal		**Lateral petal**		**Ventral petal**	
LEGUMINOSAE	**abaxial**	**adaxial**	**abaxial**	**adaxial**	**abaxial**	**adaxial**
CAESALPINOIDEAE						
Cercis crown node						
Tribe Cercidae						
Cercis canadensis	TRS	**TRS**	**TRS**	TRS	**TRS**	TRS
C. yunnanensis	TRS	**TRS**	**TRS**	TRS	**TRS**	TRS
Bauhinia divaricata	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**
B. natalensis	**TRS**	TRS	**TRS**	TRS	**TRS**	TRS
B. petersiana	**TRS**	**TRS**^{s}	**TRS**	**TRS**^{s}	**TRS**	**TRS**^{s}
B. tarapotensis	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**
B. tomentosa	**PCS**	TRS	**PCS**	TRS	**PCS**	TRS
B. variegata	**PCS**	**PCS**	**PCS**	**PCS**	**PCS**	**PCS**
Tylosema fassoglensis	PCS^{t}	**PCS**^{t}	PCS^{t}	**PCS**^{t}	PCS^{t}	**PCS**^{t}
Caesalpioid crown node						
Tribe Detarieae						
* *Brownea ariza*	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**
B. capitella	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**
Tamarindus indica	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**^{t}	**PKR**^{t}
Umtizia crown node						
Tribe Caesalpineae						
**Gleditsia triacanthos*	TRS^{t}	**TRS**^{t}	TRS^{t}	**TRS**^{t}	TRS^{t}	**TRS**^{t}
Tribe Cassieae						
Cassia emarginata	**TRS**st	**TRS**	**TRS**st	**TRS**	**TRS**^{s}	**TRS**
**C. grandis*	**PKR**^{t}	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**
C. fistula	**PKR**st	**PKR**	**PKR**st	**PKR**	**PKR**st	**PKR**
C. javanica	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}
**C. javanica*	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}	**PKR**^{t}
C. nodosa	**PKR**	**PKR**^{s}	**PKR**	**PKR**	**PKR**	**PKR**
C. roxburghii	**PKR**^{t}	**PKR**	**PKR**	**PKR**	**PKR**	**PKR**
Chamaecrista lineata	TRSst	**TRS**^{t}	TRSst	**TRS**^{t}	TRS^{t}	**TRS**^{t}
Senna alata	**PCS**	PKR	**PCS**	PKR	**PCS**	PKR
S. corymbosa	PKR	**PKR**	PKR	**PKR**	PKR	**PKR**
S. ligustrina	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**
S. mexicana	PKR	**PKR**	**PKR**	**PKR**	PKR	**PKR**
**S. mucronifera*	TRS^{t}	**TRS**^{t}	TRS^{t}	**TRS**^{t}	TRS^{t}	**TRS**^{t}
**S. nicaraguensis*	TRS	**TRS**	TRS	**TRS**	TRS	**TRS**
**S. pallida*	TRS^{t}	**TRS**^{t}	TRSst	**TRS**st	TRSst	**TRS**st
S. polyphylla	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**
**S. racemosa*	TRS	**TRS**	TRS	**TRS**	TRS	**TRS**
S. septentrionalis	TRS	**TRS**	TRS	**TRS**	TRS	**TRS**
**S. siamea*	TRS	**TRS**	TRS	**TRS**	TRS	**TRS**
**S. wislizeni*	TRS	**TRS**st	TRS	**TRS**	TRS	**TRS**
Tribe Caesalpineae						
Caesalpinia pulcherrima	PCS	**PCS**	**PCS**	**PCS**	**PCS**	**PCS**
**C. gaumeri*	TRS	TRS	TRS	TRS	TRS	TRS
Delonix regia	TRS^{t}	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**
Peltophorum pterocarpum	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**	**TRS**
Tara cacalao	**TRS**	**TRS**^{t}	**TRS**	**TRS**	**TRS**^{t}	TRS
MIMOSOIDEAE						
Mimosoid crown node						
Tribe Acacieae						
Acacia tortuosa	TRG	TRG	TRG	TRG	TRG	TRG

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial
Tribe Ingeae						
Albizia julibrissin	TRG^{t}	TRG	TRG^{t}	TRG	TRG^{t}	TRG
Calliandra haematocephala	TRG	TRG	TRG	TRG	TRG	TRG
Inga paterno	TRG^{t}	TRGst	TRG^{t}	TRGst	TRG^{t}	TRGst
Lysiloma sabicu	TRGst	TRGst	TRGst	TRGst	TRGst	TRGst
Pithecellobium arboreum	TRG	TRG	TRG	TRG	TRG	TRG
Tribe Mimoseae						
Leucaena leucocephala	TRG	TRGst	TRG	TRGst	TRG	TRGst
PAPILIONOIDEAE						
Swartzia crown node						
Tribe Swartzieae						
+*Swartzia pittieri*	**PKR**	**PKR**	-	-	-	-
Cladastris crown node						
[Tribe Sophoreae]						
Cladrastis lutea	PKR	PKR	PKR	PKR	TFS	TFS
C. sinensis	PKR	PKR	PKR	PKR	TFS	TFS
Genistioid crown node						
Tribe Crotalarieae						
Crotalaria pallida	TRS	**TRS**	**PKR**	TRS	**TFS**	**TFS**
Crotalaria sp.	TRS	**TRS**	**PKR**	PKR	**TRS**	TRS
Tribe Genisteae						
Adenocarpus decorticans	TRS	**TRS**	**TRS**	TRS	**TFS**	TFS
**Chamaecytisus albus*	TRS	**TRS**	**TRS**	TRS	**TRS**^{t}/TFS	TRS^{t}
Chamaespartium sagittale	TRS	**TRS**	**PCS**	PCS	**TFS**^{t}	TFS
Cytisus nigricans	PCS	**TRS**	**PCS**	PCS	**TFS**	TFS
Cytisus scoparius	PKR	**TRS**	**PCS**	PCS	**TFS**	TFS
Erinacea anthyllis	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
Genista lydia	TRS	**TRS**	**PCS**	PCS	**TFS**	TFS
G. pilosa	PKR	**TRS**^{t}	**PCS**	PCS	**TFS**^{t}	TFS
G. radiata	TRS^{t}	**PKR**	**PCS**	PCS	**TFS**^{t}	TFS
G. tenera	PKR	**PKR**	**PCS**	PCS	**TRS**	TFS
Laburnum x *watereri*	PCS	**PCS**	**PCS**	PCS	**TFS**	TFS
Lupinus littoralis	PCS	**PCS**	**TRS**	TRS	**TRS/TFS**	TRS/TFS
L. polyphyllus	PKR	**PKR**	**PKR**	PKR	**TRS/TFS**	TRS/TFS
**Retama rhodorhizoides*	TRS	TRS	**PCS**	TRS	**TRS**^{t}	TRS^{t}
Spartium junceum	PKR	PKR	**PCS**^{t}	PCS	**TFS**^{t}	TFS
Ulex europaeus	TRS	**PKR**	**PCS**	PCS	**TFS**	TFS^{t}
Tribe Sophoreae						
Sophora davidii	PCS	**PCS**	**PCS**	PCS	**TFS**^{s}	TFS
S. tomentosa	**PKR**^{s}	**PKR**	**PKR**	TRS^{s}	**TFS**	TFS^{s}
Tribe Thermopsideae						
Baptisia australis	PKR	**TRS**	**PKR**	TRS^{s}	TFS	TFS
Thermopsis macrophylla	PKR	TRS	PCS	PKR	TFS	TFS
Dalbergioid crown node						
Tribe Adesmieae						
+*Adesmia atacamensis*	PCS	**PCS**	**PCS**	PCS	**TFS**	TFS
Tribe Amorpheae						
+*Amorpha georgiana*	TRS	**TRS**	-	-	-	-
+*A. herbacea*	TRS	**TRS**	-	-	-	-
+*Apoplanesia paniculata*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}
+*Dalea carthagenensis*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}
+*D. eysenhardtioides*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}
+*D. greggii*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}
+*D. leporina*	PCS	**PCS**	**PCS**	PCS	**TRS**^{i}	TRS^{i}
+*Marina diffusa*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}
+*M. ghiesbreghtii*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}
+*Psorothamnus arborescens*	TRS^{i}	**TRS**^{i}	TRS^{i}	TRS^{i}	**TRS**^{i}	TRS^{i}

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial
Tribe Dalbergieae						
+*Andira galeottiana*	PKR	**PCS**	**PCS**	PCS	**TRS**	TRS
+*A. inermis*	PCS	**PCS**	**PCS**	TRS	**TRS**	TRS
+*Aeschynomene americana*	PCS	**PCS**	**PCS**	PCS	TRS/TFS[t]	TRS/TFS
+*A. ciliata*	PCS	**PCS**	**PCS**	PCS	**TRS**	TFS
+*A. fascicularis*	TRS	**TRS**[t]	**PCS**	PCS	**TFS**	TFS
**Arachis hypogaea*	TRS	**TRS**	**TRS**	TRS	**TFS**	TFS
Brya ebenus	PKR	**PKR**	**PKR**	**PKR**	**TRS**	**TRS**
+*Dalbergia ecastaphyllum*	PCS	**PCS**	**PCS**	PCS	**TRS**	TRS
+*D. brownei*	PKR	**PKR**	**PCS**	TRS	**TRS**	TRS/TFS
+*D. glabra*	PCS	**PCS**	**PCS**	PCS	**TRS**	TRS
+*D. glomerata*	PCS	**PCS**	**PCS**	PCS	**TRS**	TRS
+*D. stevensonii*	PCS	**PCS**	**PCS**	PCS	**TRS**	TRS
+*D. violacea*	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
**Diphysa carthaginensis*	PKR	**PKR**	**TRS**	TRS	**TFS**	TFS
+*Pterocarpus acapulcensis*	PCS	**PCS**	**TRS**	TRS	**TRS**	TRS
+*P. hayesii*	PCS	**PCS**	**PCS**	PCS	**TRS**	TRS
Stylosanthes hamata	TRS	**TRS**	**PCS**	TRS	**TFS**	TFS
**Tipuana tipu*	TRS	TRS	PKR	PKR	TRS	TRS
Mirbelioid						
Tribe Mirbelieae						
+*Daviesia ulicifolia*	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
+*Gastrolobium floribundum*	PKR	**PKR**	**PKR**	PKR	**TRS**	TRS
+*G. subcordatum*	PKR	**PKR**	**PKR**	PKR	**TRS**	TRS
+*Gompholobium grandiflorum*	PKR	**PKR**	**PCS**	PCS	**TRS**	TRS
+*G. virgatum*	PKR	**PKR**	**PKR**	PKR	**TRS**	TRS
+*Isotropis cuneifolia*	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
+*Mirbelia dilatata*	PKR	**PKR**	**PKR**	PKR	**TRS**	TRS
+*Oxylobium scandens*	PKR	**PKR**	**TRS**	PKR	**TRS**	TRS
Milletioid crown node						
Tribe Desmodieae						
Desmodium incanum	PCS	**PCS**	**PCS**	TRS	**TRS**	**TRS**
Lespedeza thunbergii	PCS	**PCS**	**PCS**	PCS	**TFS**	TFS
Tribe Indigofereae						
Indigofera decora	TRS[i]	**TRS**[i]	TRS[i]	TRS[i]	**TRS**[i]	TRS[i]
I. kirilowii	TRS[i]	**TRS**[i]	TRS[i]	TRS[i]	**TRS**[i]	TRS[i]
Tribe Phaseoleae						
**Canavalia rosea*	PKR	**PKR**	**TRS**	TRS	**TRS**	TRS
Centrosema virginianum	PCS[t]	**PCS**	**PCS**[t]	PCS	**TFS**[t]	TRS/TFS
Clitoria fairchildiana	TRS[t]	**PCS**[t]	**TRS**[t]	**TRS**[t]	**TFS**[t]	**TFS**
C. ternatea	TRS	**PCS**	**PKR**	TRS	**TFS**	TFS
**Erythrina carnea*	PCS	PCS	PKR	PKR	PKR	PKR
**E. crista-galli*	PCS[s]	PCS	PCS	PCS	PCS[s]	PCS[s]
**E. indica*	PCS	**PCS**	**PCS**	PKR	**PKR**	PKR
**Erythrina* sp.	PCS	PCS	PKR	PKR	PKR	PKR
**E. corallodendron*	PCS	PKR	PKR	PKR	PKR	PCS
Galactia striata	PKR	**PKR**	**PKR**	PKR	**TRS**	TRS
Glycine max	PKR	**PKR**	**PCS**	PCS	**TRS**	TRS
**Vigna elegans*	TRS[s]	**PKR**	**TRS**	TRS	**TFS**	TFS
V. luteola	PKR[s]	**PKR**	**PKR**	PKR	**TFS**	**TFS**
Phaseolus coccineus	PCS	**PCS**	**PCS**	PCS	**TFS**	TFS
P. vulgaris	PCS	**PCS**	**PCS**	PCS	**TFS**[t]	TFS
Tribe Psoraleeae						
***Bituminaria bituminosa*	PCS	PCS	PKR	PCS	TRS	TRS

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial
Tribe Millettieae						
***Piscidia piscipula**	PKR[t]	**PKR**	**PKR**	PKR	**TFS**	TFS
Robinoid crown node						
Tribe Loteae						
Anthyllis hermanniae	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
Coronilla valentina	PCS	**PCS**	**PCS**	PCS	**TRS/TFS**	TFS
C. varia	PCS	**PCS**	**PCS**	TRS	**TFS**	TFS
Hosackia chihuahuana	PCS	**PCS**	**PCS**	TRS	**TRS/TFS**	TFS
Lotus arenarius	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. burttii	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. corniculatus	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. eriosolen	TRS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. filicaulis	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. hirsutus	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. japonicus Gifu B-129	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
**L. japonicus* Gifu B-129	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. japonicus MG 20	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
Tribe Robineae						
**Coursetia caribaea*	PKR	**PKR**	**PKR**	TRS	**TRS**	TFS
**Gliricidia maculata*	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
+G. sepium	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
Robinia hispida	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
R. pseudoacacia	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
Tribe Sesbanieae						
**Sesbania grandiflora*	TRS	**TRS**	**PKR**	TRS	**TFS**	TFS
**S. herbacea*	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
S. punicea	PKR	**PKR**	**TRS/PKR**	TRS/PKR	**TFS**	TFS
IRLC crown node						
Tribe Cicereae						
Cicer arietinum	TRS[i]	**TRS[i]**	**TRS[i]**	**T**RS[i]	**TRS[iii]**	TRS[iii]
Tribe Fabeae						
Lathyrus japonicus	TRS[i]	**TRS[i]**	**TRS[i]**	**T**RS[i]	**TRS[iii]**	TRS[iii]
L. latifolius	TRS[i]	**TRS[i]**	**TRS[i]**	**T**RS[i]	**TRS[iii]**	TRS[iii]
L. odoratus	TRS[i]	**TRS[i]**	**TRS[i]/PKR**	**T**RS[i]	**TRS[iii]**	TRS[iii]
L. sativus	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[iii]**	TRS[iii]
L. sylvestris	TRS[i]	**TRS[i]** ‡	**TRS[ii]**	TRS[ii]	**TRS[iii]**	TRS[iii]
L. venetus	PKR	**PKR**	**PKR**	PKR	**TRS[iii]**	TRS[iii]
L. vernus	TRS[i]	**TRS[i]** ‡	**PKR**	PKR	**TRS[iii]**	TRS[iii]
Lens culinaris	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
Medicago sativa	TRS	PCS	PCS	PCS	TRS	TRS
M. lupina	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
Melilotus albus	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
M. officinalis	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
Ononis spinosa	TRS[t]	**TRS**	**TRS**	TRS	**TFS**	TFS
Pisum sativum	TRS[i]	**TRS[i]**	**TRS[ii]**	TRS[ii]	**TRS[iii]**	TRS[iii]
Trifolium dubium	PKR	**PCS**	**TRS[i]**	TRS[i]	**TRS[it]**	TRS[i]
T. hybridum	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
T. incarnatum	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
T. repens	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[iii]**	TRS[iii]
T. wormskioldii	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[iii]**	TRS[iii]
Vicia cracca	TRS[i]	**TRS[i]**	**PCS**	TRS[ii]	TRS[i]	**TRS[i]**
V. faba	**TRS[i]**	**TRS[i]**	**PCS**	PCS	**TRS[iii]**	**TRS[iii]**
V. hirsuta	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[iii]**	TRS[iii]
V. nigricans	TRS[i]	**PCS**	**PCS**	TRS[i]	**TRS[i]**	TRS[i]
V. sativa	TRS[i]	**PCS**	**PCS**	TRS[i]	**TRS[i]**	TRS[i]
Tribe Galegeae						
Astragalus crassicarpus	TRS[i]	**TRS[i]**	TRS[i]	TRS[i]	**TRS[i]**	TRS[i]
Tribe Millettieae						
Wisteria brachybrotis	PKR	**PKR**	**PKR**	TRS[ii]	**TFS**	TFS

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	abaxial	**adaxial**	**abaxial**	**adaxial**	**abaxial**	**adaxial**
W. floribunda var . *violacea*	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
W. floribunda var. *rosea*	PKR	**PKR**	**PKR**	PKR	**TFS**	TFS
W. sinensis	PKR	**PKR**	**PKR**	TRS[ii]	**TFS**	TFS

Table 3.3 Classification of the epidermal types observed in Leguminosae.

Major type group	Major epidermal type	Abbreviation	Figure	Example
Papillose	Papillose conical cells with striations	PCS	3 D, J, P	*Lotus japonicus* (standard)
	Papillose knobby cells with a rugose sculpture	PKR	3 E, K, Q	*Robinia pseudoacacia* (standard and lateral)
	Papillose lobular cells with striations	PLS	3 F, L, R	*Lathyrus venetus* (standard)
Tabular	Tabular rugose cells with longitudinal striations	TRS	3 B, H, N	*Wisteria sinensis* (wings)
	Tabular rugose cells with a granulose sculpture	TRG	3 A, G, M	*Calliandra haematocephala* (all petals)
	Tabular flat cells with longitudinal striations	TFS	3 C, I, O	*Lotus japonicus* (keel)
	Note: TRS is the most variable type. The main subtypes may be distinguished as follows: (i) cells elongated with dense striation (ii) cells more or less isodiametric with dense striation and (iii) isodiametric or elongated cells with less dense striations			

Table 3.4 Distribution of the major epidermal cell types within sampled Papilionoideae. PCS= papillose conical cells, PKR= papillose knobby cells, TRS= tabular rugose cells with striations, TFS= tabular flat cells with striations.

Petal	PCS		PKR		TRS		TFS	
	Abaxial	**Adaxial**	**Abaxial**	**Adaxial**	**Abaxial**	**Adaxial**	**Abaxial**	**Adaxial**
Dorsal	38	45	42	40	53	48	0	0
Lateral	44	33	38	32	48	66	0	0
Ventral	1	2	4	3	61	61	63	64

Figure 3.1 Diversity of flower symmetry in the Leguminosae. (A) Zygomorphic flowers of *Cercis canadensis* (Caesalpinioideae). (B) Flowers of *Bauhinia tomentosa* (Caesalpinioideae). (C) Radially symmetric flower of *Brownea capitella* (Caesalpinioideae). (D) Zygomorphic flowers of *Tara cacalao* (Caesalpinioideae). (E) Asymmetric enantiostylous flower in *Cassia emarginata* (Caesalpinioideae). (F) Zygomorphic flower with the dorsal petal differentiated with respect to the other petals in *Caesalpinia pulcherrima* (Caesalpinioideae). (G) Radial flower with reduced petals in *Inga paterno* (Mimosoideae). (H) Zygomorphic flowers in *Lespedeza thunbergii* (Papilionoideae) (with the ventral petal more exposed), (I) *Clitoria ternatea* (Papilionoideae) (the dorsal petal is pointing downwards), (J) *Sesbania punicea* (Papilionoideae), and (K) *Lathyrus sylvestris* (Papilionoideae) (ventral petals enclosed).

Figure 3.2 Four axes of variation considered in the study of the epidermal types and their distribution on each petal. A) *Lotus corniculatus*, three types of petals in zygomorphic papilionoid flowers, a) dorsal, b) lateral and c) ventral, within an adaxial-abaxial axis within the flower. B) Abaxial-adaxial surface within the petal. The abaxial side is exposed in lateral (b) and ventral (c) petals in most papilionoids. However, the adaxial surface is exposed in most standard petals (a). C) *Senna corymbosa* with three types of petals, (a) dorsal, (b) lateral and c) ventral within an adaxial-abaxial axis within the flower. Further axes are: proximal-distal and medio-lateral axes within D) the dorsal petal, E) lateral petal and F) ventral petal in *Lotus corniculatus*. The base of the dorsal petal (claw) in *L. corniculatus* has been separated from the rest of the petal.

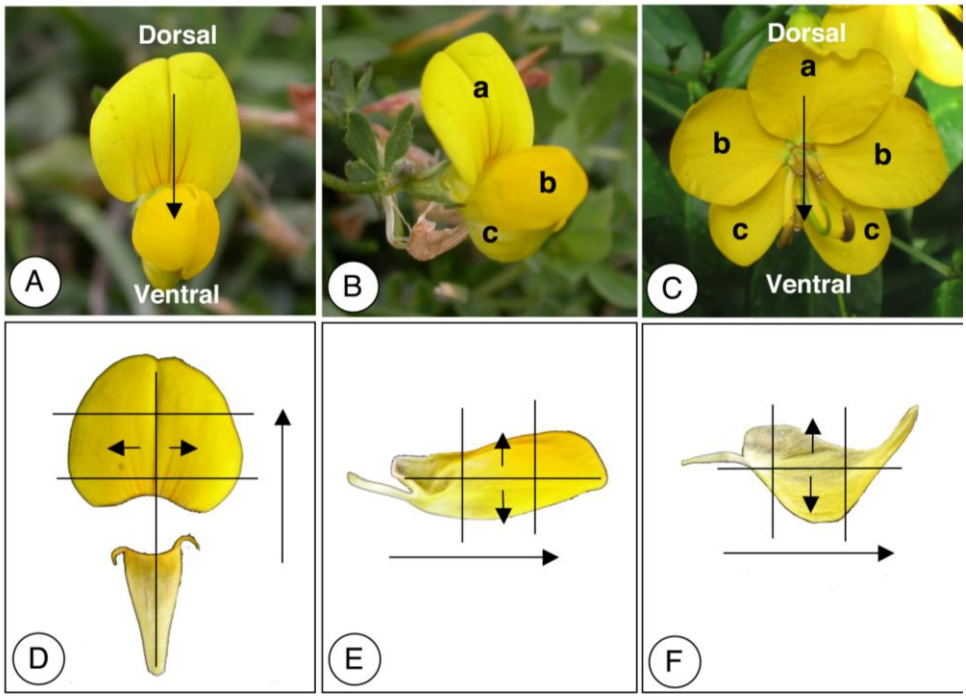


Figure 3.3 Classification of the major epidermal types in Leguminosae. (A, G, M) tabular rugose cells with granulose sculpture (TRG) in *Calliandra haematocephala* (Mimosoideae). (B and H) tabular rugose cells with striation (TRS) in *Wisteria sinensis* (Papilionoideae) and (N) in *Lotus japonicus* (Papilionoideae). (C and I) tabular flat cells with striations (TFS) in *W. sinensis* and (O) in *Lotus japonicus*. (D, J, P) papillose conical cells (PCS) in the dorsal petal of *Lotus japonicus*. (E, K, Q) papillose knobby cells (PKR) in the dorsal and lateral petals of *Robinia pseudoacacia* (Papilionoideae). (F and L) papillose lobular cells (PLS) in the dorsal petal of *Lathyrus venetus* and (R) in *Lathyrus sylvestris* (Papilionoideae). This latter epidermal type was only observed in these two species. All images correspond to the adaxial side of the petal. Scale bar 50 µm (A-F), 100 µm (G-L) and 20 µm (M-R).

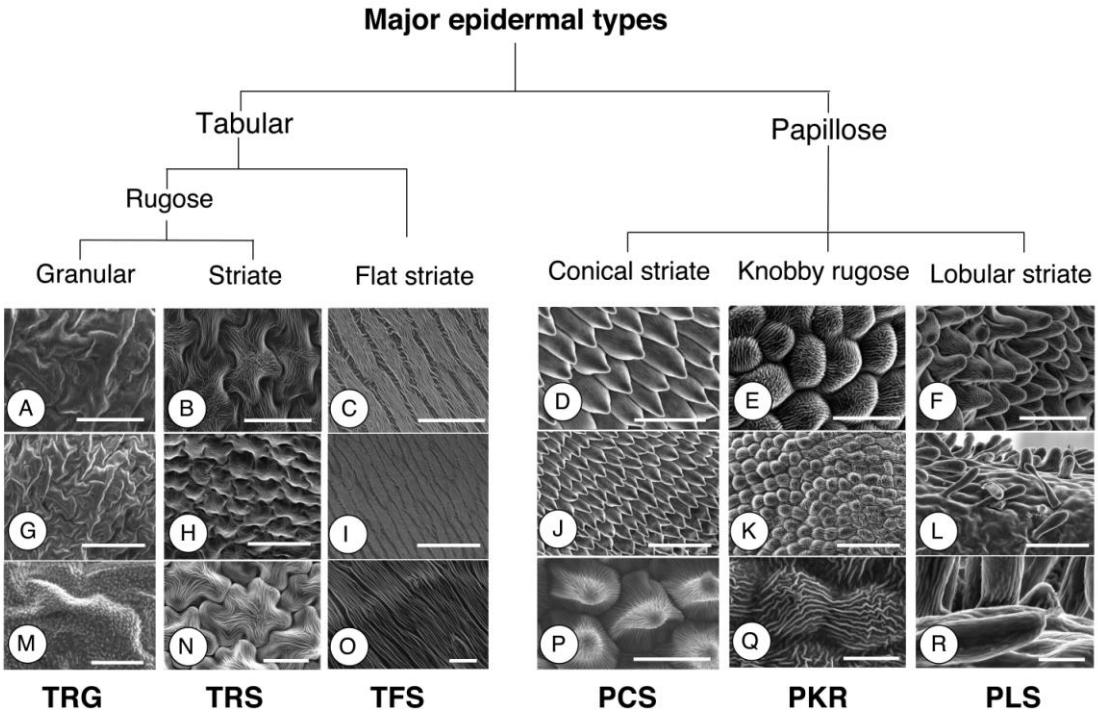


Figure 3.4 Minor epidermal types within the tabular rugose cells with striations (TRS). Minor epidermal variants within TRS are designated as i, ii and iii. (A-C) variation among minor epidermal types in *Pisum sativum* (Papilionoideae) enables each petal type to be distinguished. Variation within minor epidermal types distinguished the ventral but does not distinguish between the lateral and dorsal petals in (D-F) *Vicia hirsuta* (Papilionoideae), (G-I) *Trifolium repens* (Papilionoideae). Variation among minor epidermal types does not allow clear characterization of the three petal types in (J-L) *Melilotus officinalis* (Papilionoideae) and (M-O) *Cercis canadensis* (Caesalpinioideae). All illustrations correspond to the adaxial side of the petal, except H, I, K, L, N and O, which correspond to the abaxial side. All scale bars 50 µm.

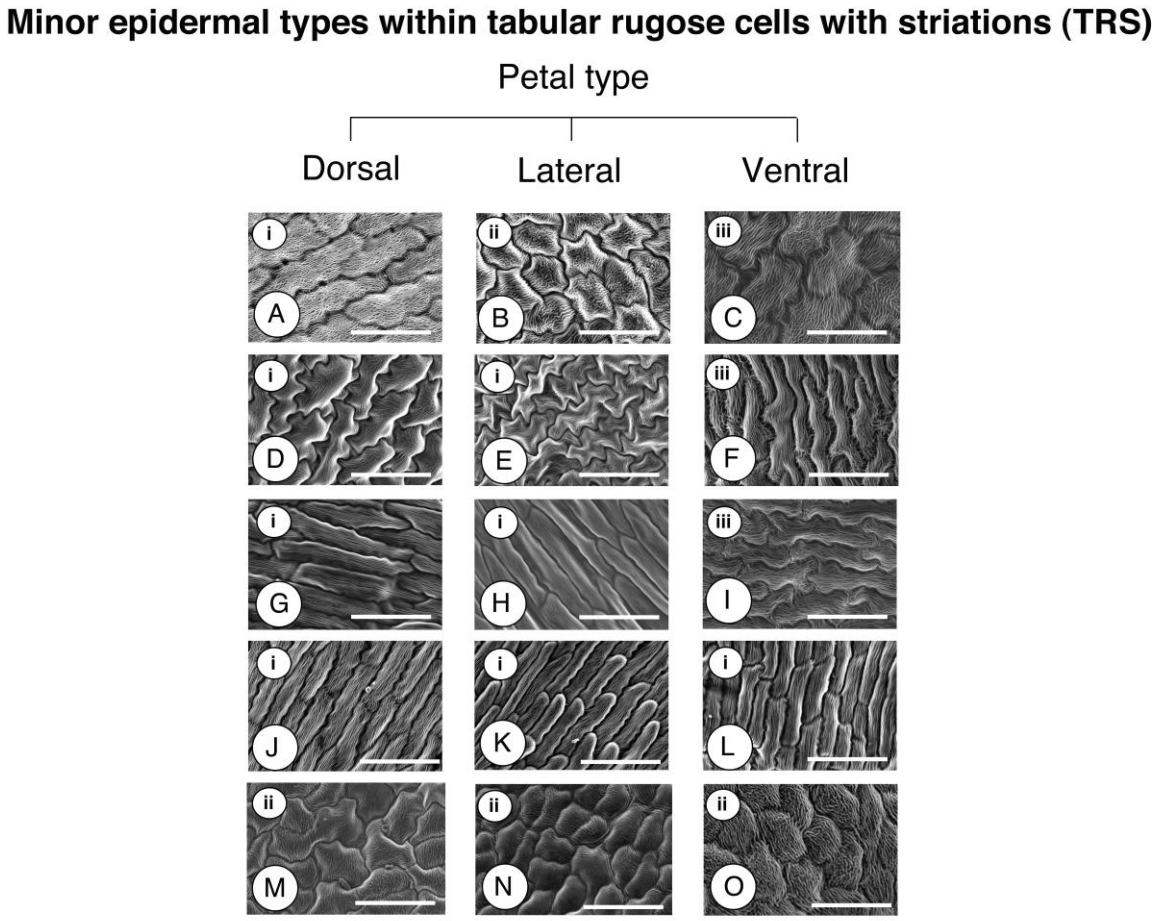


Figure 3.5 Distribution of the epidermal types along the dorsiventral (adaxial-abaxial) axis within the flower. A) micromorphological variation in *Lotus burttii* (as in almost all Loteae) (Papilionoideae), B) lack of micromorphological variation of major epidermal types in *Cassia roxburghii* (Caesalpinioideae) with only PKR, C) *Cassia emarginata* (Caesalpinioideae) with only TRS, and D) *Senna alata* (Caesalpinioideae) with only PCS on all petals. All petals have the adaxial side shown, except *L. burttii,* where the abaxial side is presented on lateral and ventral petals and *Senna alata* where images shown the abaxial side. Scale bars A) 20 µm, B) 50 µm, C) 100 µm, D) 50 µm.

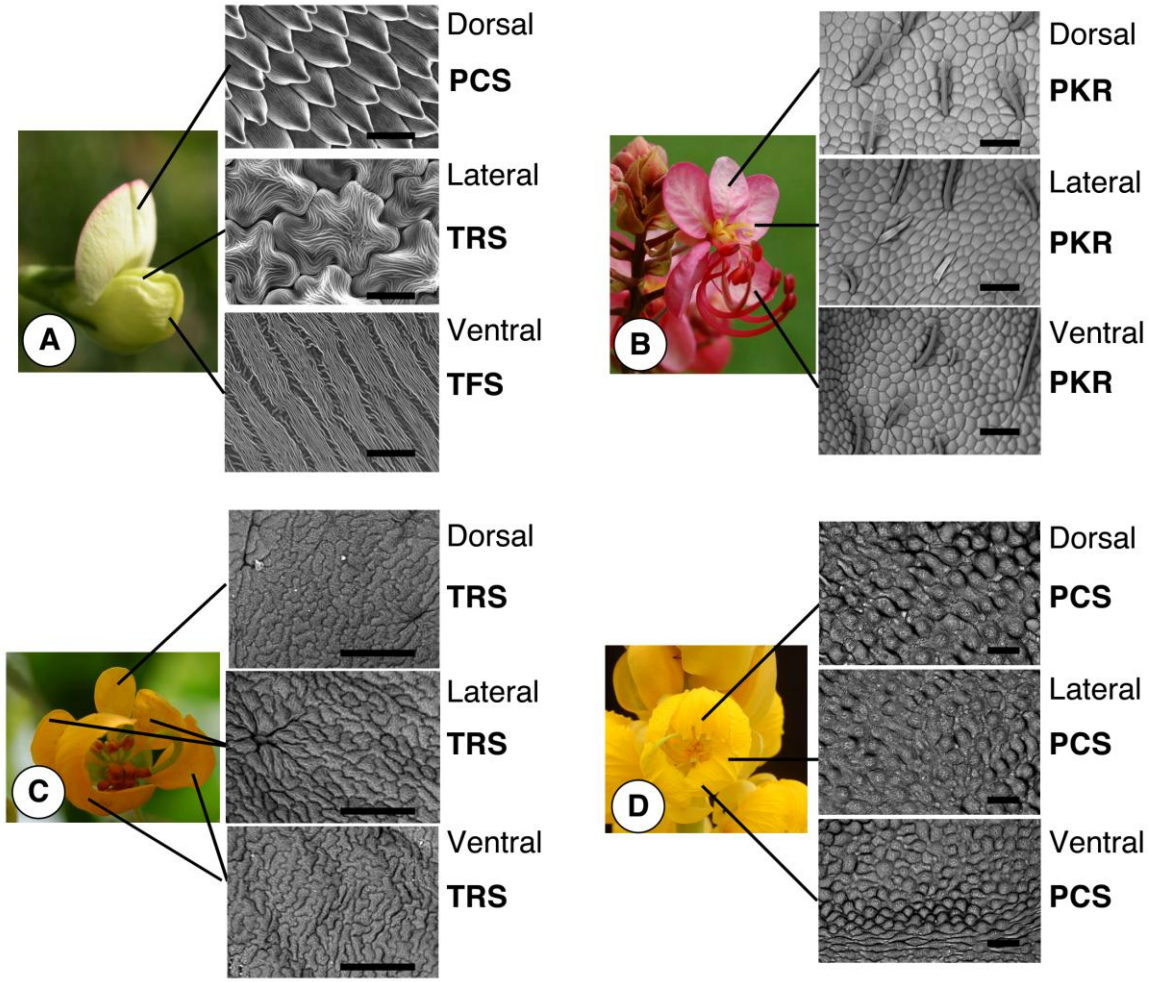


Figure 3.6 Schematic representation of the phylogenetic relationships within Leguminosae showing the typical distribution of the major epidermal types observed. This figure is intended to summarise the main patterns but it should be noted that rare variant patterns may occur in clades as well as those listed. The general epidermal surface observed within the flower is given in relation to the dorsiventral axis within the flower, using the representation: dorsal/lateral/ventral petal. Clades with an asterisk contain lineages with loss of papillose cells (PCS and PKR). The numbers following the asterisk indicate the number of losses under parsimony and ML, respectively. -- lack of this type of petal (Tree according to Wojciechowski et al., 2004; Lavin et al., 2005; Lewis et al., 2005). IRLC=inverted repeat-lacking clade.

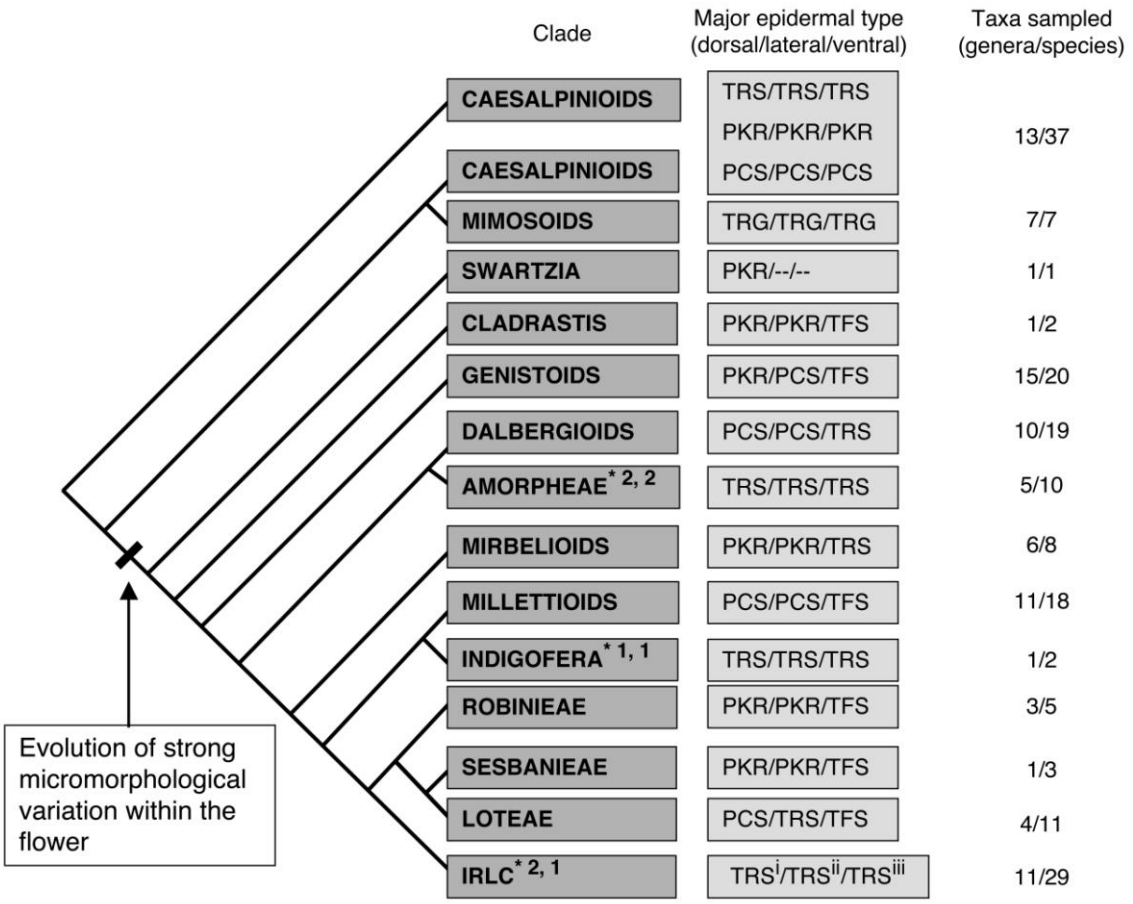
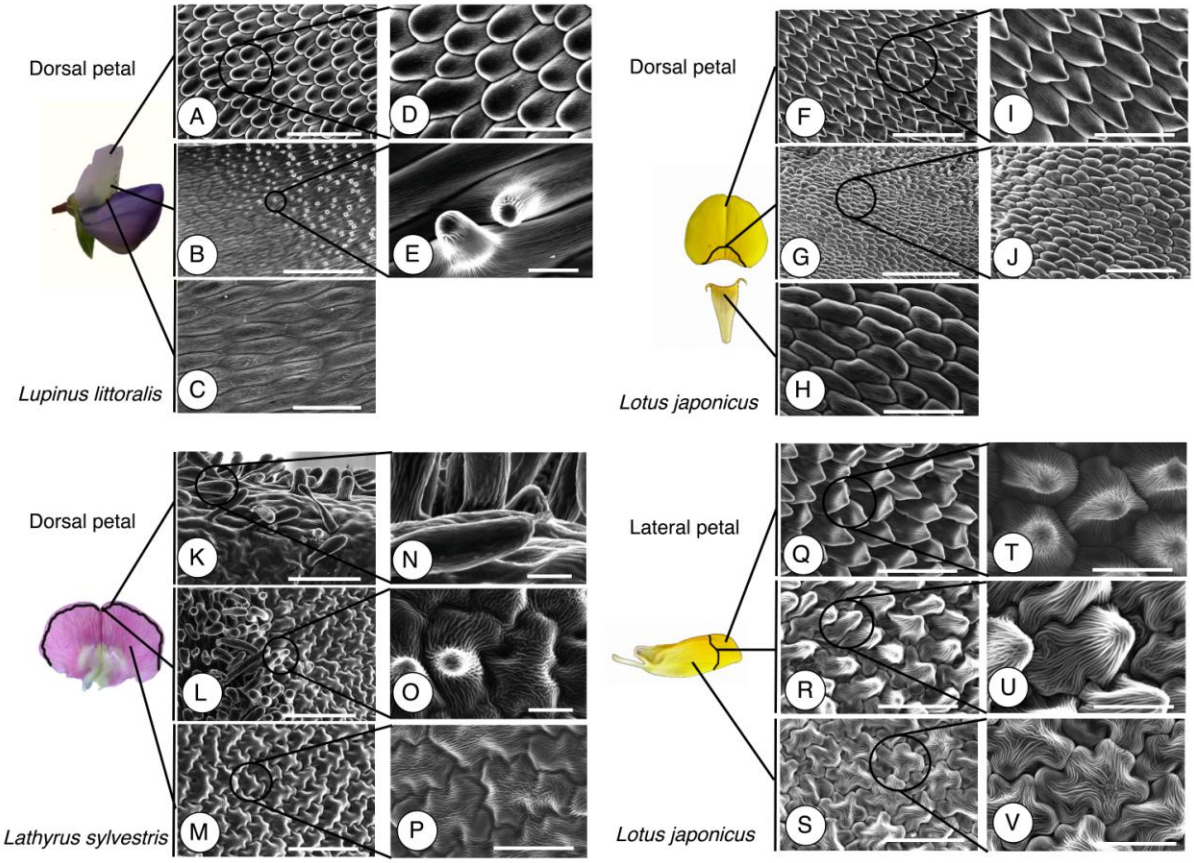


Figure 3.7 Distribution of epidermal types along a proximo-distal axis within the same petal. (A-E) *Lupinus littoralis* with transitions from poorly differentiated cells at the base of the adaxial side in the dorsal petal (C), to papillose cells (PCS) on the central part and apex of the petal (A,B). (F-J) the abaxial side of the dorsal petal in *Lotus japonicus* with transitions from poorly differentiated cells at the base of the petal (H) to PCS in the central and distal regions (G, H). A photograph of the standard petal of *Lotus japonicus* is shown besides the images with the claw separated and shown below. (K-P) the adaxial side of the dorsal petal in *Lathyrus sylvestris* has a transition zone from TRS to PLS on the borders of the petal with a transition zone where cells have a mixture of morphological features of both epidermal types. (Q-V) the abaxial side of the lateral petal in *Lotus japonicus* where TRS is mainly observed at the base and in the central part of the petal and there is a transition zone from TRS to PCS where the cells have a mixture of both epidermal types. Scale bars 500 µm in B; 200 µm in G; 100 µm in A, C, F, J-M; 50 µm in D, H, I, O, P, Q-S; 20 µm in E, N, T-V.


Dorsal petal
A
D
B
E
C
Lupinus littoralis
Dorsal petal
F
I
G
J
H
Lotus japonicus
Dorsal petal
K
N
L
O
M
P
Lathyrus sylvestris
Lateral petal
Q
T
R
U
S
V
Lotus japonicus

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4 The origin of bird pollination in Macaronesian *Lotus* (Loteae, Leguminosae)[1]

4.1 Introduction

Macaronesia consists of five volcanic archipelagos (Azores, Madeira, the Salvage Islands, the Canary Islands and Cape Verde) as well as a region of African mainland (southern Morocco and the former Spanish West Africa) known as the "Macaronesian enclave" (Fig. 4.1). The Macaronesian flora has a high degree of endemism, 20% overall (Humphries, 1979) and 40% in the Canary Islands alone (Santos-Guerra, 1999). It also has a diverse range of altitudinal zones (0-3200m), island ages (ranging from 0.8 to 21 Ma) and distances from main continental areas of Europe or Africa, ranging from 95 to 1600 km (Carracedo, 1994; Carracedo et al., 2002; Humphries, 1979).

Bird pollination has evolved in this region in the lineages of at least eleven endemic plant species from six genera, *Canarina* and *Musschia* (Campanulaceae), *Isoplexis* (Scrophulariaceae), *Echium* (Boraginaceae), *Lotus* (Fabaceae) and *Lavatera* (Malvaceae). These species possess several features associated with opportunistic nectar-feeding birds, including red-orange flowers, abundant dilute nectar, diurnal anthesis, extended flower life span, and loss of scent and landing platform (Dupont et al., 2004; Olesen, 1985, 1988; Olesen and Valido, 2003a; Ollerton et al., 2009; Rodriguez and Valido, 2008; Valido et al., 2004).

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Recent studies have suggested at least five passerine birds, the Canarian Chiffchaff (*Phylloscopus canariensis*), the Blue tit (*Parus caeruleus*), the Sardinian warbler (*Sylvia melanocephala*), canary (*Serineus canarius*) and chaffinch (*Fringilla coelebs*), as the main pollinators of these species (Ollerton et al., 2009; Rodriguez and Valido, 2008; Sletzer, 2005; Valido et al., 2002). Despite the relatively large number of lineages with a bird pollination syndrome in this region, to date there are only two studies that demonstrate the role of these opportunistic nectar feeders as effective pollinators: in *Isoplexis* and *Canarina* (Ollerton et al., 2009; Rodriguez and Valido, 2008). In the remaining groups only foraging observations have been reported.

Several hypotheses have been suggested to explain the origin and maintenance of this pollination syndrome in Macaronesia: 1) the "de novo specialist" hypothesis states that presumably extinct specialist nectarivorous birds on the islands exerted selective pressures on the flowers, followed by their further maintenance by opportunistic birds after the extinction of the specialist birds (Olesen, 1985; Vogel et al., 1984); 2) the "relict" hypothesis suggests that the selection and evolution took place in mainland areas before the plant taxa colonized the islands, once in the islands the specialist bird was replaced by non-specialist nectarivorous passerines (Valido et al., 2004); 3) the "de novo opportunistic" hypothesis suggests that current opportunistic birds acted as selective agents on the floral traits on the islands (Dupont et al., 2004; Valido et al., 2004).

To date, evidence from *Lavatera* and *Canarina* (Fuertes-Aguilar et al., 2002), and *Isoplexis* (Bräuchler et al., 2004; Rodriguez and Valido, 2008) suggests that these floral features are plesiomorphic on the island and may be relictual. It is therefore likely that the evolution of these flower features occurred on the mainland before the colonization of Macaronesia. However, in the remaining cases (*Lotus, Musschia* and *Echium*) this is still unresolved (Valido et

al., 2004) and it is unknown when these flower features evolved within these groups. Bird-pollinated *Echium* (Bohle et al., 1996; Dupont and Skov, 2004; Valido et al., 2002) and *Lotus* (Allan et al., 2004) have derived positions within entomophilous clades and this may indicate a recent origin in the Canary Islands. However, in *Lotus* it has not yet been possible to determine unequivocally the phylogenetic origin of bird pollination or the most closely related entomophilous species, due to incomplete sampling and the low resolution of the internal nuclear ribosomal transcribed spacer region (ITS) used in previous studies (Allan et al., 2004; Degtjareva et al., 2006).

Bird pollination occurs in four species within Macaronesian *Lotus* (Olesen, 1985; Ollerton et al., 2009; Valido et al., 2004). These four species are placed in a group commonly referred to as section *Rhyncholotus* (Monod) D.D. Sokoloff (Degtjareva et al., 2006; Olesen, 1985; Sandral et al., 2006; Valido et al., 2004). They have several floral traits associated with the bird pollination syndrome. These traits include: large flowers, large quantities of dilute nectar (mainly composed of hexose sugars), red-orange flower color, long lived flowers, upward orientation of the flower, and change in the relative size and shape of petals (Dupont et al., 2004; Olesen, 1985; Ollerton et al., 2009; Valido et al., 2004). The syndrome is associated with pollination by opportunistic nectar-feeders. Two birds, the Canarian chiffchaff and the blue tit, have been reported foraging in cultivated individuals of at least two of these plant species (Ollerton et al., 2009; Sletzer, 2005). These four bird-pollinated species seem to have a derived position within the entomophilous species-rich section *Pedrosia* (Lowe) Christ (Allan et al., 2004) .

The species of sections *Pedrosia* and *Rhyncholotus* represent an example of an island radiation within Macaronesia. The group comprises about 40 species of mainly perennial herbs with a high diversity of vegetative and flower features. The main diagnostic feature common to

both sections (*Pedrosia* s.l.) is the presence of a tooth (also mentioned as forked style) on the ventral side of the style (Kramina and Sokoloff, 1999). This flower feature has been used to distinguish *Pedrosia* s.l. from other sections within *Lotus*. Members of *Pedrosia* s.l. occur in a wide range of habitats ranging from rocky coast to mountain pine forest at an elevation above 1200 m. They are represented in all five archipelagos as well as in mainland Africa and Europe (Sandral et al., 2006) (Fig. 4.1 and 4.2). *Pedrosia* s.l. is also characterized by a high degree of endemism; some of its species are restricted to specific habitats within one island. At least seven species within the group are considered critically endangered (CE), one species endangered (E) and one vulnerable (V) (Bañares et al., 2004).

Taxonomically, both groups have been variously united as *Pedrosia* s. l. (*Rhyncholotus* + *Pedrosia*), or considered as separate sections (Brand, 1898), subgenera (Bentham, 1865; Monod, 1980) or genera (Christ, 1888; Kunkel, 1974). The only characteristics separating *Rhyncholotus* from *Pedrosia* are those associated with floral adaptations to bird pollination (Perez de Paz, 1990; Sandral et al., 2006).

Currently, *Pedrosia* and *Rhyncholotus* are recognized as a two separate sections within *Lotus* (Degtjareva et al., 2006), the former with about 36 species and the latter with four species. Previous analyses have not completely clarified their relationships and the current phylogenetic evidence suggests that *Pedrosia* is paraphyletic, with *Rhyncholotus* embedded in one of the clades within *Pedrosia* (Allan et al., 2004; Degtjareva et al., 2008; Degtjareva et al., 2006). The addition of morphology to the nrITS phylogeny in the most recent analysis of the group recovered *Rhyncholotus* as monophyletic (Degtjareva et al., 2006) within a paraphyletic *Pedrosia*, but the relationships within *Pedrosia* s.l. are generally still poorly resolved. Given that the four *Rhyncholotus* species are embedded within *Pedrosia*, I will refer to the four bird-pollinated species as the "rhyncholotus group".

This lack of resolution may be partly due to an incomplete sampling of the group, and partly because this group is a recent island radiation with low levels of variation at the nrITS locus. This lack of resolution in the current phylogenetic analyses has hindered taxonomic decisions (Sandral et al., 2006) and more accurate interpretations of island colonization (Allan *et al.*, 2004). This limitation in resolution has also prevented further studies of the evolution of bird pollination and traits associated with this pollination syndrome transition (Allan et al., 2004; Dupont et al., 2004; Sandral et al., 2006).

4.1.1 Objectives of the study

My aims in this chapter are (i) to clarify the phylogenetic relationships within *Pedrosia* s.l. with special regard to determining the sister-group relationship of the bird-pollinated species, (ii) to test the monophyly of the bird-pollinated group, and (iii) to assess the timing and ecological context of the origin of bird pollination in this group.

4.2 Materials and methods

4.2.1 Taxon sampling

Here, I analyze a nested series of three data sets with different sample numbers and gene regions.

(1) ITS analysis. First I made a preliminary analysis with a very comprehensive sampling using the nuclear ribosomal ITS region alone. This represented, with at least one sample, almost every taxon or population that has been recognized at the species level with this group. I also included samples from populations that may represent new species based on morphological and/or genetic evidence (Oliva-Tejera et al., 2005; Oliva-Tejera et al., 2006). This preliminary sampling included 125 samples in total, 118 from the ingroup, representing 37 described species,

two subspecies, one variety and four undescribed new species. Of these samples, 92 are new sequences generated in this analysis. In this analysis I also included 26 sequences from previous phylogenetic analyses deposited in GenBank (Allan et al., 2004; Allan et al., 2003; Degtjareva et al., 2008; Degtjareva et al., 2006) (Table 4.2). I was able to include at least two samples each for 31 of the 41 species considered (75%) with the purpose of having a good representation of the geographical distribution of some species and also being able to explore the potential intraspecific sequence variation of this gene region, a feature that has been suggested previously in *L. creticus* (Allan et al., 2004; Degtjareva et al., 2006; Sandral et al., 2006). This analysis allowed all species that are broadly related to section Rhyncholotus to be detected and used in subsequent analyses.

(2) Four-gene analysis. As a result of the previous analysis a subset of samples was selected for more detailed analysis with more gene regions. I included 54 samples (all newly collected for this analysis) representing 39 species, two subspecies, three varieties and one undescribed species within *Pedrosia* s.l. (Table 4.1). This data set was analyzed with four gene regions, one nuclear (ITS) and three plastid gene regions (*matK*, *trnH-psbA* and *CYB6*). This data set was analyzed both separately (nuclear or plastid only) and in combination.

(3) Six-gene analysis. Finally, I analyzed a data set of 21 samples (19 from the ingroup) with six gene regions, four nuclear (ITS, *LjCYC1, 2* and *3*) and two plastid (*trnH-psbA* and *matK*). *CYB6* was not use in this analysis due to its lack of variability. *LjCYC1, 2* and *-3* are developmental genes (transcription factors of the TCP family) for which further details are given in Chapter 6. This data set was used to determine the closest relatives of the bird-pollinated species. It comprises 15 species and included several accessions of the closest relatives that I identified in previous analyses (Table 4.1).

Only three species, *L. chazalei*, *L. tibesticus* and *L. loweanus* were not available for the analyses described above and were not included. *Lotus chazalei* is distributed in the coastal region of Mauritania, extreme SW Sahara and extreme SW Morocco, *L. tibesticus* is an endemic from Chad and *L. loweanus* is an endemic to the Island of Porto Santo, Madeira (Fig. 4.1). I do not believe that these are critical species to determine the closest relative of the bird-pollinated species (rhyncholotus group). There is strong morphological evidence that they are closely related to other species in the analysis, *L. tibesticus* to *L. jolyi*, *L. loweanus* to *L. argyrodes* group and *L. chazalei* to *L. assakensis* group, but not to rhyncholotus (Sandral et al., 2006).

Outgroup selection

I selected four species from *Lotus* section *Lotus* as an outgroup. This group contains the model legume *Lotus japonicus* and three related species. This group was chosen as some of the gene regions used in this analysis were first isolated and studied in detail in *L. japonicus*. Additionally, I also included two species from a different genus in the Loteae, *Hosackia*, which occur only in North America and are much less closely related to the Macaronesian *Lotus*.

4.2.2 DNA Extraction and sequence data analysis

Genomic DNA was extracted from either fresh leaves, silica gel dried leaf material or voucher specimens (Table 4.1 and 4.2) following a modification of the cetyl-trimethyl-ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). In total I sequenced and analyzed four nuclear and three plastid regions.

4.2.3 Nuclear regions

The ITS region was amplified (30 cycles of 96 °C for 1 min, 49°C for 1 min, 72°C for 1 min) using primer 4 and 5 (White et al., 1990). I included three homologues of the *CYCLOIDEA* gene from *Lotus japonicus*. *Lotus japonicus CYCLOIDEA1* (*LjCYC1*) was amplified (95 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C 2 min, and 72 °C for 2 min) using a combination of two specific and two general primers. The region that encompasses the TCP domain was amplified using CYC.1.1F and LEGCYCR1 and the region with the R domain using LEGCYCF and CYC1.1R. *LjCYC2* was amplified, either using a combination of two specific primers LC2.1F and LC2.1R (95 °C for 2 min, 30 cycles of 94 °C for 45 s, 50-57 °C for 1 min and 72 °C 2 min, and 72 °C for 2 min) or a combination of LC2.1F and LEGCYCR for the region that includes the TCP domain and LEGCYCF and LC2.1R for the region that includes the R domain (95 °C for 2 min, 30 cycles of 94 °C for 45 s, 55 °C for 1 min and 72 °C 2 min, and 72 °C for 2 min). *LjCYC3* was amplified with two primer combinations, the region including the TCP domain was amplified (95 °C for 2 min, 30 cycles of 94 °C for 30 s, 45 °C for 1 min and 72 °C 2 min, and 72 °C for 7 min) using the primers CYC3.2F and LEGCYCR1 (Citerne et al., 2003). The region that contains the R domain was amplified with LEGCYCF and CYC3.1R (Table 4.3).

4.2.4 Plastid regions

The cytochrome B6 (*CYB6*) was amplified (94 °C for 2 min, 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C 2 min, and 72 °C for 5 min) using the primer CYB6-F and CYB6-R (Choi et al., 2006b). The intergenic region *trnH-psbA* (Kress et al., 2005) and the *matK* gene

were amplified (94 °C for 3 min, 35 cycles of 94 °C for 30 s, 45 °C for 1 min and 72 °C 2 min, and 72 °C for 2 min) using the primers matKX and matK3.2, respectively (Table 4.3). Each locus was amplified, sequenced and the raw sequence data were imported to Sequencher 4.1 for editing and combining of contig sequences. Consensus sequences were imported to Se-Al ver. 1.0 (Rambaut, 1996) and aligned manually using conserved regions in order to identify homologues sequences among species. Gaps in the ITS, *trnH-psbA*, and *LjCYC1*, *2* and *3* were codified as characters (Simmons and Ochoterena, 2000).

4.2.5 Phylogenetic analyses

Phylogenetic analyses were performed using maximum parsimony (MP), maximum likelihood (ML) and the Bayesian method. Parsimony analysis was conducted using PAUP* ver. 4.0b10* (Swofford, 2001) assuming unordered characters and equal character weighting. Heuristic searches were performed with 10000 random stepwise-addition replicates TBR branch-swapping, and MULTREES optimization was used. Consistency index (excluding uninformative characters) and retention index were also calculated (Farris, 1989; Kluge and Farris, 1969). Branch support was analyzed with bootstrap (Felsenstein, 1985) using a simple addition sequence and 10 000 replicates with all parameters similar as used in the MP strategy.

Additionally, I also analyzed the same data sets using maximum likelihood as implemented in Garli Version 1.0 (Zwickl, 2006) and Bayesian analysis using Mr. Bayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best-fit model for each region was determined using the AIC as implemented in jMODELTEST ver. 0.1.1. (Posada, 2008). I used the GTR +G + I; lset nst = 6 rates = invgamma and the HYK models. Bayesian analyses were run using four Monte Carlo chains, a random tree as a starting point, sampling every 1, 000 generations, and continuing for 5,000,000 generations. ML analyses with

Garli were run using the same two models mentioned above with stepwise staring tree topology, two search rep and 100 bootstrap.

4.2.6 Dating the origin of bird pollination

Divergence times within the Macaronesian *Lotus* were estimated using the program Beast v1.5.4 (Drummond and Rambaut, 2007), which estimates branch lengths, topologies, substitution parameter models and dates simultaneously. I ran this analysis using two data sets, a combined matrix of 52 samples and four gene regions (ITS, *matK*, *trnH-psbA* and *CYB6*) with a total of 2092 bp, and a data set of six genes (ITS, *LjCYC1*, *2* and *3*, *matK*, *trnH-psbA* and *CYB6*) and 21 samples. I used a constant-rate Yule (speciation process) prior and all other priors and operators were the default settings. Four independent runs were performed using the uncorrelated lognormal relaxed-clock model (Drummond et al., 2006) for 50,000,000 generations. Trees and parameters were sampled every 5,000 generations yielding a total of 10,000 trees, with a burn in of 5, 000,000. All analyses were run using the HYK+gamma and GTR +G + I; lset nst = 6 rates = invgamma substitution model. The Beast file was created using the BEAUti program v 1.5.4 (part of the program BEAST). The performance of each run was further analyzed with the program Tracer. Mean parameter estimates and 95% highest posterior probabilities (HPDs) were determined by analyzing the Beast tree files with TreeAnnotator v 1.5.4 (Drummond and Rambaut, 2007). Trees were visualized and edited with Figtree v1.3.1. This analysis was constrained with the best hypothesis of relationship (topology) of this group obtained from MP and ML in previous analyses.

Macaronesia lacks reliable fossil records and instead I used well-known geological estimates of maximum island age as my calibration points (Fig. 4.1 and 4.2). For this particular analysis I used three calibration points. This group has several endemic species on different

islands and I used the distribution of two endemics, *L. sessilifolius* subsp. *villossisimus* (El Hierro, 1.12 Ma) and *L. sessilifolius* subsp. *sessilifolius* (La Palma 1.77 Ma) as calibration points (Ancochea et al., 1994; Carracedo, 1994). Both species are endemic to these islands and they were not able to colonize and diversify previous to the emergence of these two islands. The ages of La Palma and El Hierro have been used previously as calibration points in other plants groups (Kim et al., 2008b; Percy et al., 2004). The third calibration point of 21 Ma was based on the age of the oldest island, Fuerteventura, as an upper limit for the colonization of the Canary Islands (Carracedo, 1994) and therefore an upper limit for the age of the most common recent ancestor (MRCA) for the species of this archipelago. This approach of using multiple maximum ages has been used in several analyses within this archipelago, both in animals and plants (Anderson et al., 2009; Cox et al., 2010; García-Maroto et al., 2009; Kim et al., 2008b; O'Leary, 2009), and it has been suggested that is less prone to inaccurate date estimations (Anderson et al., 2009).

4.3 Results

4.3.1 Major clades of *Pedrosia* s.l. in Macaronesia

My analysis using the large data matrix of 125 ITS accessions recovered four major clades within the Macaronesian *Lotus* section *Pedrosia* and the rhyncholotus group (Fig. 4.3, Clades A-D), and no differences were observed when GenBank sequences where excluded or included from this analysis, The same topology was recovered with the data set of 54 accessions. Clade A contains all the Cape Verde species, which form a monophyletic group together with one African lineage of three species. Clade B includes species from the Canary Islands, Madeira and the Azores. The Madeiran and the Azorean species are located in an early divergent position within this clade together with samples of an undescribed species from Tenerife. The four bird-pollinated species (rhyncholotus) are all in clade B, and are unresolved with other five species of

Pedrosia s.s. referred to as the "*Lotus sessilifolius* group". The level of variation within the ITS phylogeny does not indicate unequivocally whether the bird-pollinated species are monophyletic or shed any light on the exact sister group relationship of rhyncholotus (Fig. 4.3). This lack of resolution is despite the fact that the ITS nuclear region is more variable that the plastid regions (Table 4.4).

Clade C also includes species mainly from the Canary Islands, one species from the Salvage Islands, one from Madeira and three African species. Two of these African species form a sister group of the remaining species within this clade and the remaining species are divided into two other subclades. Of these, subclade I includes exclusively Canary Island species with a mountain distribution, whereas subclade II included one species from Madeira and another species restricted to the Salvage Islands. Finally, another early divergent clade consists of an African species (*Lotus jolyi*) (clade D). The inclusion of indels (two in the ITS matrix for section *Pedrosia* s.l.) in the analysis increased the resolution within clade C. No additional improvement in resolution was observed in other clades.

Less resolved topologies were recovered with the separate analyses of the three plastid regions *matK, trnH-psbA* and *CYB6*, and even the combined analysis of the three plastid regions resulted in a less resolved and well-supported phylogeny than the ITS analysis alone (Fig. 4.4). This combined plastid analysis recovered only one group (clade A) observed with the ITS phylogeny and there was an incongruence of the placement of four species (*L. callis-viridis, L. kunkelii, L. arinagensis* and *L. emeroides*) between clades B and C. This incongruence is probably due to "soft" incongruence, given the low levels of sequence variation and the lack of resolution within the three plastid regions. Only one "hard" incongruence was detected in the Cape Verdean clade (clade A), in which contrasting topologies in the two data sets have moderate bootstrap support. However, importantly for this study, the position of the four bird-

pollinated species was consistent in all analyses and the same species, *L. sessilifolius* and *L. mascaensis*, were recovered in both data sets as the closest relatives of the ornithophilous species.

The combined analysis of these four regions (ITS, *trnH-psbA, matK* and *CYB6*) resulted in a more highly resolved phylogeny with the recovery of the same major clades as in the ITS analysis. The position of the four bird-pollinated species is the same, whether in separate analyses (nuclear and plastid), or in combination. The four bird-pollinated species are placed together within clade B, and they are revealed to be closely related to two species of the *L. sessilifolius* group, *L. sessilifolius* and *L. mascaensis*, and one undescribed species from Tenerife (Fig. 4.5). However, there is still a lack of resolution of the relationships among these species and it is still not possible to unequivocally identify the sister group of the bird-pollinated species.

The above results are all based on maximum parsimony analyses. However, similar results were obtained when the same data sets were analyzed with maximum likelihood or Bayesian analyses, and only minor discrepancies were observed within the four major clades obtained from MP analyses. The only major difference was the placement of *L. jolyi*. All MP analyses placed this species as the sister group of all Macaronesian *Lotus*, while Bayesian analysis place it as a sister clade of the Cape Verde clade (clade A) and ML placed it as the sister species of clades B and C. However, in all analyses the support for the placement of this species is low and further studies should be considered to address its position.

4.3.2 Recovery of the sister group of rhyncholotus using a six gene region analysis with the inclusion of *CYCLOIDEA* homologues

The previous combined analysis of four regions identified two species (*L. sessilifolius* and *L. mascaensis*) as the closest relatives of the bird-pollinated species within clade B. A further

analysis of a targeted data set using three additional nuclear regions and two plastid regions was therefore undertaken. One plastid region used in previous analyses (*CYB6*) was abandoned as it had no variation at this level. The addition of three *CYCLOIDEA* homologues increased the resolution within this clade. The three *CYCLOIDEA* homologues gave similar topologies when analyzed separately and in combination using either MP, ML or Bayesian analyses. Among these three paralogues, *LjCYC1* seems to be the most variable copy (Table 4.5). For this sample set the combined analysis of ITS and the two plastid regions (*matK* and *trnH-psbA*) gave less resolution than the three *CYCLOIDEA* copies together, and most of the resolution within clade B in this analysis was obtained from the three *CYCLOIDEA* homologues.

The combined analysis of the six regions increased the resolution and indicates that *L. sessilifolius* is likely the closest relative of the bird-pollinated species. I sampled representatives from all of the geographic distribution (samples from four islands) and intraspecific taxonomic groups (two subspecies and one variety) within this species and it appears that *L. sessilifolius* s.l. is likely a paraphyletic species with respect to the rhyncholotus group. However, with these data sets I was unable to identify unequivocally the population or populations closest to the four bird-pollinated species (Fig. 4.6A-B).

4.3.3 Dating the origin of bird-pollinated *Lotus* in the Canary Islands

Calibration of the Macaronesian *Lotus* indicates that this group likely colonized this region in three events between 6 to 4.3 Ma between late Miocene and early Pliocene. Under this scenario the earliest colonization occurred in the Cape Verdean archipelago, circa 6.1 (0.50-7.29) Ma (Clade A) with the latest in the Canary Island, circa 4.3 Ma (0.58-6.38) (Fig. 4.9).

The four species with the bird pollination syndrome (rhyncholotus group) appears to be of recent origin with the most recent common ancestor (MRCA) for *L. sessilifolius* and the

rhyncholotus group dated at 2.2 Ma and an age of for the bird-pollinated rhyncholotus species (MRCA) of 1.2 Ma (Fig. 4.7).

The six gene chronogram (Fig. 4.10) gives an age (MRCA) for the *L. eremiticus*/*L. pyranthus* group of 0.67 Ma and of *L. berthelotii*/*L. maculatus* of 1.11 Ma, and a date of the whole *L. sessilifolius* group, including rhyncholotus, of 2.09 Ma (Table 4.6). The evolution of bird pollination is therefore associated with a recent radiation of Macaronesian *Lotus* (the *L. sessilifolius* group) that started the last 2 Ma in the islands of Tenerife, La Palma, El Hierro and to a lesser extent in Gran Canaria around the beginning of the Pleistocene.

4.4 Discussion

4.4.1. Closest relative of the four bird-pollinated species

A previous study has suggested either species of the *L. argyrodes* group from Madeira and the Azores (4 spp) (Fig. 4.7A-C) or members of the *L. sessilifolius* group (5 spp) (Fig. 4.7D-I) as the most closely relatives of the bird-pollinated rhyncholotus (Sandral et al., 2006). Most members of these two groups have relatively larger flowers compared to the rest of the *Pedrosia* s.s. and both groups have the ability to modify flower colour after anthesis by producing anthocyanidins similar to those observed in red-orange, bird-pollinated flowers (see Chapter 7). However, previous phylogenetic analyses were based on less comprehensive sampling in both of these two groups (Allan et al., 2004; Degtjareva et al., 2006) and the determination of the closest relative of rhyncholotus remained unclear.

My data set of four gene regions (ITS, *trnH-psbA, matK* and *CYB6*) (Fig.4.4) indicates that three species, *L. mascaensis, L. sessilifolius* and *Lotus* sp. nov. 1 from Tenerife are likely the closest relatives of the rhyncholotus group. The first two species, *L. mascaensis, L. sessilifolius,* share some morphological features with the bird-pollinated species. Both species have flowers in

a somewhat upward orientation, an intermediate inclination between the rest of *Pedrosia* s.s. and the rhyncholotus group. Additionally, the leaflets of both species are filiform or linear (Fig. 4.7D-I), a feature present also in rhyncholotus (Fig. 4.8). In contrast, *Lotus* sp. nov. 1 does not share these features, the leaflets are oblong to lanceolate and the flowers are held in a horizontal position, a feature commonly observed in the rest of *Pedrosia* s.s. This undescribed species shares leaflet morphology with the *L. argyrodes* group (4 spp.) (Fig. 4.7A-C) and *L. emeroides* from La Gomera, in all of which the three distal leaflets are longer than the two basal.

My data set of six gene regions (ITS, *LjCYC1, 2* and *3*, *trnH-psbA*, *matK* and *CYB6*) specifically supports *L. sessilifolius* as the closest relative of the four bird-pollinated rhyncholotus (Fig. 4.6A and B). This species exits as a complex group which is distributed in four islands (La Palma, La Gomera, Tenerife and El Hierro) within the Canary Island archipelago (Sandral et al., 2006) and it is the most widely distributed species in *Lotus* subg. *Pedrosia* (Fig. 4.2). *Lotus sessilifolius* has been divided into three subtaxa (two subspecies and one variety), *L. sessilifolius* subp. *sessilifolius* from Tenerife, La Gomera and La Palma, *L. sessilifolius* subsp. *villosissimus* from El Hierro (Sandral et al., 2006) and *L. sessilifolius* var. *pentaphyllus* from Tenerife (Fig. 4.7G-I) (Bramwell and Bramwell, 2001). According to the six gene data set, *L. sessilifolius* represents a paraphyletic group from which rhyncholotus evolved. Similar topologies were obtained with ML and Bayesian analyses and the position of this species as the closest relative of the rhyncholotus group is well supported in these analyses (Fig. 4.6A-B). The relationship between individual accessions of this species and rhyncholotus is not unequivocally resolved, but in all analyses I recovered *L. sessilifolius* var. *pentaphyllus* as the earliest diverging accession within this group (Fig. 4.6A and B).

My results from the six gene data set raises the possibility that bird pollination may have a double origin (Fig. 4.6A and B), although there is no statistical support for this, and on

morphological grounds a single origin would seem to be more likely because of the many striking similarities between all four bird-pollinated species (Fig. 4.8). Further analyses are required to fully determine whether bird pollination has a single or double origin in this particular group.

4.4.2. How old is the bird pollination syndrome in Macaronesian *Lotus*?

The phylogenetic results reported here, support the scenario that the floral features associated with bird pollination in Macaronesian *Lotus* (rhyncholotus group) evolved relatively recent. All my phylogenetic analyses place the four species of this group in a highly derived position within the section *Pedrosia*, apparently nested within a single species (Fig. 4.6A-B).

All my dating analyses strongly suggest that the clade containing the four rhyncholotus species evolved within the last 2 Ma representing a recent island radiation, postdating the Tertiary-Quaternary boundary (~2.6 Ma). Similar age estimates were obtained when rhyncholotus was constrained to be monophyletic in both data sets (Table 4.4). The four rhyncholotus species seem to have shared a MRCA with *L. sessilifolius* the last 2.08-2.70 Ma. The four rhyncholotus species are therefore a neoendemic lineage (Cronk, 1992; Vargas, 2007) of recent evolution in this archipelago. It is truly remarkable that a clade which has been considered in the past a separate genus should in actuality have evolved so recently (and in some analyses be apparently nested within a single extant species, *L. sessilifolius*). These results, however, should be considered with caution. I calibrated the phylogenetic trees using the ages of the islands as upper bound limits and the current distribution of endemics species. It is possible, although unlikely, that these species could have been more widespread distributed in the past and their current distribution does not reflect previous historic ranges. Therefore, using the current distribution on endemics might underestimate the ages obtained.

Bird pollination in *Echium* likely occurred within the same time window as in *Lotus*. The bird-pollinated *E. wildpretii* seems to have diverged in the last 1 Ma from the melittophilous *E. pinianana* (García-Maroto et al., 2009). In contrast, bird pollination in *Lavatera*, *Canarina* and *Isoplexis* probably evolved earlier, as they represent apparently older lineages (Bräuchler et al., 2004; Fuertes-Aguilar et al., 2002; Rodriguez and Valido, 2008); although no phylogenetic dating studies have been carried out in these groups. The age estimates reported here for *Lotus* together with the *Echium* estimate are the only age estimations for the evolution of bird pollination in Macaronesia.

Outside Macaronesia, much older dates have been inferred for the evolution of bird pollination in various groups studied, such as *Alloxylon*, *Telopea* and *Embothrium* (Proteaceae), where bird pollination apparently originated in the early Eocene (52.8 Ma ± 6.8) (Barker et al., 2007).

Under this scenario of recent evolution, it is likely that these floral features evolved in the Canary Islands *Lotus* under the selective pressure of opportunistic passerine birds ("de novo opportunistic" hypothesis) (Dupont et al., 2004; Valido et al., 2004). There are records of floral visits by two passerine birds, the Canarian chiffchaff and the blue tit (Ollerton et al., 2009; Sletzer, 2005) in this group. However, there has been no rigorous analysis of the effectiveness of these two bird species as pollinators in the four species of rhyncholotus. In fact, these observations have only been made in cultivated populations, and only in one species, *L. berthelotii* and its hybrid with *L. maculatus*. There are no records of bird visits to the other three species of rhyncholotus, either in cultivation or in individuals from wild populations. Therefore, the role of these birds as selective agents for the evolution of these floral traits remains largely uninvestigated, and this is complicated by the rarity of the plants in the wild. Age colonizations of some passerines in Macaronesia (including *Parus*, *Serineus* and *Sylvia*) have been estimated

between 0.008 to 2.3 Ma (using divergence times calculated after rate estimates of 2 % sequence divergence per Ma) (reviewed in Dietzen, 2007). In particular, the polytipic *Parus caeruleus* contains at least 15 subspecies distributed from Macaronesia, Europe and Africa. Four endemic subspecies are distributed in the Canary Island archipelago, *P. caeruleus* subsp. *ombriosus* (El Hierro), *P. caeruleus* subsp. *palmensis* (La Palma), *P. caeruleus* subsp. *teneriffae* (La Gomera, Tenerife, Gran Canaria) and *P. caeruleus* subsp. *degener* (Fuerteventura, Lanzarote), that colonized and radiated within this archipelago within the last 0.3- 1.5 Ma (Dietzen, 2007). This passerine group seems to have radiated more or less at the same time as the rhyncholotus group. At least one of the putatively pollinators of rhyncholotus seems to have recently colonized and radiated in this archipelago, thus supporting the de novo opportunistic explanation for the origin of this pollination syndrome. Another explanation is that specialist bird were once distributed in the Canary Island archipelago, and after their extinction, opportunistic passerine birds might have occupied their niche. However, there is no current fossil evidence to support this.

All four species of rhyncholotus are considered endangered in the wild with much reduced populations (Caceres et al., 2004a, b; Gómez and Coello, 2004; Ojeda and Marrero, 2004), which is partially responsible for the lack of more comprehensive analyses.

What is then the selective advantage of bird pollination in rhyncholotus, and the other plant lineages in Macaronesia? Birds are more reliable pollinators under harsh environmental conditions and /or unpredictable environment, such as high altitudes (Stiles, 1978). Birds also have a higher range of mobility than insects, and therefore are more effective pollinators over long distances in isolated populations. It has been shown in *Penstemon* (Plantaginaceae) (Kramer et al., 2011) and *Streptocarpus* (Gesneriaceae) (Hughes et al., 2007a) that bird-pollinated species possess a greater functional connectivity among distant populations than bee-pollinated species. Bird-pollinated species have less genetic structure that their bee-pollinated counterparts,

evidence of more effective gene flow among isolated distant populations. The four bird-pollinated rhyncholotus have small, isolated populations, and the foraging behaviour of birds would increase population connectivity.

4.4.3. The availability of new niches and the evolution of ornithophily in *Lotus*

The current distribution of the four bird-pollinated species suggests that this syndrome may have evolved as new habitats became available due to recent volcanic activity and the lowering of the relative sea level in the islands of Tenerife, El Hierro and La Palma. *Lotus berthelotii* and *L. maculatus* are distributed in the central parts of Tenerife (Gómez and Coello, 2004; Hind, 2008; Martín and Berriel, 2007; Ojeda and Marrero, 2004), which were formed in the last 3 Ma, when volcanic activity united the older regions of Anaga, Teno and Adeje (Carracedo et al., 2002). No recorded observations exist of populations of these two species in these three older regions in Tenerife. The other two species, *L. pyranthus* and *L. eremiticus* are endemics of La Palma (Caceres et al., 2004a; Coello, 2007; Gonzáles et al., 2004; Medina, 2008), an island that emerged in the last 1.7 Ma (Carracedo, 1994).

However, it must be borne in mind that their current distributions may not reflect their original distribution, either in the geological past, or before European settlement and the increase of agricultural activity (and, more recently, development for tourism). All four species were described relatively recently. *Lotus berthelotii* was the first species described of the group in 1881 by Masferre-Arquimbau (Masferrer-Arquimbau, 1881) and the last species, *L. pyranthus* was described two decades ago (Perez de Paz, 1990). Therefore, we have virtually no information on the distribution of these plants prior to the twentieth century.

Compared to radiations driven by key morphological innovations, such as spur length in columbines (Hodges, 1997), the availability of novel habitats may have been a more important

diversification factor for this group, and consequently for the evolution of bird pollination in rhyncholotus. The emergence of new volcanic terrain and new islands may have had effects on bird behaviour that facilitated the evolution of bird pollination. Birds on islands tend to have a reduced interspecific competition compared to those in continental habitats (Crowell, 1962; MacArthur et al., 1972). As a result birds on islands tend to increase their dietary range, and this may have led to the inclusion of nectar as an additional food source (Valido et al., 2004). This phenomenon of niche widening due to relaxed interspecific competition in islands has been also reported in lizards (Olesen and Valido, 2003b). Interestingly, a lizard (*Gallotia galloti* Oudart, Lacertidae) has also been reported foraging in flowers of *L. maculatus* and *L. berthelotii* (Ollerton et al., 2009); however, their role as an effective pollinator has not been fully assessed.

Table 4.1 Samples included in the phylogenetic analysis of the two data sets with 21 samples only (*) and with the 54 samples within *Pedrosia* s.l. JAO= Jardín de Aclimatación de la Orotava, JBCVC= Jardín Botánico Canario "Viera y Clavijo", UBC= University of British Columbia. T= Tenerife, GC= Gran Canaria, G= La Gomera, P=La Palma, H=El Hierro, CV=Cape Verde, M=Madeira

Taxon	**Collection information**	**Voucher, herbarium or GenBank**
Outgroup		
Hosackia chihuahuana S. Watson	Cultivated UBC # PI 262405 Mexico	*Ojeda 79*/UBC
Hosackia gracilis Benth.	Cultivated UBC	-
Lotus japonicus MG20 (Regel) K. Larsen	Cultivated UBC	*Ojeda 69*/UBC
**Lotus japonicus* Gifu B129 (Regel) K. Larsen	Cultivated UBC	*Ojeda 70*/UBC
Lotus filicaulis Durieu	Cultivated UBC	*Ojeda 71*/UBC
Lotus corniculatus L.	Vancouver, BC	*Ojeda 46*/UBC
Lotus burttii Borsos	Cultivated UBC seeds from Univ. Miyazaki	*Ojeda 72*/UBC
Ingroup		
***Lotus* section *Pedrosia* (Lowe) Christ**.		
Lotus arborescens Lowe ex Cout.	Cultivated JBCVC # 164/06, Morro Cove Roche Sao Nicolao CV	F. Oliva / A. Marrero / JBCVC / *Ojeda 180*/ UBC
**Lotus arenarius* Brot.	Cultivated UBC # PI 631956, Kourigba, Morocco	*Ojeda 78*/UBC
**Lotus arinagensis* Bramwell.	DNA bank # 651 cultivated JBCVC # 138/01	J. Cruz / A. Roca / JBCVC
**Lotus argyrodes* R.P Murray	Cultivated JBCVC # 5485B/UPM/07 (Banco Germoplasma UPM, Dr. Gómez Campo) Ponta Pargo, M	F. Oliva / A. Marrero / JBCVC /*Ojeda 189*/UBC
Lotus assakensis Brand	Voucher, Tarfaya-Tan Tan Sahara, Africa	Molero 1992 (Fernandez Casas 13699)
**Lotus azoricus* P. W. Ball	Cultivated JAO # 161-00	ORT # 36336
Lotus bollei Christ [=*L. purpureus* sensu Sandral et al]	Cultivated JBCVC # 163/06, Baia das Gatas on Monte Verde, Sao Vicente, CV	F. Oliva / A. Marrero / JBCVC /*Ojeda 182*/UBC
Lotus brunneri Webb in Hooker	Cultivated JBCVC # 514B/07, Mocete Negro (between St[a] María and Pedro Lume) Sal, CV	F. Oliva / A. Marrero / JBCVC /*Ojeda 181*/UBC
Lotus callis-viridis Bramwell & D.H. Davis	DNA Bank # 654, cultivated JBCVC**,** # 369/04	A. Roca / B. Navarro / A. Marrero / JBCVC
Lotus campylocladus Webb & Berthel.	Arona-Ifonche, T	*Ojeda 210*
Lotus creticus L.	Cultivated JBCVC # 64/05 (# 339/97) Cabo Pino.	B. Navarro /JBCVC / *Ojeda 188*/UBC
Lotus dumetorum Webb ex R. P. Murray	Mirador Jardina, Mercedes, Anaga, T Teno Alto, Teno, T	*Ojeda 213*/UBC *Ojeda 228*/UBC
Lotus emeroides R. P. Murray	Inchora, G	*Ojeda 207*/UBC
Lotus eriosolen (Maire) Mader & Podlech	Cultivated UBC # PI 631959, Ouarzate, Morocco	*Ojeda 243*/UBC
L. erythrorhyzus Bolle	Fuerteventura	A. Santos
L. genistoides Webb. *(nom. nudum)*	DNA Bank # 655, cultivated JBCVC # 330/02	F. Oliva / J. Navarro / J. Naranjo / B.

Taxon	Collection information	Voucher, herbarium or GenBank
Lotus glaucus Sol.	Cultivated JBCVC # 235B/07, Porto Moniz, M	F. Oliva / A. Marrero / JBCVC *Ojeda 187*/UBC
Lotus hillebrandii Christ	DNA Bank # 656, cultivated JBCVC # 42/B, Llano las Chozas, P	P. Maya / V. Montelongo / J. Naranjo / R. Febles / JBCVC
Lotus holosericeus Webb & Berthel.	DNA Bank # 657, cultivated JBCVC # 334/02, Pilancones, GC	F. Oliva, / J. Naranjo / J. Navarro / I. Santana / B. Vilches / JBCVC
Lotus jacobaeus L.	DNA Bank # 658, cultivated JBCVC # 46/03, Bordeira bei Piorno Fogo CV, 2100 m.	T.Leyens
Lotus jolyi Battand.	Voucher, Province Guelmin, Morocco	*S.L. Jury & T.M. Upson 20480*/RNG
**Lotus kunkelii* (Esteve) Bramwell & D. H. Davis	DNA Bank # 3805, Barranco Jinamar, GC	F. Oliva / J. Navarro / J. Caujapé / N. Cabrera /JBCVC
Lotus lancerottensis Webb & Berth.	DNA Bank # 3823 Villaverde, Betancuira, F	F. Oliva / J. Navarro /JBCVC
Lotus latifolius Brand	DNA Bank # 1812, Ctra. Porto Novo, Santo Antao, CV	A. Marrero / R Almeida / J. Caujapé/ JBCVC
Lotus leptophyllus (Lowe) K. Larsen	Barranco Guayedra, T	*Ojeda 170*/UBC
**Lotus macranthus* Lowe	Voucher, M	ORT # 36675
Lotus maroccanus Ball	Voucher, Talouine, Morocco	*S.L. Jury 14471*/RNG
Lotus mascaensis Burchard	DNA Bank # 659, Cultivated JBCVC # 133/M Valle de Masca, Teno, T	M. Aleman /JBCVC *Ojeda 200*/UBC
Lotus pseudocreticus Maire, Weiller & Wilczek	Cultivated JAO 468-00	*Ojeda 231*/UBC
Lotus purpureus Webb	Cultivated JAO # 130-99	ORT # 36670
Lotus salvagensis R.P. Murray	Voucher, SG	ORT # 35118
**Lotus sessilifolius* D.C. subsp. *villosissimus* (Pitard) Sandra & Sokoloff	Las Playas, S from Parador, H	*Ojeda 196*/UBC
**Lotus sessilifolius* D.C. subsp. *sessilifolius*	Poris de Abona, T Punta Llana, G Playa Pocito, Mazo, P	*Ojeda 225*/UBC *Ojeda 208*/UBC -
**Lotus sessilifolius* DC. var. *pentaphyllus* (Link) D. H. Davis	San Juan-Guia de Isora, T	*Ojeda 205*/UBC
Lotus spartioides Webb & Berthel.	DNA Bank # 662, cultivated JBCVC # 337/02, Chira-Pinar Santiago, GC	F. Oliva / J. Navarro / J. Naranjo / B. Navarro / I. Santana / B.Vilches /JBCVC
Lotus tenellus (R. Lowe) Sandral, Santos & D.D. Sokoloff	Arachico, Ermita San Roque, T	*Ojeda 246*/UBC
**Lotus* sp. nov. 1	South Roque dos hermanos, Anaga, T	*Ojeda 193*/UBC
***Lotus* section *Rhyncholotus* (Monod) D.D. Sokoloff**		
**L. berthelotii* Masf. var. *berthelotii*	Ifonche, T Cultivated UBC, commercial plant	- *Ojeda 238*/UBC
**Lotus eremiticus* A. Santos	DNA Bank # 3838, Garafia, P	JCVC 366-04
**L. maculatus* Breitf.	DNA Bank # 660, Puertito Sauzal, T	Jaén 08/04 JCVC
**Lotus pyranthus* P. Perez	DNA Bank # 661 JBCVC 210/99 DNA Bank # 3842	*Ojeda 175*/UBC -

Table 4.2 Species included in the phylogenetic analysis with the nuclear ribosomal ITS region only. GenBank sequences and new sequences generated in this analysis. I excluded six sequences from GenBank (*L. dumetorum* AY294294, *L. campylocladus* AF450196, *L. creticus* AF450192, *L. arinagensis* FJ411112, *L. loweanus* FJ 411117, *L. mascaensis* FJ411118) due to the ambiguities in the sequences and/or suspected misidentification. JAO= Jardín de Aclimatación de la Orotava, JBCVC= Jardín Botánico Canario "Viera y Clavijo", UBC= University of British Columbia. T= Tenerife, GC= Gran Canaria, G= La Gomera, P=La Palma, H=El Hierro, CV=Cape Verde, M=Madeira

Taxon	**Collection Info.**	**Voucher, herbarium or GenBank**
Outgroup		
Hosackia chihuahuana S. Watson	Cultivated UBC # PI 262405 Mexico	*Ojeda 79*/UBC
Hosackia gracilis Benth.	Cultivated UBC	-
Lotus japonicus MG20 (Regel) K. Larsen	Cultivated UBC	*Ojeda 69*/UBC
Lotus japonicus Gifu B-129 (Regel) K. Larsen	Cultivated UBC	*Ojeda 70*/UBC
Lotus filicaulis Durieu	Cultivated UBC	*Ojeda 71*/UBC
Lotus corniculatus L.	Vancouver, BC	*Ojeda 46*/UBC
Lotus burttii Borsos	Cultivated UBC seeds from Univ. Miyazaki	*Ojeda 72*/UBC
Ingroup		
***Lotus* section *Pedrosia* (Lowe) Christ.**		
Lotus arborescens Lowe ex Cout.	Cultivated JBCVC # 164/06, Morro Cove Roche Sao Nicolao CV	F. Oliva / A. Marrero / JBCVC / *Ojeda 180*/ UBC
Lotus arenarius Brot.	Cultivated UBC # PI 631956, Kourigba, Morocco Cultivated UBC # PI 631780, Tiznir, Morocco GenBank GenBank	- *Ojeda 78*/UBC AF450193 (Allan et al., 2003) AF218528 (Allan et al, 2004)
Lotus arinagensis Brawm.	DNA bank # 651 cultivated JBCVC # 138/01 Barranco Viejo, Arinaga, GC DNA bank # 652, Cultivated JBCVC # 55/02 Carretera faro Arinaga, GC Playa Arinaga, GC	J. Cruz / A. Roca / JBCVC J. Naranjo / J. Navarro / F. Oliva / JBCVC *Ojeda 202*/UBC
Lotus argyrodes R.P Murray	Cultivated JBCVC # 5485B/UPM/07 (Banco Germoplasma UPM, Dr. Gómez Campo) Ponta Pargo, M Voucher, Punta San Lorenzo, M	F. Oliva / A. Marrero / JBCVC /*Ojeda 189*/UBC ORT # 37806
Lotus assakensis Brand	Voucher, Tarfaya-Tan Tan Sahara, Africa GenBank GenBank DNA Bank # 1084 Tiznir, Aglou Plage, Morocco DNA Bank # 1085 North Agadir, Tamri, Morocco	Molero 1992 (Fernandez Casas 13699) DQ160277 (Degtjareva et al., 2006) AF450204 (Allan et al., 2003) A. Marrero JBCVC A. Marrero JBCVC
Lotus azoricus P. W. Ball	Genbank , Cult. JAO # 161-00, Azores	AY294293 (Allan et al., 2004), ORT # 36336
Lotus bollei Christ [=*L. purpureus* sensu Sandral et al]	Cultivated JBCVC # 163/06, Baia das Gatas on Monte Verde, Sao Vicente, CV	F. Oliva / A. Marrero / JBCVC /*Ojeda 182*/UBC
Lotus brunneri Webb in Hooker	Cultivated JBCVC # 514B/07, Mocete Negro (between Stª María and Pedro Lume) Sal, CV	F. Oliva / A. Marrero / JBCVC /*Ojeda 181*/UBC
Lotus callis-viridis Bramwell & D.H. Davis	DNA Bank # 654, cultivated JBCVC**,** # 369/04 Andén Verde, GC	A. Roca / B. Navarro / A. Marrero / JBCVC *Ojeda 169*/UBC
Lotus campylocladus Webb & Berthel.	Road to Cañada Teide, T Arona-Ifonche, T	*Ojeda 206*/UBC *Ojeda 210*/UBC
Lotus creticus L.	Cultivated JBCVC # 64/05 (# 339/97) Cabo Pino. Cult # PI 308978, Israel Cult # PI 505409, Spain GenBank	B. Navarro /JBCVC / *Ojeda 188*/UBC *Ojeda 241*/UBC *Ojeda 242*/UBC DQ160279 (Degtjareva et al., 2006)
Lotus dumetorum Webb ex R. P. Murray	Mirador Jardina, Mercedes, Anaga, T Teno Alto, Teno, T	*Ojeda 213*/UBC *Ojeda 228*/UBC
Lotus emeroides R. P. Murray	Inchora, G Epina, G Genbank	*Ojeda 207*/UBC *Ojeda 209*/UBC AY294295 (Allan et al., 2004)

Taxon	**Collection Info.**	**Voucher, herbarium or GenBank**
Lotus eriosolen (Maire) Mader & Podlech	Cult. UBC # PI 631959, Ouarzate, Morocco Cult. UBC # PI 631784, Tiznir, Morocco GenBank (labeled as *L. maroccanus*) GenBank	*Ojeda 244*/UBC *Ojeda 243*/UBC AF450181 (Allan et al., 2003) DQ160281 (Degtjareva et al., 2006)
L. erythrorhyzus Bolle	Fuerteventura GenBank	A. Santos AY294296 (Allan et al., 2004)
L. genistoides Webb. *(nom. nudum)*	DNA Bank # 655, cultivated JBCVC # 330/02	F. Oliva / J. Navarro / J. Naranjo / B.
Lotus glaucus Sol.	Cultivated JBCVC # 235B/07, Porto Moniz, M Cult. JAO # 19-05	F. Oliva / A. Marrero / JBCVC *Ojeda 187*/UBC *Ojeda 233*/UBC
Lotus hillebrandii Christ	DNA Bank # 656, cultivated JBCVC # 42/B, Llano las Chozas, P Mirador Isora, H Todoque-Playa Naos, P Genbank	P. Maya / V. Montelongo / J. Naranjo / R. Febles / JBCVC *Ojeda 198*/UBC *Ojeda 232*/UBC AY294298 (Allan et al., 2004)
Lotus holosericeus Webb & Berthel.	DNA Bank # 657, cultivated JBCVC # 334/02, Pilancones, GC	F. Oliva, / J. Naranjo / J. Navarro / I. Santana / B. Vilches / JBCVC
Lotus jacobaeus L.	DNA Bank # 658, cultivated JBCVC # 46/03, Bordeira bei Piorno Fogo CV, 2100 m. DNA Bank # 2089 Ribeira Monte espia Fogo CV DNA Bank # 2181 Fogo, CV DNA Bank # 2126 Ribeira-Campana Fogo, CV Genbak	T.Leyens A. Marrero / R. Almeida / J. Caujapé Marrero et al JCVC Marrero et al JCVC AY294299 (Allan et al., 2004)
Lotus jolyi Battand.	Voucher, Province Tan Tan, Morocco Voucher, Province Guelmin, Morocco GenBank	*S.L. Jury & T.M. Upson 20503*/RNG *S.L. Jury & T.M. Upson 20480*/RNG DQ166240 (Degtjareva et al., 2006)
Lotus kunkelii (Esteve) Bramwell & D. H. Davis	DNA Bank # 3804, Barranco Jinamar, GC DNA Bank # 3805, Barranco Jinamar, GC Cultivated JBCVC # 217/07 (# 497/99)	- F. Oliva / J. Navarro / J. Caujapé / N. Cabrera /JBCVC M. Aleman / JBCVC/ *Ojeda 176*/UBC
Lotus lancerottensis Webb & Berth.	DNA Bank # 3823, Villaverde, La Matilla, DNA Bank # 3825 Corralejo, F Voucher, L GenBank	F. Oliva / J. Navarro /JBCVC - ORT # 36458 AY294300 (Allan et al., 2004)
Lotus latifolius Brand	DNA Bank # 1812, Ctra. Porto Novo, Santo Antao, CV	A. Marrero / R Almeida / J. Caujapé/ JBCVC
Lotus leptophyllus (Lowe) K. Larsen	Genbank Barranco Guayedra, T	AY294301 (Allan et al., 2004) *Ojeda 170*/UBC
Lotus macranthus Lowe	Voucher, Pico Branco, Porto Santo, M Voucher, M	ORT # 33596 ORT # 36675
Lotus maroccanus Ball	Voucher, Talouine, Morocco Voucher, Marrakech, Morocco	*S.L. Jury 14471*/RNG *Fernandez Casas 13737*/RNG
Lotus mascaensis Burchard	DNA Bank # 659, Cultivated JBCVC # 133/M DNA bank # 3844, Masca, T Valle de Masca, Teno, T Punta Teno, Teno, T GenBank	M. Aleman /JBCVC - *Ojeda 200*/UBC *Ojeda 230*/UBC AY294302 (Allan et al., 2004)
Lotus pseudocreticus Maire, Weiller & Wilczek	Cult. JAO 468-00 Genoank	*Ojeda 231*/UBC DQ160284 (Degtjareva et al., 2006)
Lotus purpureus Webb	Cult. JAO # 130-99 Genbank	ORT # 36670 AY294303 (Allan et al., 2004)
Lotus salvagensis R.P. Murray	Voucher, Salvage Grande, SG	ORT # 35118

Taxon	Collection Info.	Voucher, herbarium or GenBank
Lotus sessilifolius D.C. subsp. *villosissimus* (Pitard) Sandral & Sokoloff	Las Playas, S from Parador, H	*Ojeda 196*/UBC
Lotus sessilifolius D.C. subsp. *sessilifolius*	Poris de Abona, T Punta Llana, G Playa Pocito, Mazo, P	*Ojeda 225*/UBC *Ojeda 208*/UBC -
Lotus sessilifolius DC. var. *pentaphyllus* (Link) D. H. Davis	San Juan-Guia de Isora, T	*Ojeda 205*/UBC
Lotus spartioides Webb & Berthel.	Tamadaba, GC Pinar Pajonales, GC GenBank DNA Bank # 662, cultivated JBCVC # 337/02, Chira-Pinar Santiago, GC Presa las Niñas, GC Llanos de la Pez, GC	F Oliva/J. Caujapé /R. Jaén /JBCVC *Ojeda 217*/UBC F Oliva/J. Caujapé /R. Jaén /JBCVC *Ojeda 216*/UBC AY294304 (Allan et al., 2004) F. Oliva / J. Navarro / J. Naranjo / B. Navarro / I. Santana / B.Vilches /JBCVC F Oliva/J. Caujapé /R. Jaén /JBCVC, *Ojeda 211*/UBC F Oliva/J. Caujapé /R. Jaén /JBCVC, *Ojeda 191*/UBC
Lotus tenellus (R. Lowe) Sandral, Santos & D.D. Sokoloff	GenBank, T GenBank, T (labeled as *L. glaucus*) Arachico, Ermita San Roque, T	AY294305 (Allan et al., 2004) AY294297 (Allan et al., 2004) *Ojeda 446*/UBC
Lotus sp. nov. 1	Playa de los Roques, Tagana, Anaga, T Punta Hidalgo, Anaga, T South Roque dos hermanos, Anaga, T Teno Alto, Teno, T	*Ojeda 215*/UBC A. Santos *Ojeda 193*/UBC *Ojeda 194*/UBC
Lotus sp. nov. 2	Cortijo de San Ignacio, GC	*Ojeda 203*/UBC
Lotus sp. nov. 3	Punta Góngora, GC	*Ojeda 167*/UBC
***Lotus* section *Rhyncholotus* (Monod) D.D. Sokoloff**		
L. berthelotii Masf.	DNA Bank # 3832, Arico, T Ifonche, T Cultivated UBC, commercial plant GenBank	- - *Ojeda 238*/UBC AY204306 (Allan et al., 2004)
Lotus eremiticus A. Santos	Cultivated JAO 430-95 DNA Bank # 3838, Garafia, P GenBank	- JBCVC 366-04 AY294307 (Allan et al., 2004)
L. maculatus Breitf.	DNA Bank # 660, cultivated JBCVC # 43/99, ex horto Puertito Sauzal, T Cultivated UBC, commercial plant DNA Bank # 3840 Sauzal, T GenBank	R. Almeida / JBCVC *Ojeda 239* - AY294308 (Allan et al., 2004)
Lotus pyranthus P. Perez	DNA Bank # 661, cultivated JBCVC, # 210/99 DNA Bank # 3842, cultivated Vivero Ceplam, Bco. Cultivated JAO 124/01 GenBank	J. Cruz/ JBCVC / *Ojeda 175*/UBC F. Oliva / E. Ojeda *Ojeda 226*/UBC AY294309 (Allan et al., 2004)

Table 4.3 Nuclear and plastid regions used in the three data sets analyzed in this study.

Locus	Primer sequence (5'--3')	Region	Size (bp)	Reference
Nuclear				
ITS	ITS F5 GGAAGGAGAAGTCGTAACAAG ITS R4 TCCTCCGCTTATTGATATGC	Intergenic ribosomal region	660	White et al., 1990
LjCyc1	Cyc1.1F TTCTCCTTCACCATACCC Cyc1.1R TTGGATACATAGGGAAGG	*Cycloidea* homologue	935	This study
LjCyc2	LC2.1F TCCCTTTCAGCTCAAGCCCTTACCC LC2.1R GAAGTCATCTCTTGGCGCCTCACC	*Cycloidea* homologue	932	Cronk, unpubl.
LjCyc3	CYC3.2F ACTCCATTAACCCTTTCCS CYC3.1R CCTGCTTCCTTATTAGGGATTGC	*Cycloidea* homologue	982	This study
LEGCYC	LEGCYCF TCAGGGSYTGAGGGACCG LEGCYCR TCCCTTGCTCTTGCTCTTGC	Region between the TCP and R domains in Legumes		Citerne et al., 2003
Plastid				
matK	matKXf TAATTTACGATCAATTCATTC matk 3.2R CTTCCTCTGTAAAGAATTC	Maturase K	980	Kress et al., 2005
CYB6	CYB6F CTTTTTGTTTTGAGCCGTACGAGATGA CYB6R AAGTCATAGCAAAACCCGTCGCTACT	Cytochrom e B6	183	Choi et al., 2006
trnH-psbA	trnH GGCGCATGGTGGATTCACAAATC psbA GTTATGCATGAACGTAATGCTC	Intergenic region	367	Kress et al., 2005

Table 4.4 Information of the four nuclear and plastid regions used in the phylogenetic reconstruction of *Pedrosia* s.l. with the ITS only including GenBank sequences[a], excluding ITS sequences from GenBank[b] and with the ITS data set combined with the plastid regions[c]. *Using maximum parsimony.

	ITS			**Plastid**			**Plastid combined**	**All four regions**
	ITS[a]	ITS[b]	ITS[c]	*trnH-psbA*	*matK*	*CYB6*		
Number of samples	125	100	54	54	54	54	54	54
Aligned length (bp)	621	621	626	385	898	183	1465	2093
Number of indels (ingroup)	2	2	2	2	0	0	1	2
Number of constant sites	390	403	446	265	848	175	1288	1735
Number of parsimony informative sites	105	103	100	77	23	4	107	209
Number of parsimony informative sites excluding outgroup	23	22	16	7	8	1	17	34
Number of trees recovered*	303	285	239	135	56	8	37	451

Table 4.5 Gene regions used with the 21 sample data set used to identify the closest relative species of the four rhyncholotus species within clade B. Variability of each region when analyzed separate and in combination.

	Nuclear regions				**Plastid regions**		**All combined**
	ITS	*LjCYC1*	*LjCYC2*	*LjCYC3*	*trnH-psbA*	*matK*	
Number of samples	21	21	21	21	21	21	21
Aligned length (bp)	615	879	851	880	340	898	4461
Number of indels (ingroup)	0	11	4	2	2	0	19
Number of constant sites	55	826	783	825	326	895	4193
Number of parsimony informative sites	4	29	27	12	6	3	80
Number of parsimony informative sites excluding outgroup	2	24	26	7	6	3	67
Number of trees recovered	1	30	24	60	18	20	374

Table 4.6 Date of origin (MRCA) in Ma of various clades based on two data sets. Values obtained for each clade when rhyncholotus constrained or unconstrained to be monophyletic.

Clade	**4-gene chronogram**		**6-gene chronogram**	
	Constrained	Unconstrained	Constrained	Unconstrained
[*L. sessilifolius* + rhyncholotus]	**2.20**	**2.08**	**2.70**((0.62-4.17)	**2.09**(0.62-4.13)
[Rhyncholotus]	**1.20**(0.32-1.70)	-	**1.72**(0.29-3.3)	-
[*L. eremiticus*/*L. pyranthus*]	-	-	**0.58**(0.06-1.81)	**0.67**(0.06-1.90)
[*L. berthelotii*/*L. maculatus*]	-	-	**1.45**(0.06-1.80)	**1.11**(0.07-2.21)

Figure 4.1 Geographical distribution of *Lotus* section *Pedrosia* and *Rhyncholotus* in the Macaronesian region, including five Atlantic volcanic archipelagos (Madeira, Azores, the Salvage islands, the Canary Islands and Cape Verde islands), Europe and Africa. Each archipelago with the No. of species/ No. of endemic species. The age of current above-sea level for each island according to Carracedo et al. (2002). The phytogeographic region of Macaronesia is indicated in dashed lines, including a portion of Africa mainland denominated as the "Macaronesian enclave" (Kim et al. 2008).

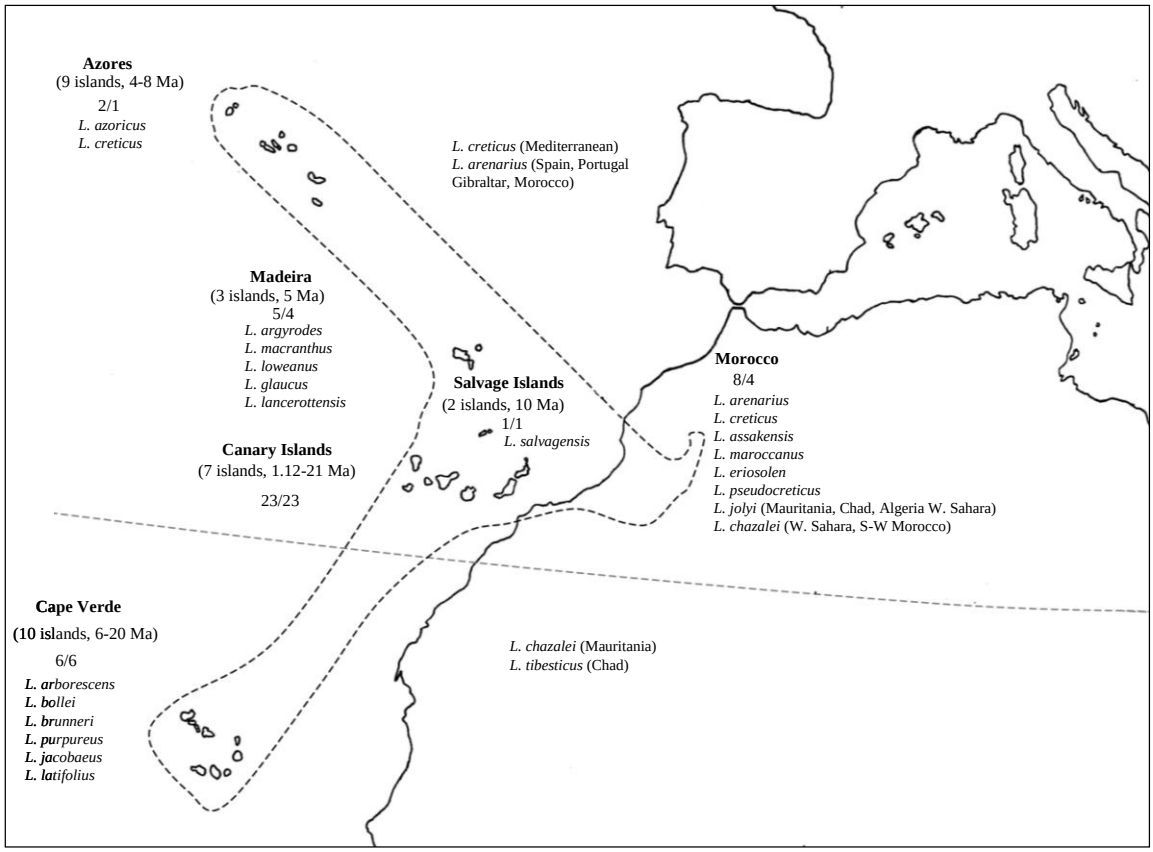


Figure 4.2 Geographical distribution of *Lotus* sections *Pedrosia* and *Rhyncholotus* in the Canary Island archipelago, with the oldest ages of the subaerial volcanism of each island according to Carracedo et al. (2002). Species in black are bird-pollinated species from the rhyncholotus group. No. of species/No. of endemic species on each island.

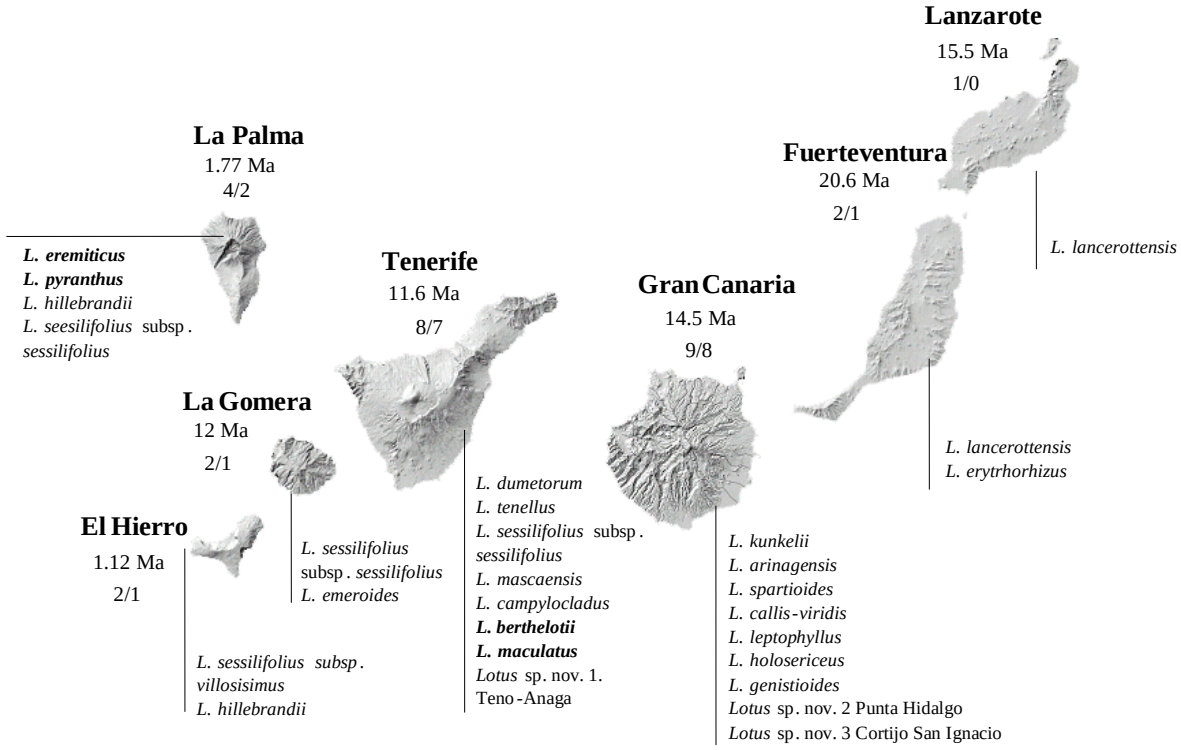


Figure 4.3 Strict consensus tree of the phylogenetic relationships using parsimony within *Pedrosia* s.l. using ITS DNA sequences. The four clades recovered are labeled (clade A-D). * indicate species groups previously recognized in *Lotus* according to Sandral et al (2006). Values above branches represent bootstrap values/Bayesian support.

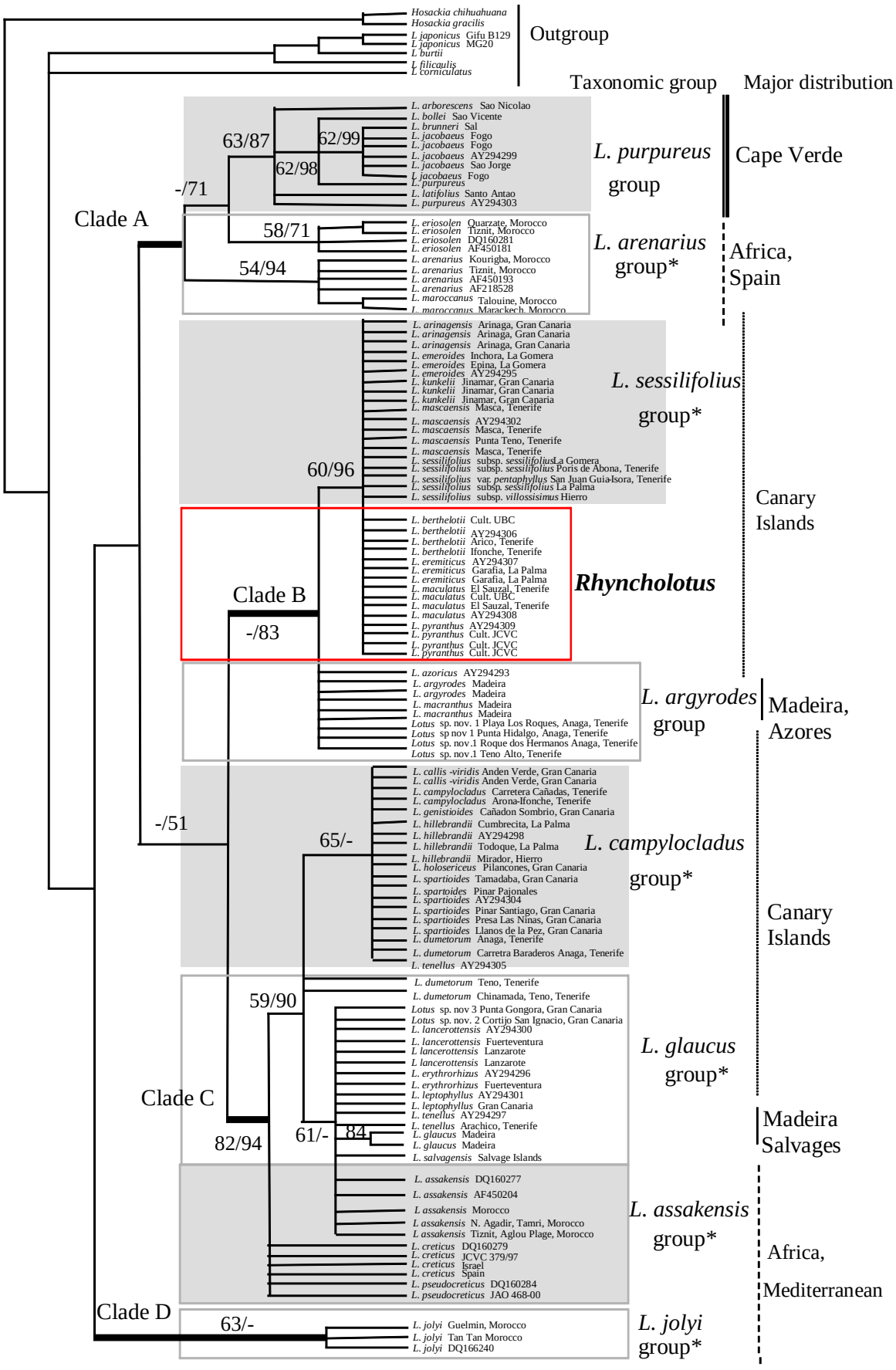

Hosackia chihuahuana
Hosackia gracilis
L japonicus Gifu B129
L japonicus MG20
L burtii
L filicaulis
L corniculatus
Outgroup
Taxonomic group
Major distribution
L. arborescens Sao Nicolao
L. bollei Sao Vicente
L. brunneri Sal
L. jacobaeus Fogo
L. jacobaeus Fogo
L. jacobaeus AY294299
L. jacobaeus Sao Jorge
L jacobaeus Fogo
L. purpureus
L. latifolius Santo Antao
L. purpureus AY294303
63/87
62/99
62/98
-/71
L. purpureus group
Cape Verde
Clade A
L. eriosolen Quarzate, Morocco
L. eriosolen Tiznit, Morocco
L. eriosolen DQ160281
L. eriosolen AF450181
L. arenarius Kourigba, Morocco
L. arenarius Tiznit, Morocco
L. arenarius AF450193
L. arenarius AF218528
L. maroccanus Talouine, Morocco
L. maroccanus Marackech, Morocco
58/71
54/94
L. arenarius group*
Africa, Spain
L. arinagensis Arinaga, Gran Canaria
L. arinagensis Arinaga, Gran Canaria
L. arinagensis Arinaga, Gran Canaria
L. emeroides Inchora, La Gomera
L. emeroides Epina, La Gomera
L. emeroides AY294295
L. kunkelii Jinamar, Gran Canaria
L. kunkelii Jinamar, Gran Canaria
L. kunkelii Jinamar, Gran Canaria
L. mascaensis Masca, Tenerife
L. mascaensis AY294302
L. mascaensis Masca, Tenerife
L. mascaensis Punta Teno, Tenerife
L. mascaensis Masca, Tenerife
L. sessilifolius subsp. sessilifoliusLa Gomera
L. sessilifolius subsp. sessilifolius Poris de Abona, Tenerife
L. sessilifolius var. pentaphyllus San Juan Guia-Isora, Tenerife
L. sessilifolius subsp. sessilifolius La Palma
L. sessilifolius subsp. villossisimus Hierro
60/96
L. sessilifolius group*
Canary Islands
L. berthelotii Cult. UBC
L. berthelotii AY294306
L. berthelotii Arico, Tenerife
L. berthelotii Ifonche, Tenerife
L. eremiticus AY294307
L. eremiticus Garafia, La Palma
L. eremiticus Garafia, La Palma
L. maculatus El Sauzal, Tenerife
L. maculatus Cult. UBC
L. maculatus El Sauzal, Tenerife
L. maculatus AY294308
L. pyranthus AY294309
L. pyranthus Cult. JCVC
L. pyranthus Cult. JCVC
L. pyranthus Cult. JCVC
Clade B
-/83
Rhyncholotus
L. azoricus AY294293
L. argyrodes Madeira
L. argyrodes Madeira
L. macranthus Madeira
L. macranthus Madeira
Lotus sp. nov. 1 Playa Los Roques, Anaga, Tenerife
Lotus sp nov 1 Punta Hidalgo, Anaga, Tenerife
Lotus sp. nov.1 Roque dos Hermanos Anaga, Tenerife
Lotus sp. nov.1 Teno Alto, Tenerife
L. argyrodes group
Madeira, Azores
L. callis -viridis Anden Verde, Gran Canaria
L. callis -viridis Anden Verde, Gran Canaria
L. campylocladus Carretera Cañadas, Tenerife
L. campylocladus Arona-Ifonche, Tenerife
L. genistioides Cañadon Sombrio, Gran Canaria
L. hillebrandii Cumbrecita, La Palma
L. hillebrandii AY294298
L. hillebrandii Todoque, La Palma
L. hillebrandii Mirador, Hierro
L. holosericeus Pilancones, Gran Canaria
L. spartioides Tamadaba, Gran Canaria
L. spartoides Pinar Pajonales
L. spartioides AY294304
L. spartioides Pinar Santiago, Gran Canaria
L. spartioides Presa Las Ninas, Gran Canaria
L. spartioides Llanos de la Pez, Gran Canaria
L. dumetorum Anaga, Tenerife
L. dumetorum Carretra Baraderos Anaga, Tenerife
L. tenellus AY294305
-/51
65/-
L. campylocladus group*
Canary Islands
L. dumetorum Teno, Tenerife
L. dumetorum Chinamada, Teno, Tenerife
Lotus sp. nov 3 Punta Gongora, Gran Canaria
Lotus sp. nov. 2 Cortijo San Ignacio, Gran Canaria
L. lancerottensis AY294300
L. lancerottensis Fuerteventura
L lancerottensis Lanzarote
L lancerottensis Lanzarote
L. erythrorhizus AY294296
L. erythrorhizus Fuerteventura
L. leptophyllus AY294301
L. leptophyllus Gran Canaria
L. tenellus AY294297
L. tenellus Arachico, Tenerife
L. glaucus Madeira
L. glaucus Madeira
L. salvagensis Salvage Islands
59/90
82/94
61/-
84
Clade C
L. glaucus group*
Madeira Salvages
L. assakensis DQ160277
L. assakensis AF450204
L assakensis Morocco
L assakensis N. Agadir, Tamri, Morocco
L assakensis Tiznit, Aglou Plage, Morocco
L. creticus DQ160279
L. creticus JCVC 379/97
L. creticus Israel
L. creticus Spain
L. pseudocreticus DQ160284
L. pseudocreticus JAO 468-00
L. assakensis group*
Africa, Mediterranean
Clade D
63/-
L. jolyi Guelmin, Morocco
L. jolyi Tan Tan Morocco
L. jolyi DQ166240
L. jolyi group*

Figure 4.4 Strict consensus tree based on a combined analysis of three plastid regions (*trnH-psbA*, *matK* and *CYB6*) using maximum parsimony. Values above branches indicate bootstrap values.

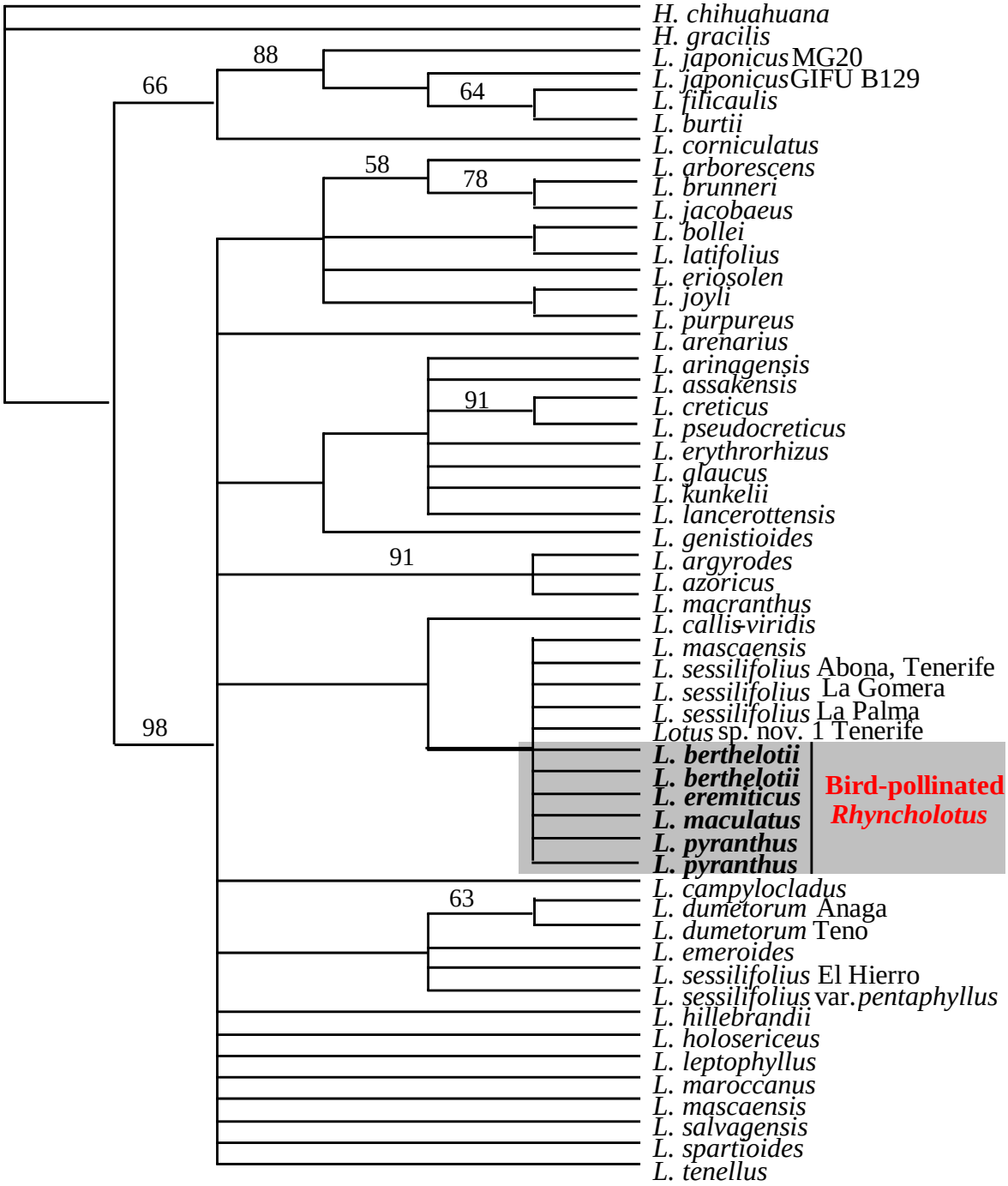


Figure 4.5 Majority tree recovered from a combined analysis of one nuclear (ITS) and three plastid gene regions (*matK, trnH-psbA* and *CYB6*) using maximum parsimony. Branches with an arrow indicate clades not observed in the strict consensus. Values above branches represent bootstrap from MP/posterior probabilities support.

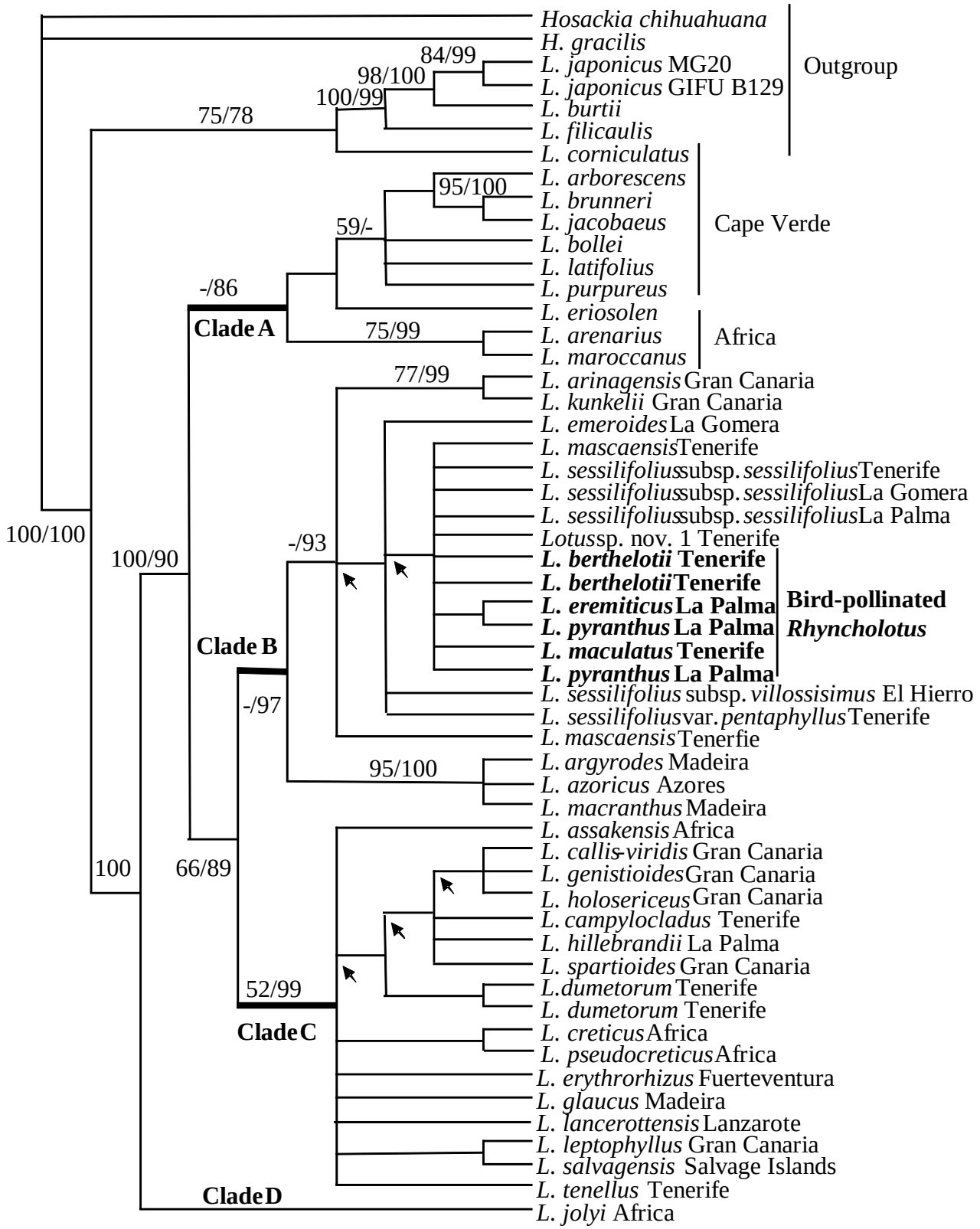

Hosackia chihuahuana
H. gracilis
L. japonicus MG20
L. japonicus GIFU B129
L. burtii
L. filicaulis
L. corniculatus
L. arborescens
L. brunneri
L. jacobaeus
L. bollei
L. latifolius
L. purpureus
L. eriosolen
L. arenarius
L. maroccanus
L. arinagensis Gran Canaria
L. kunkelii Gran Canaria
L. emeroides La Gomera
L. mascaensis Tenerife
L. sessilifolius subsp. sessilifolius Tenerife
L. sessilifolius subsp. sessilifolius La Gomera
L. sessilifolius subsp. sessilifolius La Palma
Lotus sp. nov. 1 Tenerife
L. berthelotii Tenerife
L. berthelotii Tenerife
L. eremiticus La Palma
L. pyranthus La Palma
L. maculatus Tenerife
L. pyranthus La Palma
L. sessilifolius subsp. villossisimus El Hierro
L. sessilifolius var. pentaphyllus Tenerife
L. mascaensis Tenerfie
L. argyrodes Madeira
L. azoricus Azores
L. macranthus Madeira
L. assakensis Africa
L. callis-viridis Gran Canaria
L. genistioides Gran Canaria
L. holosericeus Gran Canaria
L. campylocladus Tenerife
L. hillebrandii La Palma
L. spartioides Gran Canaria
L. dumetorum Tenerife
L. dumetorum Tenerife
L. creticus Africa
L. pseudocreticus Africa
L. erythrorhizus Fuerteventura
L. glaucus Madeira
L. lancerottensis Lanzarote
L. leptophyllus Gran Canaria
L. salvagensis Salvage Islands
L. tenellus Tenerife
L. jolyi Africa
Outgroup
Cape Verde
Africa
Bird-pollinated Rhyncholotus
84/99
98/100
100/99
75/78
95/100
59/-
-/86
Clade A
75/99
77/99
100/100
100/90
-/93
Clade B
-/97
95/100
100
66/89
52/99
Clade C
Clade D

Figure 4.6A. Maximum parsimony strict consensus tree based on four nuclear regions (ITS, *LjCYC 1, 2* and *3*) and two plastid regions (*matK* and *trnH-psbA*) using maximum parsimony. Values above branches indicate bootstrap values/ Bayesian support. Values below 50 are not indicated. Arrows indicate clades recovered in MP, Bayesian and ML analyses using this data set.

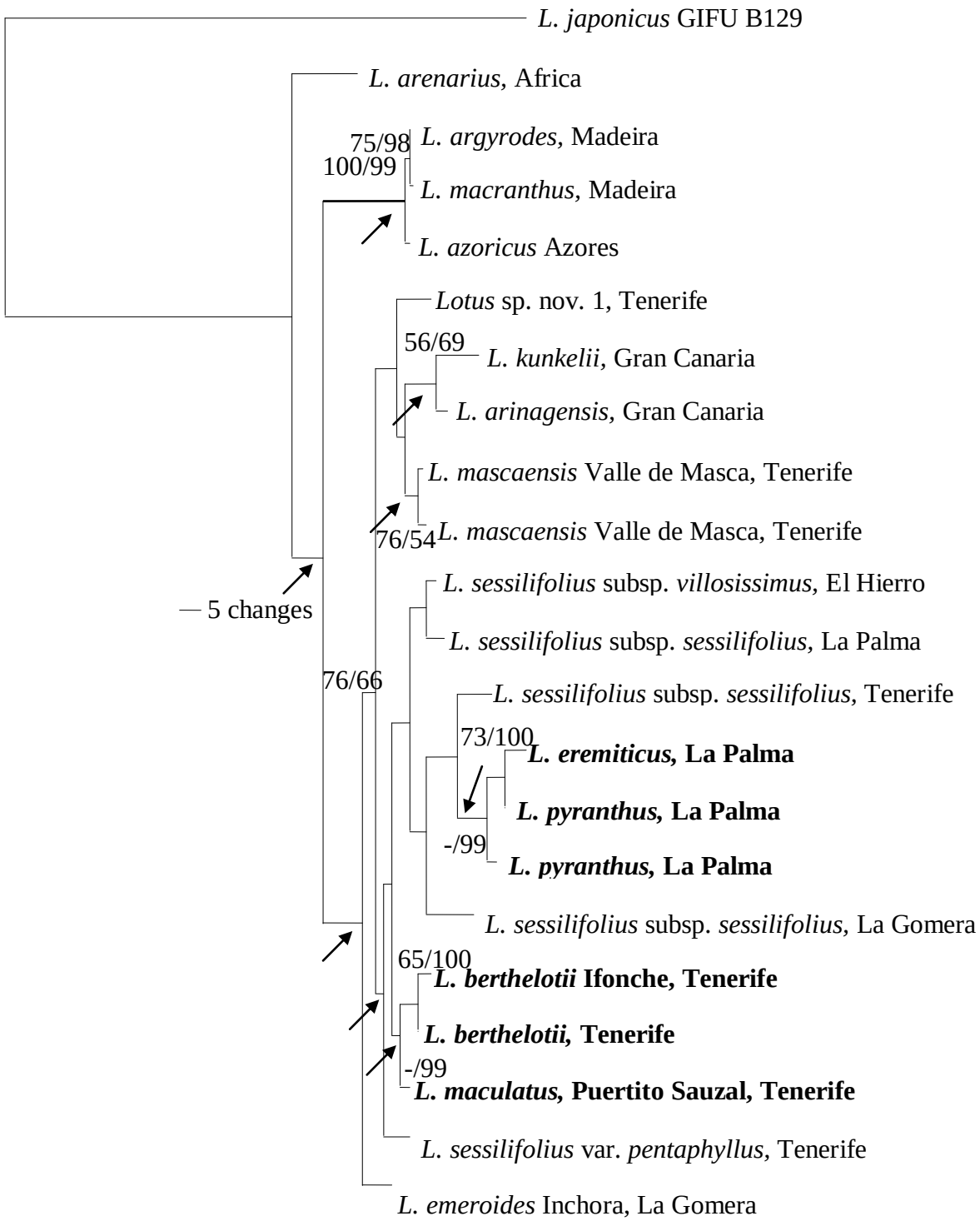


Figure 4.6B. Maximum likelihood tree recovered using a data set of six genes and 21 samples with Garli.

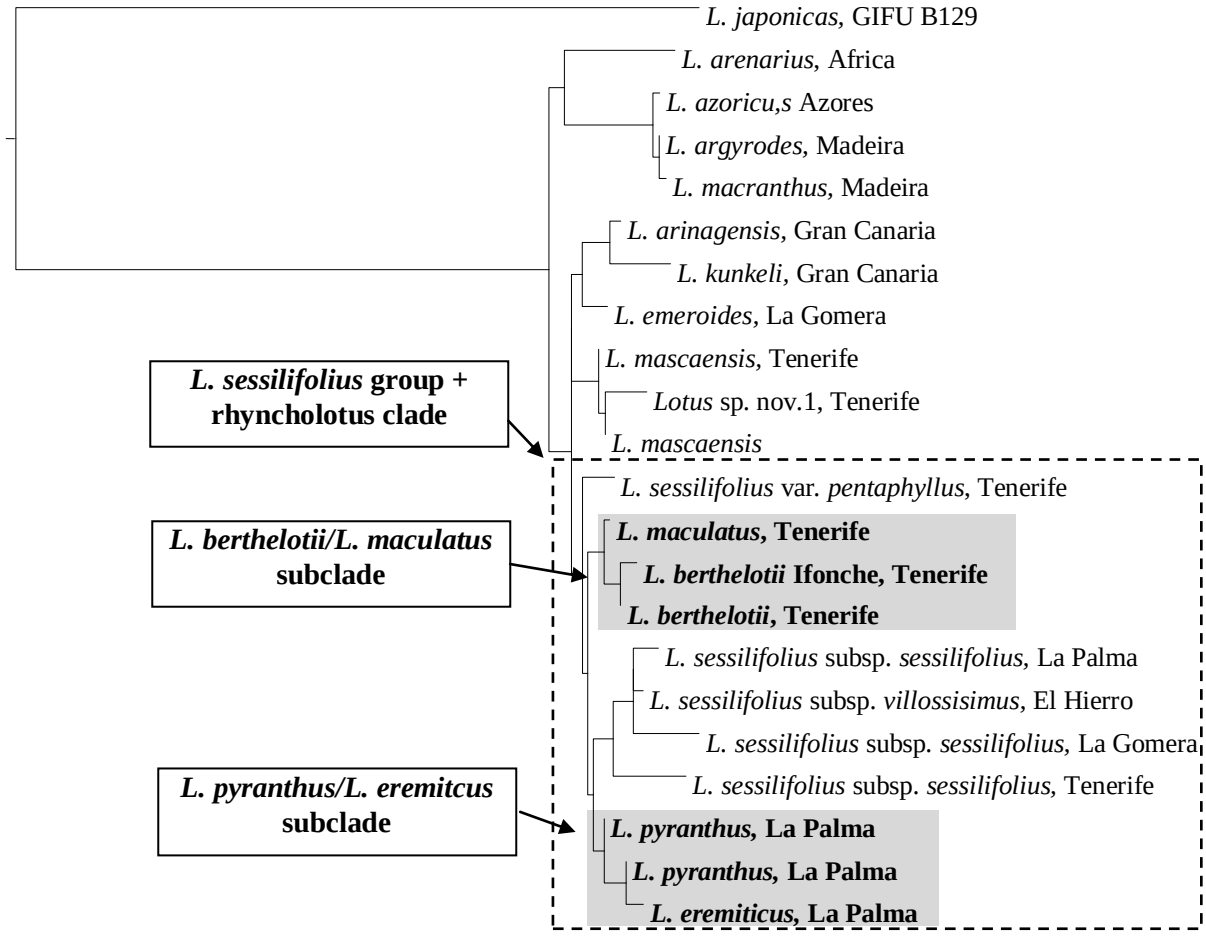


Figure 4.7 Flower and leaflet morphology in *L. argyrodes* (A-C), *L. mascaensis* (D-F) and *L. sessilifolius* var. *pentaphyllus* (G-I).

Figure 4.8 Flower and leaflet morphology in the four bird-pollinated species. Both groups are differentiated by minor morphological features including the number of flowers per inflorescence, the position and orientation of the dorsal petal and the position of the lateral petal. In the species from La Palma the lateral petal is fused at the tip and covers the tip of the ventral petal. In contrast, species from Tenerife have a ventral petal more exposed.

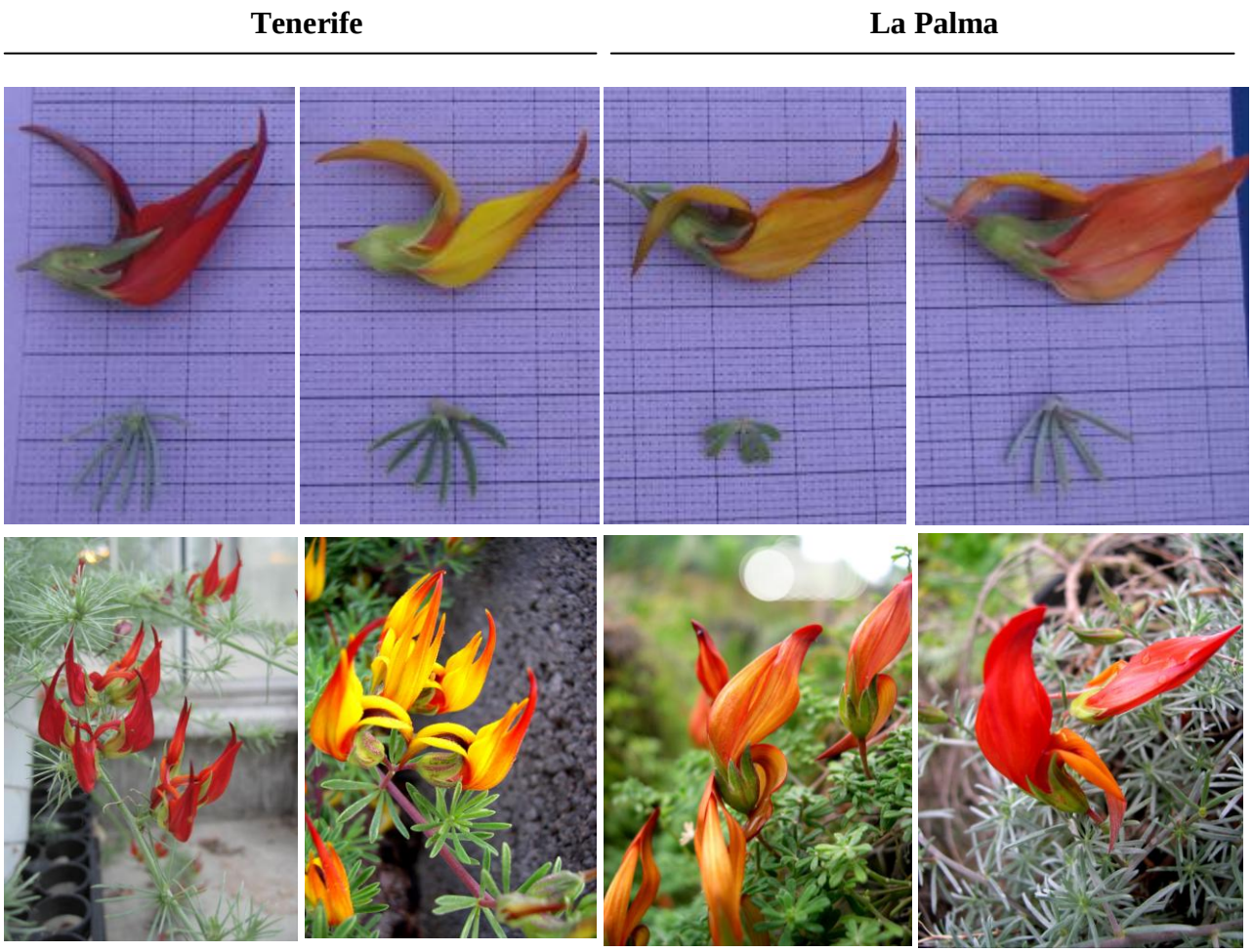


Figure 4.9 Chronogram obtained for the evolution of bird pollination in Macaronesian *Lotus* under a Bayesian relaxed clock uncorrelated clock model using Beast and applied to the combined data set of 52 samples and using a data set of four gene regions (ITS, *matK*, *trnH-psbA* and *CYB6*). Upper limits of the ages of La Palma (1.77 Ma), El Hierro (1.12 Ma), and Fuerteventura (21 Ma) were used as calibration points (black circles). Bird-pollinated species are shown in red branches. Ages estimates with their 95% credibility intervals are shown on nodes. Values on grey squares represent bootstrap values from MP/posterior probabilities inferred from the Bayesian inference. Major geological events between the Miocene and Pleistocene are indicated with arrows at the bottom. Clades named after the groups recovered with ITS and the four gene data analyses.

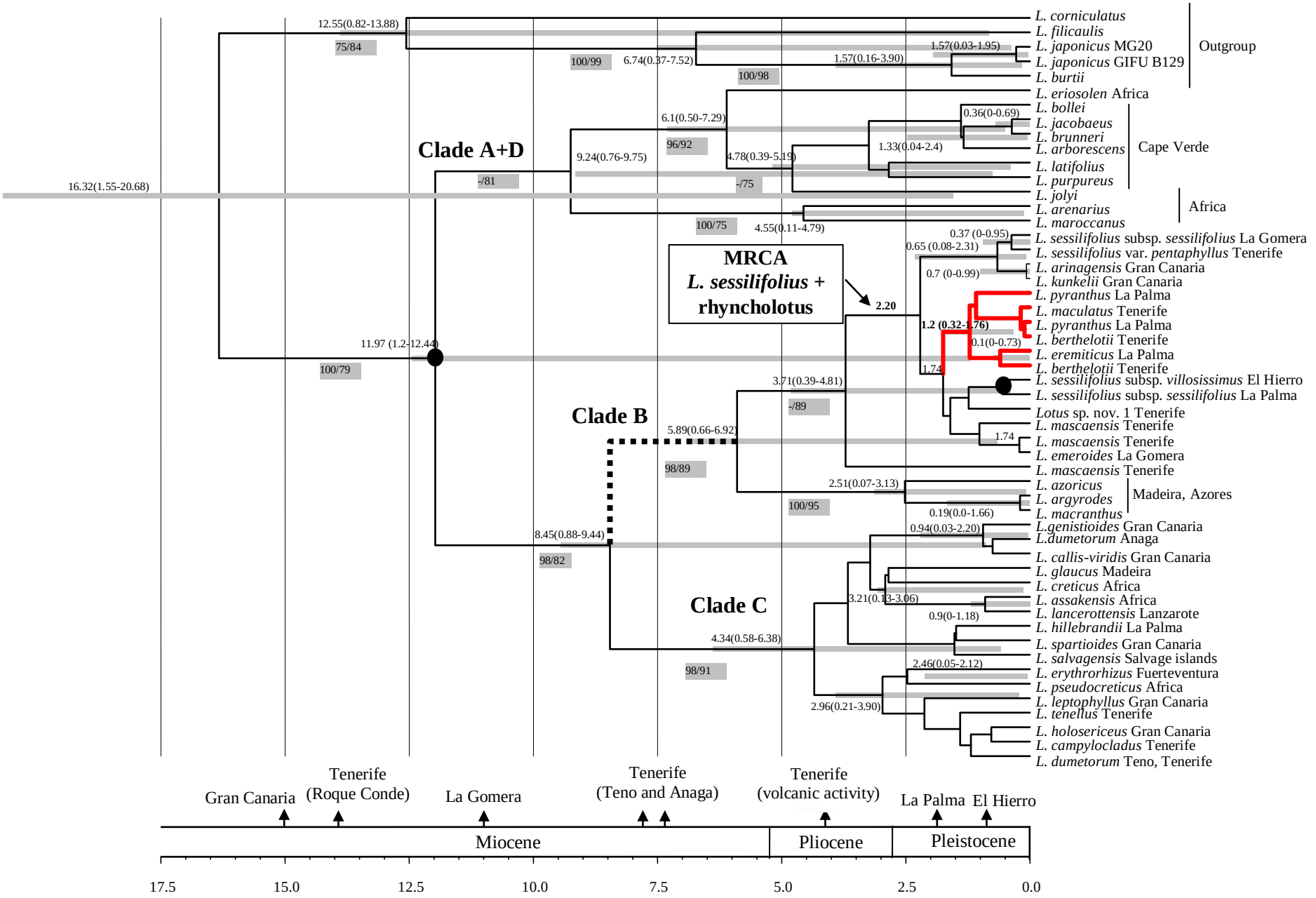
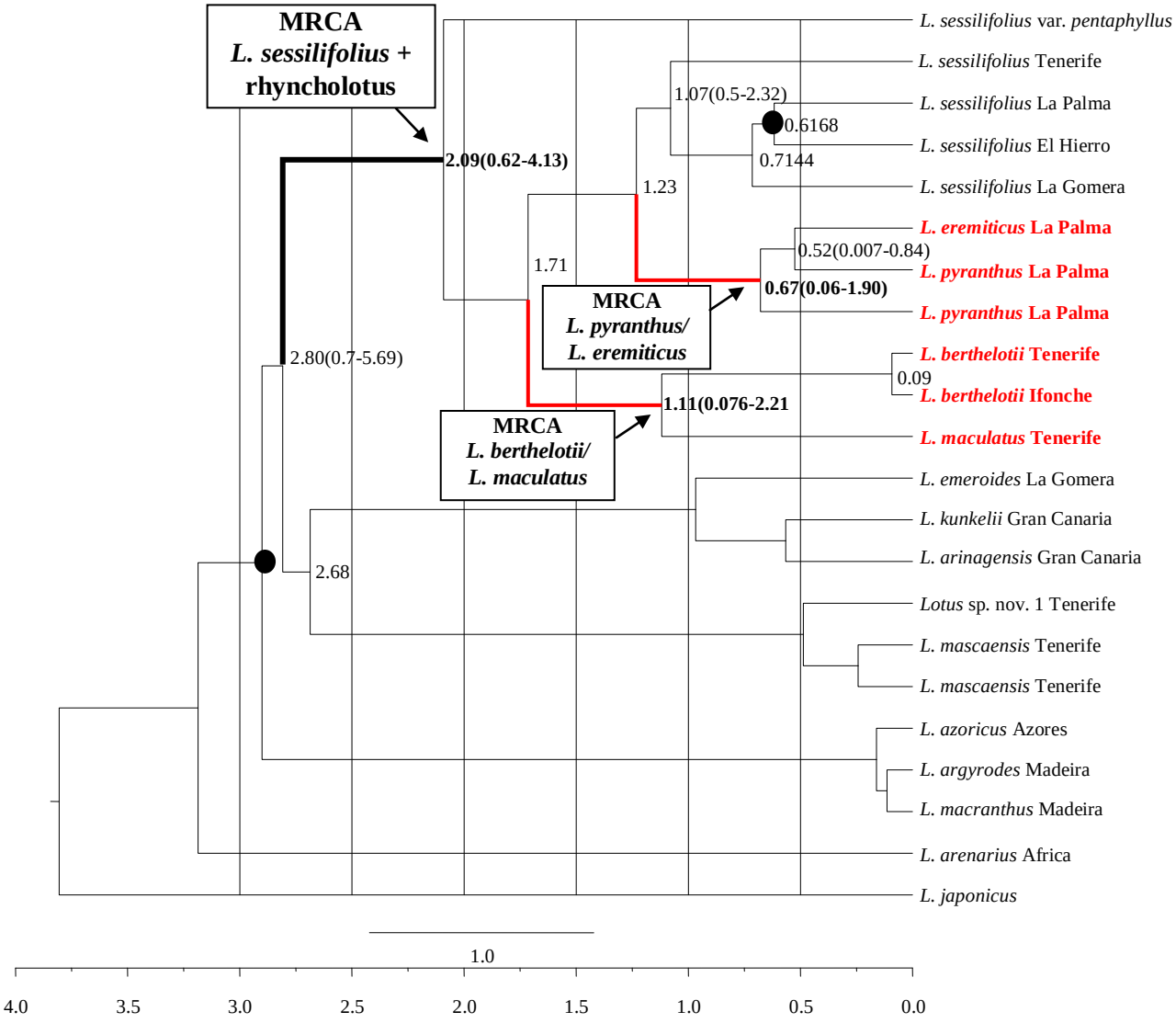

12.55(0.82-13.88)
75/84
100/99
6.74(0.37-7.52)
1.57(0.03-1.95)
1.57(0.16-3.90)
100/98
L. corniculatus
L. filicaulis
L. japonicus MG20
L. japonicus GIFU B129
L. burtii
Outgroup
L. eriosolen Africa
6.1(0.50-7.29)
96/92
0.36(0-0.69)
L. bollei
L. jacobaeus
L. brunneri
L. arborescens
1.33(0.04-2.4)
Cape Verde
Clade A+D
9.24(0.76-9.75)
4.78(0.39-5.19)
L. latifolius
L. purpureus
16.32(1.55-20.68)
-/81
-/75
L. jolyi
L. arenarius
L. maroccanus
Africa
100/75
4.55(0.11-4.79)
0.37 (0-0.95)
L. sessilifolius subsp. sessilifolius La Gomera
0.65 (0.08-2.31)
L. sessilifolius var. pentaphyllus Tenerife
MRCA
L. sessilifolius +
rhyncholotus
2.20
0.7 (0-0.99)
L. arinagensis Gran Canaria
L. kunkelii Gran Canaria
L. pyranthus La Palma
L. maculatus Tenerife
1.2 (0.32-1.76)
L. pyranthus La Palma
0.1(0-0.73)
L. berthelotii Tenerife
11.97 (1.2-12.44)
L. eremiticus La Palma
1.74
L. berthelotii Tenerife
100/79
3.71(0.39-4.81)
L. sessilifolius subsp. villosissimus El Hierro
-/89
L. sessilifolius subsp. sessilifolius La Palma
Clade B
Lotus sp. nov. 1 Tenerife
5.89(0.66-6.92)
1.74
L. mascaensis Tenerife
L. mascaensis Tenerife
L. emeroides La Gomera
98/89
L. mascaensis Tenerife
2.51(0.07-3.13)
L. azoricus
L. argyrodes
Madeira, Azores
100/95
0.19(0.0-1.66)
L. macranthus
0.94(0.03-2.20)
L.genistioides Gran Canaria
8.45(0.88-9.44)
L.dumetorum Anaga
98/82
L. callis-viridis Gran Canaria
L. glaucus Madeira
L. creticus Africa
Clade C
3.21(0.13-3.06)
L. assakensis Africa
L. lancerottensis Lanzarote
0.9(0-1.18)
L. hillebrandii La Palma
4.34(0.58-6.38)
L. spartioides Gran Canaria
L. salvagensis Salvage islands
98/91
2.46(0.05-2.12)
L. erythrorhizus Fuerteventura
L. pseudocreticus Africa
2.96(0.21-3.90)
L. leptophyllus Gran Canaria
L. tenellus Tenerife
L. holosericeus Gran Canaria
L. campylocladus Tenerife
L. dumetorum Teno, Tenerife
Gran Canaria
Tenerife
(Roque Conde)
La Gomera
Tenerife
(Teno and Anaga)
Tenerife
(volcanic activity)
La Palma
El Hierro
Miocene
Pliocene
Pleistocene
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0

Figure 3.10 Chronogram of the rhyncholotus group using a six gene data set and analyzed with Beast using El Hierro (1.12 Ma), La Palma (1.77 Ma) and the age of Gran Canaria (14.5 Ma) as upper age estimates (black circles). Node ages are indicated above branches with 95% HDP intervals.



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5 DNA barcoding of plant island radiations and its applicability in species recognition and conservation in Macaronesian *Lotus* section *Pedrosia* and *Rhyncholotus* (Loteae, Leguminosae)[1]

5.1 Introduction

DNA barcoding is a procedure for species identification and recognition that uses a standard sequence of DNA (Hebert et al., 2003). It has proved successful in a variety of animal groups based on the mitochondrial gene cytochrome c oxidase subunit 1 (COI or *cox1*), which has sufficient variation for species discrimination (> 95%) (Hajibabaei et al., 2006; Kerr et al., 2007; Smith et al., 2008; Ward et al., 2005) in animals, but not plants (Fazekas et al., 2009). Plant barcoding is more complicated as the plant mitochondrial genome evolves at a slower rate than in animals. However, the search for an appropriate barcode region in plants has turned to the plastid genome as this is the fastest evolving genomes and because the plant nuclear genome is difficult to work with in general. A variety of combinations of plastid regions have been tested and such studies have generally found relatively moderate levels (~70%) of species discrimination (CBOL, 2009; Fazekas et al., 2008; Fazekas et al., 2009; Kress and Erickson, 2007).

After a period of debate and the testing of several regions in a variety of combinations (Kress and Erickson 2007; Sass et al. 2007; Lahaye et al. 2008; Newmaster et al. 2008; Newmaster and Ragupathy 2009; Seberg and Petersen 2009), the suggested barcode regions for plants, *matK* and *rbcL*, were recently recommended by the CBOL Plant working group (CBOL, 2009).

[1]A version of this chapter will be submitted for publication: Ojeda, I. Santos-Guerra, A., Oliva-Tejera, F. Jaen Molina, R. Caujape, J. Marrero, A. and Cronk. Q.C.B. BNA barcoding plant island radiations and its applicability in species recognition and conservation in Macaronesian *Lotus* section *Pedrosia* and *Rhyncholotus* (Loteae, Leguminosae).

This two-locus combination is able to discriminate about 72% of the samples included in this study to species level with the remaining samples assigned to congeneric species groups.

The internal transcribed spacer of the nuclear ribosomal repeat (ITS) has been used as a barcode in fungi (Druzhinina et al. 2005; Kõljalg et al. 2005; Tedersoo et al. 2008). In plants, this region has also been tested in some particular groups (Chase et al. 2005; Kress et al. 2005; Gemeinholzer et al. 2006; Kress and Erickson 2007; Okuyama and Kato 2009).

Despite the lower levels of species discrimination obtained in plant barcoding in comparison to animals, it is evident that there are numerous practical applications of plant barcoding in a variety of fields, such as taxonomy, ecology, floristic studies, industry and conservation. However, it is also clear that DNA barcoding in plants will not reach the high levels of species discrimination achieved in animals. Nevertheless, in certain applications is potentially a useful tool for species recognition (Kondo et al., 2007; Kress et al., 2009; Newmaster et al., 2008; Ragupathy et al., 2009; Soininen et al., 2009; Song et al., 2009b).

Many of the studies that have tested regions as barcodes in plants have focused their attention on large data sets that span the entirety of land plants or at least angiosperms (CBOL, 2009; Fazekas et al., 2008; Ford et al., 2009; Kress and Erickson, 2007; Lahaye et al., 2008). Their purpose has been the assessment of the universal applicability of the regions in species discrimination. However, it has been argued that the recovery rate of DNA barcode drop in (i) some groups with complex biology, due to a variety of phenomena, such as hybridization, polyploidy, introgression, and (ii) closely related species within the same genus, or in recently evolved groups, such as those of island radiations. To date, the level of species discrimination within the same genus has been tested in a number of cases (Newmaster et al., 2008; Newmaster and Ragupathy, 2009; Sass et al., 2007; Yao et al., 2009). In most studies, the level of species identification within the same genus is promising. However, some of these examples included

either few species within the same genus and/or well diverged species groups. There is emerging evidence that at least some closely related groups will be problematic for barcoding (Fazekas et al., 2009; Sass et al., 2007; Seberg and Petersen, 2009; Spooner, 2009).

When individual genera have been sampled more extensively, the percentage of species discrimination tends to decrease, even when several regions are combined (Edwards et al., 2008; Kondo et al., 2007; Sass et al., 2007; Seberg and Petersen, 2009; Yao et al., 2009). However, the applicability of species recognition of the recommended barcode regions in very recently evolved groups, such as those of an island radiation, has not being tested extensively. It is unclear if the levels of DNA variation within these two regions, *matK* + *rbcL*, will allow species discrimination. Here I present the assessment of five plastid regions suggested as barcodes in previous studies (*matK, rpoC1, rpoB, trnH-psbA* and *rbcL*) and the nuclear ribosomal internal transcribed spacer regions, ITS1 and ITS2.

Here I tested these regions in a group of *Lotus* species that radiated in the Macaronesian region (Azores, Madeira, Cape Verde, Canary Islands and Salvage Islands). The group has its highest diversification in the Canary Islands and in mainland Morocco. This Macaronesian *Lotus* assemblage comprises 41 described species divided into two sections: *Pedrosia* and *Rhyncholotus* (Degtjareva et al., 2006). The section *Pedrosia* comprises 37 recognized species while section *Rhyncholotus* comprises only four species: *Lotus berthelotii*, *L. maculatus* both from Tenerife and *L. pyranthus* and *L. eremiticus* from La Palma. It should be noted that section "*Rhyncholotus*" is in fact nested phylogenetically within section *Pedrosia* and possibly within a single species (see Chapter 4). The two groups are distinguished from each other by a distinctive flower morphology associated with different pollination syndromes, but within each group vegetative features are more useful for species recognition and identification (Sandral et al., 2006). Species identification

using morphology is not always straightforward and a critical examination of a number of morphological features by a specialist will often be necessary for accurate identification.

Many species are restricted to specific habitats, such as pine forest and lowland scrub. Furthermore, about 70% of the species are endemic to single islands. Thus, the group is highly susceptible to habitat destruction and at least 10 species are listed under some category of conservation threat, ranging from rare to critically endangered (Bañares et al., 2004; Martín et al., 2008; VV.AA., 2000) (Table 5.1).

5.1.1 Objectives of the study

The aims of this study are to answer the following questions: (1) are these DNA barcode regions variable enough to discriminate species in recently evolved groups? (2) is there any potential applicability of these barcoding regions in conservation?, and (3) are these regions variable enough to separate between the two sections *Pedrosia* and *Rhyncholotus*?

5.2 Materials and methods

5.2.1 Taxon sampling

The sampling includes 78 accessions within the ingroup representing all the species currently described within the sections *Pedrosia* and *Rhyncholotus*, except three species (*L. loweanus, L. chazalei* and *L. tibesticus*) that were not available for this analysis. In order to sample the intraspecific variation and geographical distribution of some species, I included more than one accession for 27 of the 38 species analyzed (71%). This analysis also included samples from some populations that based on previous molecular and morphological analyses (Oliva-Tejera et al., 2005; Oliva-Tejera et al., 2006; Sandral et al., 2006), may represent four new

undescribed species within the section *Pedrosia* (Table 5.2). For comparison, I also included five accessions from *Lotus* section *Lotus* (Table 5.2).

5.2.2 Selecting barcode regions

Several regions have been previously suggested and tested as barcode markers (Fazekas et al., 2008; Kress and Erickson, 2007, 2008a; Kress et al., 2005; Lahaye et al., 2008; Newmaster et al., 2006; Newmaster et al., 2008). For this analysis I included five plastid regions, *rbcL*, *trnH-psbA*, *matK*, *rpoB* and *rpoC1*, including the recently recommended two-locus barcode *matK* + *rbcL* (CBOL, 2009), along with the nuclear ribosomal ITS region, which has been assessed in some plant groups (Chase et al., 2005; Kress and Erickson, 2007; Kress et al., 2005).

5.2.3. Molecular analysis

Genomic DNA was extracted from either fresh leaves, silica-gel dried leaf material or voucher specimens following a modification of the procedure of Doyle and Doyle (1987). Amplification was carried out with the following PCR conditions for all the plastid regions: 94°C for 3 min., 30 cycles of 94°C for 3 min., 45°C for 1 min. and 72°C for 2 min., with a final cycle of 72°C for 5 min. The nuclear ribosomal intergenic spacer ITS was amplified using the following conditions: 94°C for 3 min., 30 cycles of 94°C for 1 min., 55°C for 1 min. and 72°C for 1.5 min, with a final cycle of 72°C for 5 min. Each locus was sequenced and the raw sequence data were imported to Sequencher 4.1 for base-coding, editing and construction of contig sequences. Consensus sequences were imported to Se-Al ver. 1.0 (Rambaut, 1996) and aligning was made manually using conserved regions. Each region was analyzed separately and in two-pair combinations. The analyses were carried both excluding and including missing sequences. In all the gene regions I

included the entire sequence, except for a small inversion of 3 bp in the intergenic *trnH-psbA* region 214-217 position that was excluded from all the analyses.

5.2.4 Assessment of the barcode regions

Three parameters have been suggested for the official barcodes: universality, sequence quality and coverage, and discrimination (CBOL, 2009). I evaluated these three parameters in the six regions tested within this group.

Amplification success: I estimated the percentage of amplification success on the first trial with the sample set of this group as an indicator of universality, using the same PCR amplification and conditions.

Sequence quality and coverage: I estimated the percentage of bidirectional sequences with few or no ambiguous bases.

Discrimination: I evaluated discrimination at two levels: species discrimination and discrimination of informal taxonomic groups following according to previous taxonomic analysis based on morphological features (Sandral et al., 2006). I considered that a species was discriminated when it was grouped with samples of the same species (in the case of species with more than one sample) forming a monophyletic group or when it position was resolved fully resolved (in the case of unique samples).

Nine informal taxonomic groups at the infrageneric level have been suggested within the Macaronesian assemblage (Table 5.3). I consider that a useful discrimination at this level was achieved when at least 50% of the species were assigned within the same group, forming a monophyletic group.

Each region was analyzed separately and in various combinations with Neighbor-joining (NJ) using a Kimura 2-parameter as implemented in PAUP4b10 (Swofford, 2001). This is the standard method used in barcoding studies.

5.3 Results

5.3.1 Universality and sequence quality

All regions had above 95% of sequence success and quality, except for the *matK* region, with 83% of success, which required additional PCR runs to reach this level due to either failure of amplification or due to regions with T or A repeats that caused failure during sequencing. This last barcode region had the lowest level of bidirectional sequence quality (Table 5.5).

5.3.2 Species discrimination of the plastid regions

The *trnH-psbA* region showed the highest level of variation and species discrimination of all regions evaluated (18%), while the *rpoB* region had the lowest level of variation and species discrimination when analyzed alone (Table 5.4). The combination *trnH-psbA* + *matK* showed the highest level of discriminatory power at the species level for two-locus plastid combinations (29%). No differences were observed in species discrimination between the CBOL recommended 2-locus barcode (*matK* + *rbcL*) (Fig. 5.1) and other combinations (Table 5.4).

In this study, I achieved the identification of 14 species (37%) of the 38 species in this sample when all five plastid regions where combined (Table 5.4). Only two out of 10 species (20%) of conservation concern were identified at the species level (Fig. 5.2). Only five informal taxonomic groups were discriminated with all plastid regions combined (Table 5.4). The intergenic spacer *trnH-psbA* showed the highest levels of variation and it was the only region where we observed intraspecific variation. I found an inversion (3 bp long) at the 214-217

position. The ingroup was either AAA or TTT for this region. This inversion polymorphism is a hairpin loop structure surrounded by an inverted repeat sequence of 22 bp (47 bp in total). Surprisingly 8 out of 27 species with multiple samples showed this polymorphism within a species. This plastid region was also the only one in which we observed indels. Two indels were observed within the ingroup, one large in the position 94-104 that involved 10 bp (TAGATAAAAT) which is shared by all *Rhyncholotus* species along with four species of *Pedrosia*; the other indel consisted of a 1 bp deletion (position 194) shared mainly by all members analyzed of the *L. assakensis* group (*L. assakensis*, *L. creticus* and *L. pseudocreticus*) and most of the members of the *L. glaucus* group (*L. glaucus*, *L. salvagensis*, *L. lancerottensis* and *L. erythrorhyzus*) (Table 5.3).

5.3.3 ITS as a barcode in *Lotus*

This region showed the highest level of variability of all regions tested in this study when analyzed alone, with the identification of 26% of the species. The level of species discrimination increased when combined with the plastid regions in pairs. All two-pair combinations of ITS with the plastid regions increased the discriminatory power (Table 5.4). The addition of ITS increased the discriminatory power in this group to 52% of the species when all six regions were combined, including four species with conservation concern (*L. arinagensis, L. kunkelii, L. erythrorhyzus* and *L. genistoides*) (Fig. 5.3).

5.4 Discussion

Within Macaronesian *Lotus* I was able to identify 52% of the samples at the species level when all six regions were combined (Table 5.4). Previous studies have reported from 55% (*trnH-psbA* in *Aspalathus*) to 92% (e.g. *Crocus*) of species discrimination in several plant groups

(Edwards et al., 2008; Sass et al., 2007; Seberg and Petersen, 2009). As previously suggested, barcodes will have some limitations in closely related species (Chase and Fay, 2009), especially from a rapid and recent island radiation. A recent study in Amazonian trees using a similar amount of barcode regions as in this study found moderate levels of species discrimination (70%) but a tendency to have low levels of species discrimination in species-rich clades (González et al., 2009).

The CBOL suggested 2-locus combination (*matK* + *rbcL*) discriminated only 18% of the species (Fig. 5.2 and) and no major increase was observed with other two-pair plastid combinations (Table 5.4) My results therefore indicate that the discriminatory power of the barcode regions is low in recently evolved groups, such as those diversifying rapidly as part of an island radiation. Low level of variation has been also reported from *Scalesia* (Asteraceae) from the Galápagos (Seberg and Petersen, 2009). Similar scenarios with low species discrimination have been reported when barcoding animals on islands, such as the one reported in the genus *Copelatus* (Coleoptera) in the Fijian archipelago (Monaghan et al., 2006).

The applicability of barcodes in the identification of protected or endangered species in this group is low, as I identified four species of the ten (40%) considered under some level of threat. Taken together, these results suggest that DNA barcoding recently evolved groups in islands will remain a challenge for species identification, and are perhaps a "worst case scenario". The performance of the barcodes it is also generally low when applied at the floristic level on islands. Recent evidence from congeneric species in the Garajonay National Park in La Gomera (Jaen-Molina et al. unpubl. data) shows that *matK* and *rbcL* in combination only allowed the identification of ca. 75% cases. This result contrasts with other studies which have applied floristic barcoding approaches on diverse areas, such as at La Selva Biological Station (Kress and

Erickson, 2008b) and the 50-ha Forest Dynamic Plot on Barro Colorado (Kress et al., 2009), where species identification is above 90%.

Despite these results, it is worth noting that the low levels of species identification within the Macaronesian *Lotus*, the CBOL recommended two-locus barcode will accurately assign samples to section *Pedrosia* s.l. (*Pedrosia* + *Rhyncholotus*) as distinct from the comparison group (*Lotus* section *Lotus*). I observed high levels of variation between section *Lotus* and section *Pedrosia* s. l. in all barcoding regions. Even the less variable region unequivocally separated the two sections. However, it should be noted that *Lotus* comprises about 120 species subdivided into 14 sections (Degtjareva et al., 2006), so that further study will be needed to confirm that barcoding can distinguish all sections of *Lotus* reliably.

Another factor that may also decrease species discrimination is the existence of within-species variation for some of the analyzed regions. Of the 27 for which I included more than one sample, I observed within-species variation in the intergenic spacer *trnH-psbA* in eight species (30%) (Table 5.2). This intraspecific variation is similar to that reported for other plant groups in ITS and in some intergenic regions, including the *trnH-psbA* (Cowan et al., 2006; Edwards et al., 2008; Kress et al., 2009; Seberg and Petersen, 2009; Spooner, 2009; Vischi et al., 2006; Zhang et al., 2009).

I only observed intraspecific variation in the intergenic region of the *trnH-psbA* due to an inversion and deletions, unlike other studies in *Aspathalus* (Leguminosae) and *Helianthus argophylus* (Asteraceae) that have reported intraspecific indel variation (Chase and Fay, 2009; Edwards et al., 2008; Vischi et al., 2006).

5.4.1 ITS vs. plastid data

The low levels of interspecific variation observed in this group in the five plastid regions indicate that additional regions might be considered in particular cases. Faster evolving gene regions, preferably from the nucleus, should therefore be explored in addition to the proposed barcodes. This is supported by the fact that the addition of ITS increased the discriminatory power of these regions in combination with the plastid regions and also when all six regions were combined (Table 5.4 and Fig. 5.3). The discriminatory power for the endangered species also increased with the inclusion of the ITS region.

Previous studies have considered ITS as a potential barcode in land plants (Chase et al., 2005; Kress and Erickson, 2007; Kress et al., 2005; Okuyama and Kato, 2009). However, the ITS region has received less attention than plastid regions as a DNA barcode in some groups because of associated problems with sequencing success (González et al., 2009; Kress and Erickson, 2007) and high levels of intraspecific variation in some groups (Edwards et al., 2008; Spooner, 2009). In this analysis I observed intraspecific variation within the ITS region in only 13% of the species (five out of the 38) analyzed. The ITS region performed better than the *trnH-psbA* in species discrimination despite the intraspecific variation observed, which is probably due to the homogenizing effect of concerted evolution (Álvarez and Wendell, 2003).

It is also worth noting that the ITS variability was not homogeneous through the entire ingroup. Whereas it provided sufficient variation for identification in widely distributed species and some (presumably older clades mainly from Africa and Cape Verde) (see Chapter 3), it lacked resolving power in the most recent species groups.

However, the general lack of resolution in most phylogenies of Canarian groups undertaken with ITS calls for the exploration of additional regions from the nuclear, especially on groups of recent origin. The ETS and the 5S-NTS regions are less used in phylogenetic studies

than the ITS region, but they show higher or similar rates of evolution in some groups where these two regions have been explored (Baldwin and Markos, 1998; Kårehed et al., 2008), and are perhaps good candidates for faster evolving regions within the nuclear genome.

Table 5.1 Macaronesian *Lotus* species considered under different levels of threat. According to Red List of Spanish Vascular Flora based on the IUCN Red Data Book (IUCN) (VV. AA., 2000), the Atlas of Endangered Spanish Vascular Flora (AESVF) (Bañares et al., 2004), and the ranking according to the Top 100 endangered species of Macaronesia (Martin et al., 2008). Numbers indicate their rank under the Top 100 list, -= not considered within the 100 most endangered species. CR= critically endangered, EN= endangered, VU= vulnerable.

Species	Distribution	IUCN 2000	AESVF 2004	Top 100 in Macaronesia
L. arinagensis	Canary Islands	CR	CR	-
L. berthelotii	Canary Islands	CR	CR	7
L. callis-viridis	Canary Islands	EN	EN	-
L. dumetorum	Canary Islands	VU	-	-
L. eremiticus	Canary Islands	CR	CR	25
L. genistoides	Canary Islands	-	CR	-
L. kunkelii	Canary Islands	CR	CR	6
L. maculatus	Canary Islands	CR	CR	3
L. mascaensis	Canary Islands	VU	-	-
L. pyranthus	Canary Islands	CR	CR	-
L. spartioides	Canary Islands	-	VU	-

Table 5.2 Species from the sections *Pedrosia* and *Rhyncholotus* sampled in this analysis. Distribution: G= La Gomera, P=La Palma, T=Tenerife, GC= Gran Canaria, CV= Cape Verde, M= Madeira, H= Hierro, L= Lanzarote, F= Fuerteventura. UBC= University of British Columbia, LBCVC= Jardín Botánico Canario Viera y Clavijo, JAO= Jardín de Aclimatación de la Orotava.

Taxon	Collection information	Voucher, herbarium
Outgroup		
Lotus japonicus MG20 (Regel) K. Larsen	Cultivated from seeds at UBC	*Ojeda 69*/UBC
Lotus japonicus Gifu B-129 (Regel) K. Larsen	Cultivated from seeds at UBC	*Ojeda 70*/UBC
Lotus filicaulis Durieu	Cultivated from seeds at UBC	*Ojeda 71*/UBC
Lotus corniculatus L.	Vancouver, BC	*Ojeda 46*/UBC
Lotus burttii Borsos	Cultivated from seeds at UBC from Univ. Miyazaki	*Ojeda 72*/UBC
Ingroup		
***Lotus* section *Pedrosia* (Lowe) Christ.**		
Lotus arborescens Lowe ex Cout.	Cultivated JBCVC # 164/06 Sao Nicolao CV	*Ojeda 180*/ UBC
Lotus arenarius Brot.	Cultivated UBC # PI 631779, Casablanca, Morocco Cultivated UBC # PI 631956, Kourigba, Morocco	- *Ojeda 78*/UBC
Lotus arinagensis Brawm.	DNA bank # 651 Barranco Viejo, Arinaga, GC DNA bank # 652 Ctra faro, Arinaga, GC	José Cruz & Alicia Roca Felicia Oliva & José Naranjo
Lotus argyrodes R.P Murray	Cult. JCVC # 5435/UDH/07 Punta de Pargo, M Voucher, Punta San Lorenzo, M	*Ojeda 189*/UBC ORT # 37806
Lotus assakensis Brand	Voucher, Tarfaya-Tan Tan Sahara, Africa DNA Bank # 1084 Tiznir, Aglou Plage, Morocco	Molero 1992 (Fernández Casas 13699) -
Lotus azoricus P. W. Ball	Cultivated JAO # 161-00, Azores	ORT # 36336
Lotus bollei	Cultivated JBCVC # 163/06 Sao Vicente, CV	*Ojeda 182*/UBC
Lotus brunneri Webb in Hooker	Cultivated JBCVC # 514B/07 Sal, CV	*Ojeda 181*/UBC
Lotus callis-viridis Bramwell & D.H. Davis	DNA Bank # 654 Andén Verde, GC Andén Verde, GC	Alicia Roca & Bernardo Navarro *Ojeda 169*/UBC
Lotus campylocladus Webb & Berthel.	Road to Cañada Teide, T Arona-Ifonche, T	*Ojeda 206*/UBC *Ojeda 210*/UBC
Lotus creticus L.	Cultivated JBCVC # 64/05 Cultivated UBC, PI 505409, Spain	*Ojeda 188*/UBC *Ojeda 242*/UBC
Lotus dumetorum Webb ex R. P. Murray	Mirador Jardina, Mercedes, Anaga, T Teno Alto, Teno, T	*Ojeda 213*/UBC *Ojeda 228*/UBC
Lotus emeroides R. P. Murray	Inchereda, G Epina, G	*Ojeda 207*/UBC *Ojeda 209*/UBC
Lotus eriosolen (Maire) Mader & Podlech	Cultivated UBC # PI 631959, Ouarzate, Morocco Cultivated UBC # PI 631784, Tiznir, Morocco	*Ojeda 244*/UBC *Ojeda 243*/UBC
L. erythrorhyzus Bolle	Fuerteventura	A. Santos
L. genistoides (nom. nudum)	DNA Bank # 655 Cañadón Sombrío, GC	Felicia Oliva & José Naranjo/JBCVC
Lotus glaucus Sol.	Cult. JBCVC # 223/B/07 Porto Nurbita (??), M Cult. JAO 19-05	*Ojeda 187*/UBC *Ojeda 233*/UBC
Lotus hillebrandii Christ	DNA Bank # 656 Llanos Chozas, P Mirador Isora, H	José Naranjo & Paloma Maya*Ojeda 198*/UBC
Lotus holosericeus Webb & Berthel.	DNA Bank # 657 Pilancones, GC	F. Oliva, J. Naranjo, J. Navarro, I. Santana & B. Vilches/JBCVC
Lotus jacobaeus L.	DNA Bank # 658 Bordeira bei Piorno Fogo CV DNA Bank # 2089 Ribeira Monte espia Fogo CV	Marrero et al/JBCVC Marrero et al/JBCVC
Lotus jolyi Battand.	Voucher, Province Tan Tan, Morocco Voucher, Province Guelmin, Morocco	*S.L. Jury & T.M. Upson 20503*/RNG *S.L. Jury & T.M. Upson 20480*/RNG
Lotus kunkelii (Esteve) Bramwell & D. H. Davis	DNA Bank # 3805, Barranco Jinamar, GC Cult. JBCVC # 217/07	José Cruz & Miguel Alemán *Ojeda 176*/UBC
Lotus lancerottensis Webb & Berth.	DNA Bank # 3823 Villaverde, Betancuira, F Voucher, L	- ORT # 36458
Lotus latifolius Brand	DNA bank # 1812 Crtra. Porto Novo, CV	Marrero et al/JBCVC
Lotus leptophyllus (Lowe) K. Larsen	Barranco Guayedra, GC GC	*Ojeda 170*/UBC A. Santos
Lotus macranthus Lowe	Voucher, Pico Branco, Porto Santo, M Voucher, M	ORT # 33596 ORT # 36675
Lotus maroccanus Ball	Voucher, Talouine, Morocco Voucher, Marrakech, Morocco	*S.L. Jury 14471*/RNG *Fernández Casas 13737*/RNG
Lotus mascaensis Burchard	DNA Bank # 659, Cultivated JBCVC Valle de Masca, T	José Cruz & Ruth Jaén/JBCVC *Ojeda 200*/UBC
Lotus pseudocreticus Maire, Weiller & Wilczek	Voucher, SW Agadir Morocco Voucher, Tamri Agadir, Morocco	*F. Damblon 84/40*/RNG *Davies 53484*/RNG
Lotus purpureus Webb	Cult. JAO # 130-99	ORT # 36670
Lotus salvagensis R.P. Murray	Voucher, Salvage Grande, SGVoucher, Acantilados del NE, Salvage Islands	ORT # 35118 ORT # 35116

Taxon	Collection information	Voucher, herbarium
Lotus sessilifolius D.C. subsp. *villossisimus* (Pitard) Sandral & Sokoloff	Las Playas, S from Parador, H	*Ojeda 196*/UBC
Lotus sessilifolius D.C. subsp. *sessilifolius*	Poris de Abona, T Puntallana, G Playa Pocito, Mazo, P	*Ojeda 225*/UBC *Ojeda 208*/UBC A. Santos
Lotus sessilifolius DC. var. *pentaphyllus* (Link) D. H. Davis	San Juan-Guía de Isora, T	*Ojeda 205*/UBC
Lotus spartioides Webb & Berthel.	Tamadaba, GC Pinar Pajonales, GC DNA bank # 662 Chira-Pinar Santiago, GC Presa las Niñas, GC	*Ojeda 217*/UBC *Ojeda 216*/UBC F. Oliva, J. Naranjo, J. Navarro, I. Santana & B. Vilches/JBCVC *Ojeda 211*/UBC
Lotus tenellus (R. Lowe) Sandral, Santos & D.D. Sokoloff	Garachico, Ermita San Roque, T	*Ojeda 446*/UBC
Lotus sp. nov. ined. 1	Punta Hidalgo, T South Roque Dos hermanos, Anaga, T Teno Alto, T	- *Ojeda 193*/UBC *Ojeda 194*/UBC
Lotus sp. nov. ined. 2 (*L. leptophyllus* group)	Punta Góngora, GC	*Ojeda 167*/UBC
Lotus sp. nov. ined. 3 (*L. spartioides* group) *Lotus* sp. nov. ined. 4 (*L. sessilifolius* group)	Cortijo de San Ignacio, GC Punta Teno, Teno, T	*Ojeda 203*/UBC *Ojeda 230*/UBC
***Lotus* section *Rhyncholotus* (Monod) D.D. Sokoloff**		
L. berthelotii Masf. var. *berthelotii*	Ifonche, T Cultivated UBC, commercial plant	- *Ojeda 238*/UBC
Lotus eremiticus A. Santos	DNA Bank # 3839, cult. JBCVC (366/04), Garafia, P	Jose Cruz
L. maculatus Breitf.	DNA Bank # 660, ex horto (8/04) Puertito Sauzal, T Cult. UBC, commercial plant	Rafael Almeida/JBCVC *Ojeda 239*
Lotus pyranthus P. Perez	DNA Bank # 661 JBCVC 210/99 DNA Bank # 3842, Cult. Vivero Ceplam	*Ojeda 175*/UBC -

Table 5.3 Sections *Pedrosia* and *Rhyncholotus* and their informal classification based on morphological features. *According to Sandral et al. (2006). ¶Species not sampled in this analysis.

Section	Informal taxonomic groups below section level	Species	Distribution
Pedrosia	*Lotus purpureus* group	*L. arborescens* *L. bollei* *L. brunneri* *L. jacobaeus* *L. purpureus* *L. latifolius*	Cape Verde
	Lotus arenarius group*	*L. arenarius* *L. maroccanus* *L. eriosolen*	Africa, Spain
	Lotus sessilifolius group*	*L. sessilifolius* *L. mascäensis* *L. arinagensis* *L. emeroides* *L. kunkelii*	Canary Islands
	Lotus argyrodes group*	*L. argyrodes* *L. macranthus* *L. azoricus* *L. loweanus*¶	Azores, Madeira
	Lotus campylocladus group*	*L. callis-viridis* *L. campylocladus* *L. genistoides* *L. holosericeus* *L. hillebrandii* *L. spartioides* *L. dumetorum*	Canary Islands
	Lotus glaucus group*	*L. glaucus* *L. tenellus* *L. leptophyllus* *L. salvagensis* *L. lancerottensis* *L. erythrorhyzus*	Canary Islands, Salvage Islands and Madeira
	Lotus assakensis group*	*L. assakensis* *L. creticus* *L. pseudocreticus* *L. chazalei*¶	Africa, Mediterranean
	Lotus jolyi group*	*L. jolyi* *L. tibesticus*¶	Africa
Rhyncholotus	*Rhyncholotus* group*	*L. berthelotii* *L. eremiticus* *L. maculatus* *L. pyranthus*	Canary Islands

Table 5.4 Performance of the five plastid regions and the nuclear ribosomal ITS tested separate and in two-pair combinations. A= including all accessions, B= excluding accessions with missing sequences in two-pair combinations. * Informal sections according to Sandral et al. (2006)

	Aligned sequence (bp)	**No. of species discriminated/ endangered**		**No. of informal taxonomic groups***	
One region					
ITS	621	10/0		3	
trnH-psbA	342	7/1		4	
matK	867	7/1		4	
rpoC1	511	5/1		0	
rbcL	588	2/0		0	
rpoB	354	0/0		0	
Plastid combinations		**A**	**B**	**A**	**B**
matK + trnH-psbA	1209	11/2	13/2	4	4
matK + rpoC1	1378	10/2	10/3	4	3
rpoC1 + trnH-psbA	853	10/1	9/0	3	3
rbcL + trnH-psbA	930	7/1	9/1	3	3
matK + rbcL	1455	7/1	7/1	3	2
matK + rpoB	1221	6/1	6/0	3	2
rpoB + trnH-psbA	696	5/0	6/0	4	4
rbcL + rpoC1	1099	3/0	3/0	0	0
rpoB + rpoC1	865	5/1	5/1	0	0
rbcL + rpoB	942	4/0	3/0	1	0
All plastid combined	**2662**	**9/2**	**14/3**	**4**	**4**
ITS + plastid					
ITS + *trnH-psbA*	963	15/3	14/3	4	4
ITS + *rpoC1*	1132	12/1	11/1	3	3
ITS + *matK*	1468	11/1	7/0	4	4
ITS + *rpoB*	975	11/1	11/1	3	3
ITS + *rbcL*	1209	10/0	9/0	3	3
All six regions combined	**3283**	**20/4**	**17/3**	**4**	**4**

Table 5.5 Nuclear and plastid gene regions tested in this analysis with their specific primers and performance.

Region	Primer pair	PCR success	Sequencing success	No. indels
trnH-psbA	Fw PA Rev TH	96	98	2
matK	matK2.1F matK3.2X	83	85	0
rpoC1	rpoC1F rpoC14R	96	100	0
rbcL	80F ajf634R1	97	100	0
rpoB	rpoB2F rpoB3R	97	100	0
ITS	ITS4 ITS5	100	99	2

Figure 5.1 NJ tree generated with the combination of the CBOL recommended two-locus, *matK + rbcL*. Gray squares represent species with more than one sample and species in a square represent species with a single accession. Branches with a black square represent informal taxonomic groups identified. Species in bold belong to section *Rhyncholotus* while species not in bold are included within section *Pedrosia.* *Endangered species identified.

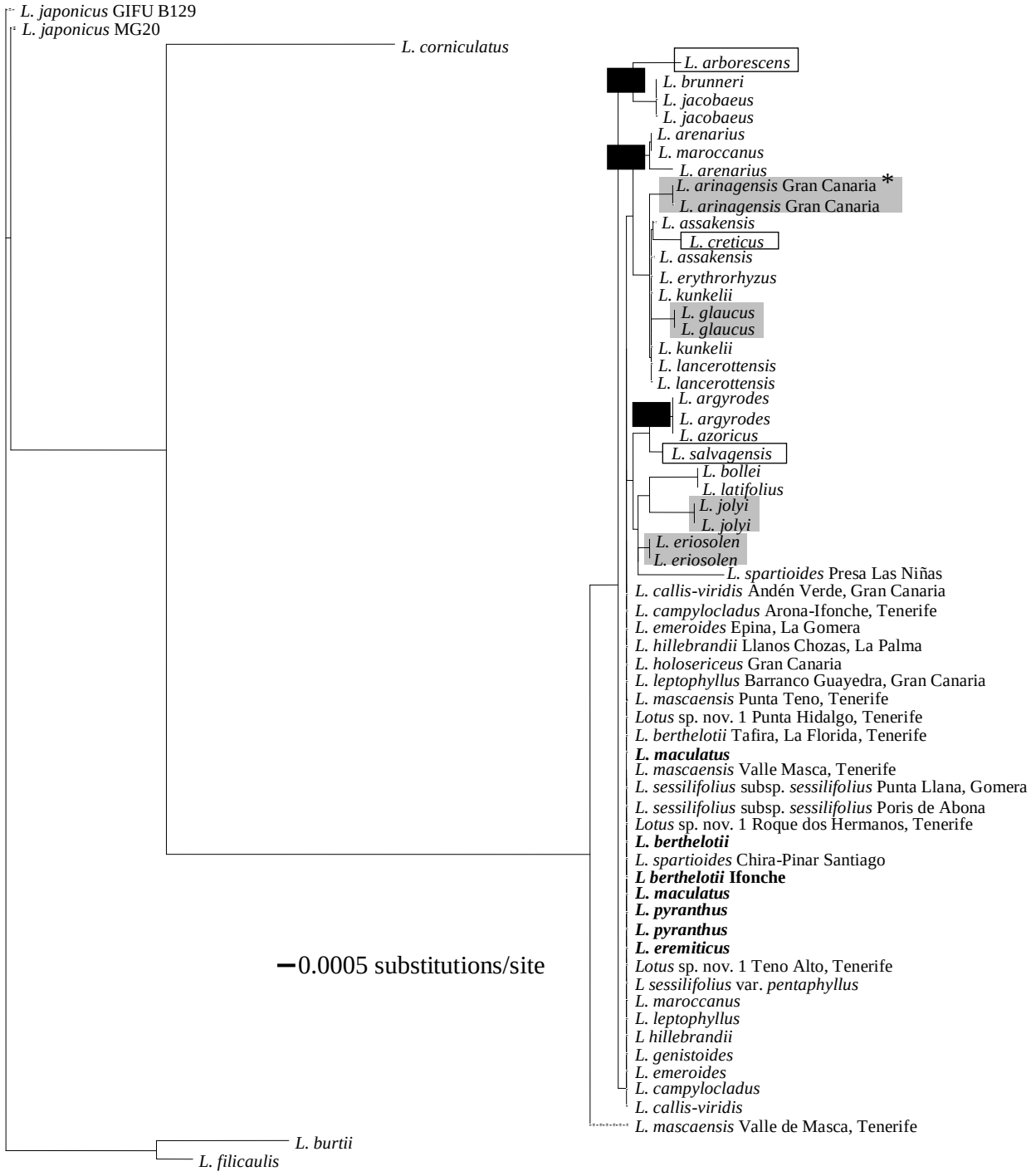

L. japonicus GIFU B129
L. japonicus MG20
L. corniculatus
L. arborescens
L. brunneri
L. jacobaeus
L. jacobaeus
L. arenarius
L. maroccanus
L. arenarius
L. arinagensis Gran Canaria *
L. arinagensis Gran Canaria
L. assakensis
L. creticus
L. assakensis
L. erythrorhyzus
L. kunkelii
L. glaucus
L. glaucus
L. kunkelii
L. lancerottensis
L. lancerottensis
L. argyrodes
L. argyrodes
L. azoricus
L. salvagensis
L. bollei
L. latifolius
L. jolyi
L. jolyi
L. eriosolen
L. eriosolen
L. spartioides Presa Las Niñas
L. callis-viridis Andén Verde, Gran Canaria
L. campylocladus Arona-Ifonche, Tenerife
L. emeroides Epina, La Gomera
L. hillebrandii Llanos Chozas, La Palma
L. holosericeus Gran Canaria
L. leptophyllus Barranco Guayedra, Gran Canaria
L. mascaensis Punta Teno, Tenerife
Lotus sp. nov. 1 Punta Hidalgo, Tenerife
L. berthelotii Tafira, La Florida, Tenerife
L. maculatus
L. mascaensis Valle Masca, Tenerife
L. sessilifolius subsp. sessilifolius Punta Llana, Gomera
L. sessilifolius subsp. sessilifolius Poris de Abona
Lotus sp. nov. 1 Roque dos Hermanos, Tenerife
L. berthelotii
L. spartioides Chira-Pinar Santiago
L berthelotii Ifonche
L. maculatus
L. pyranthus
L. pyranthus
L. eremiticus
Lotus sp. nov. 1 Teno Alto, Tenerife
L sessilifolius var. pentaphyllus
L. maroccanus
L. leptophyllus
L hillebrandii
L. genistoides
L. emeroides
L. campylocladus
L. callis-viridis
L. mascaensis Valle de Masca, Tenerife
0.0005 substitutions/site
L. burtii
L. filicaulis

Figure 5.2 NJ tree generated with the combination of all five plastid regions. Gray squares represent species with more than one sample and species in a square with a single accession. Branches with a black square represent informal taxonomic groups identified. Species in bold belong to section *Rhyncholotus* while species not in bold are included within section *Pedrosia.* *Endangered species identified.

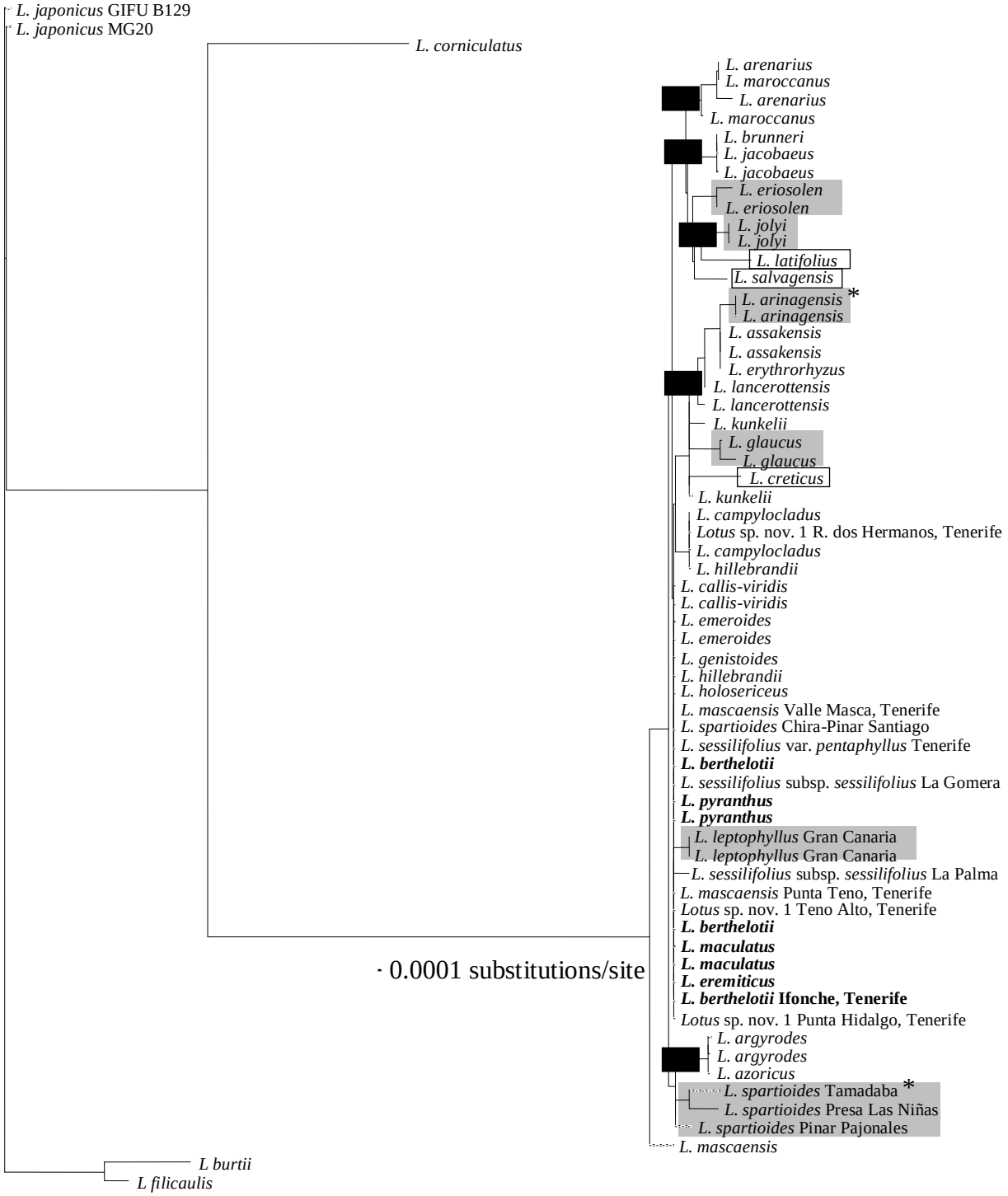
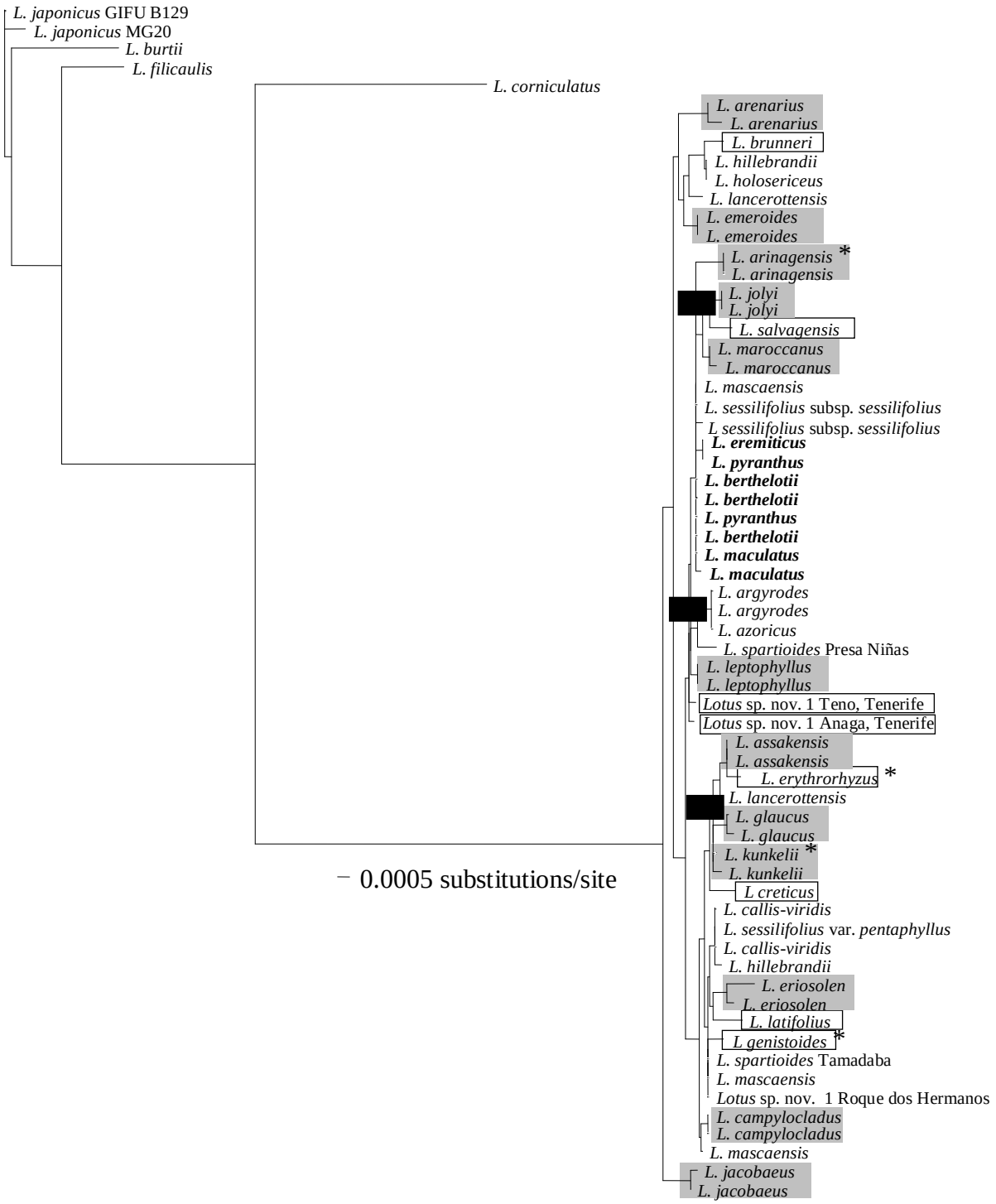

L. japonicus GIFU B129
L. japonicus MG20
L. corniculatus
L. arenarius
L. maroccanus
L. arenarius
L. maroccanus
L. brunneri
L. jacobaeus
L. jacobaeus
L. eriosolen
L. eriosolen
L. jolyi
L. jolyi
L. latifolius
L. salvagensis
L. arinagensis *
L. arinagensis
L. assakensis
L. assakensis
L. erythrorhyzus
L. lancerottensis
L. lancerottensis
L. kunkelii
L. glaucus
L. glaucus
L. creticus
L. kunkelii
L. campylocladus
Lotus sp. nov. 1 R. dos Hermanos, Tenerife
L. campylocladus
L. hillebrandii
L. callis-viridis
L. callis-viridis
L. emeroides
L. emeroides
L. genistoides
L. hillebrandii
L. holosericeus
L. mascaensis Valle Masca, Tenerife
L. spartioides Chira-Pinar Santiago
L. sessilifolius var. pentaphyllus Tenerife
L. berthelotii
L. sessilifolius subsp. sessilifolius La Gomera
L. pyranthus
L. pyranthus
L. leptophyllus Gran Canaria
L. leptophyllus Gran Canaria
L. sessilifolius subsp. sessilifolius La Palma
L. mascaensis Punta Teno, Tenerife
Lotus sp. nov. 1 Teno Alto, Tenerife
L. berthelotii
L. maculatus
L. maculatus
L. eremiticus
L. berthelotii Ifonche, Tenerife
Lotus sp. nov. 1 Punta Hidalgo, Tenerife
L. argyrodes
L. argyrodes
L. azoricus
L. spartioides Tamadaba *
L. spartioides Presa Las Niñas
L. spartioides Pinar Pajonales
L. mascaensis
· 0.0001 substitutions/site
L burtii
L filicaulis

Figure 5.3 NJ tree generated with the combination of all six regions tested (*rbcL, matK, trnH-psbA, rpoC1, rpoB* and ITS). Gray squares represent species with more than one sample and species in a square represent species with a single accession. Branches with a black square represent informal taxonomic groups identified. Species in bold belong to section *Rhyncholotus* while species not in bold are included within section *Pedrosia.*

***Endangered species identified.**


L. japonicus GIFU B129
L. japonicus MG20
L. burtii
L. filicaulis
L. corniculatus
L. arenarius
L. arenarius
L. brunneri
L. hillebrandii
L. holosericeus
L. lancerottensis
L. emeroides
L. emeroides
L. arinagensis *
L. arinagensis
L. jolyi
L. jolyi
L. salvagensis
L. maroccanus
L. maroccanus
L. mascaensis
L. sessilifolius subsp. sessilifolius
L sessilifolius subsp. sessilifolius
L. eremiticus
L. pyranthus
L. berthelotii
L. berthelotii
L. pyranthus
L. berthelotii
L. maculatus
L. maculatus
L. argyrodes
L. argyrodes
L. azoricus
L. spartioides Presa Niñas
L. leptophyllus
L. leptophyllus
Lotus sp. nov. 1 Teno, Tenerife
Lotus sp. nov. 1 Anaga, Tenerife
L. assakensis
L. assakensis
L. erythrorhyzus *
L. lancerottensis
L. glaucus
L. glaucus
L. kunkelii *
L. kunkelii
L creticus
L. callis-viridis
L. sessilifolius var. pentaphyllus
L. callis-viridis
L. hillebrandii
L. eriosolen
L. eriosolen
L. latifolius
L genistoides *
L. spartioides Tamadaba
L. mascaensis
Lotus sp. nov. 1 Roque dos Hermanos
L. campylocladus
L. campylocladus
L. mascaensis
L. jacobaeus
L. jacobaeus
0.0005 substitutions/site

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6 Temporal, but not spatial, changes in expression patterns of floral identity genes are associated with the evolution of bird pollination in *Lotus* (Leguminosae)[1]

6.1 Introduction

The understanding of the genetic and molecular basis of floral symmetry and petal identity has increased in recent years using model species (Feng et al., 2006b; Luo et al., 1996; Wang et al., 2008) and this knowledge is now being extended to non-model species. In particular *CYCLOIDEA*, a transcription factor that belongs to the Class II TCP family, is known to have a major role in petal identity in the Leguminosae. In legumes this gene family has four members at least two of these genes play a role in the determination of petal identity (Citerne et al., 2003; Feng et al., 2006b; Wang et al., 2008). *Lotus japonicus CYCLOIDEA2* (*LjCYC2*) specifies dorsal petal identity and *LjCYC3* acts to specify the identity of lateral petals. Each one of these genes is associated with a particular epidermal micromorphology, the former with papillose conical cells (PCS) and the latter with tabular rugose cells (TRS) (Chapter 4)

The tribe Loteae has been identified as one of the groups with a highly differentiated distribution of epidermal types, in which a particular epidermal type is highly associated with a specific petal identity (Ojeda et al., 2009).

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[1]A version of this chapter will be submitted for publication: Ojeda, I. Santos-Guerra, A., Oliva-Tejera, F. Jaen Molina, R. Caujape, J. Marrero, A. and Cronk. Q. C. B. Temporal, but not spatial, changes in expression patterns of floral identity genes are associated with the evolution of bird pollination in *Lotus* (Leguminosae).

Homologues of *CYCLOIDEA* have formerly been associated with shifts in floral symmetry and pollination syndrome (Citerne et al., 2006). Shifts of pollinators require the modification of several floral features associated with the attraction and reward of legitimate pollinators, or exclusion of illegitimate pollinators.

Evolutionary change in pollination syndrome involves morphological aspects of the flower (such as flower colour, flower size, length of nectar spurs, scent production, and nectar volume and composition). In a few cases, the specific genes involved in the transitions have been identified, such as *Anthocyanin 2* (*AN2*) and *flavonoid-3'-hydroxylase* (*F3'H*), or major quantitative trait loci (QTL) (Bradshaw et al., 1998; Bradshaw and Schemske, 2003; Cronk and Ojeda, 2008; Galliot et al., 2006a; Hoballah et al., 2007; Quattrochio et al., 1999; Stuurman et al., 2004; Whittall and Hodges, 2007; Zufall and Rausher, 2004).

Here I study a transition in flower morphology from melittophilous ancestors to ornithophily (specifically pollination by opportunistic passerine birds) that evolved in a group of four species within the genus *Lotus* section *Rhyncholotus* (Fig. 6.1A-D) in the islands of Tenerife and La Palma in the Canary Island archipelago. There are no modifications in flower symmetry during the evolutionary transition between these two pollination syndromes (both groups have zygomorphic flowers). The major differences between the four ornithophilous compared with the melittophilous species are the size and shape of the petals and their orientation within the flower (Fig. 6.1E-H, and Fig. 6.2D), differences in flower colour and petal micromorphology. These modifications suggest changes in relative growth rates and in the role of individual petal types on pollination attraction.

The pollination mechanism in the melittophilous species is identical to that described for *L. corniculatus* (Proctor et al., 1996). Pollination takes place when the insect lands and depresses the wings and the keel, forcing out a string of pollen from the stamens located within the keel and

placing it in the underside of the visitor (Proctor et al., 1996). Bees need a landing platform and the horizontal orientation of the flowers allows the lateral petals to play this role (Fig. 6.2A, F).

On the other hand, in the ornithophilous species the pollinator seeks nectar located at the base of the calyx and the pollen is deposited either on the top of the head or in the throat when the dorsal petal is pressed down (Fig. 6.2C, E) (Olesen, 1985). In this case, the bird needs no landing platform (as it usually forages from the ground) and a flower in vertical orientation is better suited for nectar storage and pollen placement (Fig. 6.2E). To date, only the Canarian chiffchaff (*Phylloscopus canariensis*) and the blue tit (*Parus caeruleus*), have been identified foraging in two of these four species (Ollerton et al., 2008; Stelzer, 2005).

The petal modifications in these four bird-pollinated species seem remarkable within the tribe Loteae, a group that comprises about 275 species that predominantly have a bee-pollination syndrome and a fairly uniform flower morphology. To my knowledge, this is the only confirmed case of bird pollination within this tribe. There is another report of a Costa hummingbird visiting *Hosackia rigida*, but the flower morphology of this species is not ornithophilous and these birds visit this species only in the absence of the other bird-pollinated flowers (Grant and Grant, 1968). Phylogenetic analyses in this group (Allan et al., 2004; Sandral et al., 2006) and geological evidence from La Palma and Tenerife (Carracedo et al., 2002) suggest that this pollination syndrome probably evolved recently (see Chapter 4).

5.1.1 Objectives of the study

The objectives of this chapter are: 1) to determine the distribution of epidermal types on petals of the sections *Pedrosia* and the rhyncholotus group and its relation to modification in pollinator syndromes, and 2) to analyze the association of changes in the expression of petal identity genes with alterations in petal morphology that are related to shifts in pollination.

6.2 Material and methods

6.2.1 Plant material and growth conditions

The following five species were used in the gene expression analysis: *Lotus japonicus* ecotype Gifu B129, *L. filicaulis* (bee-pollinated), *and L. sessilifolius* (bee-pollinated but closely related to the bird-pollinated species), *L. maculatus* and *L. berthelotii* (bird-pollinated). The plants were grown in the nursery in pots of 10-20 cm in diam. at 20-25 °C and were more than 6 weeks old when flowers were collected for analyses. *Lotus berthelotii* and *L. maculatus* were purchased from commercial nurseries. They were grown under the same environmental conditions as the rest of the species, except during the vernalization period of 30 days in which the temperature was reduced (1 -10°C). After this period, *L. berthelotii* and *L. maculatus* started to bloom from May-August. *Lotus sessilifolius* was propagated from seeds collected from the field in Tenerife.

For the petal micromorphology survey I analyzed a total of seven genera and 56 species within the tribe Loteae. Within *Lotus*, I analyzed representative species of 9 (out of 14) sections currently recognized within this group (Degtjareva et al., 2006). This analysis included all four species with a bird pollination syndrome and 32 (out of 36) of the currently recognized species within section *Pedrosia* with a bee pollination syndrome (Table 6.1) (Sandral et al., 2006).

6.2.2 Flower developmental stages and petal growth

Early flower development in *L. japonicus* has previously been divided into seven stages from stage 0 when floral primordia are initiated to stage 7 when the primordia of all five petals are initiated but elongation has not started (Dong et al., 2005). At this stage the veins and the characteristic epidermal type of each petal type has not differentiated (Dong et al., 2005; Feng et al., 2006b; Zhang et al., 2003). For this study I extended the classification of *L. japonicus* flower development into a further six stages, 8-13 (Fig. 6.1). I used morphological landmarks and size

relationships of the petals to characterize each stage. Further, analogous stages were established on the *Pedrosia* and the rhyncholotus group analyzed following the same landmarks.

At stage 8 no petal is visible as they are covered by the sepals. Differentiation of veins and of the characteristic epidermal type of each petal starts at this stage. At stage 9 only the dorsal petal is visible; it is still folded and completely covers the lateral and ventral petals. The dorsal petal is as long as the sepals. Veins of each petal are evident at this stage. At stage 10 the dorsal petal is still folded and covers the lateral and ventral petals; however, its size has increased and now the exposed part is as long as the sepals. At stage 11 the dorsal petal is twice the size of the sepals. It is still folded over the lateral and ventral petals, but both the lateral and ventral petals are visible. At stage 12 the three types of petals are completely exposed but their final position in the mature flower is not yet reached. At stage 13 all petals are fully developed and the final disposition of each petal in the flower is established.

Petal growth was measured as length and width of each petal during the 7-13 developmental stages.

6.2.3 Scanning electron microscopy (SEM) and light microscopy

Full open mature flowers at anthesis of each species were analyzed using a Hitachi S-2600N scanning electron microscopy (SEM) at 10 to 12 Kv of acceleration voltage or from fresh flowers preserved on ethanol 70% and analyzed with a light microcope. Some species were analyzed from herbarium specimens, re-hydrated and preserved in ethanol 70%. The distribution of the cell types was analyzed on each type of petal (dorsal, lateral and ventral) in both adaxial and abaxial sides. The epidermal types were classified following a previous study within Leguminosae (Ojeda et al., 2009).

6.2.4 RNA extraction and RT-PCR

Expression of *CYCLOIDEA* homologues was studied in five species: two from section *Lotus*, one from the section *Pedrosia* and two from the section *Rhyncholotus*. The expression patterns have been previously reported in *L. japonicus*, but only in early flower development (stage 0-7) and in mature flowers (Feng et al., 2006b). *L. filicaulis* is a closely related species of *L. japonicus* and these two species were included as a comparison of previous expression patterns reported in the model legumes *L. japonicus*. I selected *L. sessilifolius* from the section *Pedrosia* as it has been identified as one of the closely related species to the bird-pollinated species (see Chapter 4). *L. sessilifolius* has a typical melittophilous flower morphology and flower size and petal arrangement similar to *L. japonicus* (Fig. 6.2H). Finally, two species with an ornithophilous flower morphology, *L. berthelotii* and *L. maculatus*, were included from the section *Rhyncholotus* (Fig. 6.2A, C).

Tissue from each petal type and from three different developmental stages (8, 10 and 13) of each species were extracted using the Pure Link^{TM} Plant RNA Reagent form Invitrogen following manufacturer's protocol. RNA was treated with DNAse and it was visualized on an agarose gel (2%) and its quantity was measured using a Nanodrop. The RNA was converted to cDNA using the RevertAid ^{TM} H Minus First Strand cDNA Synthesis Kit from Fermentas according to manufacturer's protocol. Genomic contamination was assessed using the intron in *LjCYC2* with SL1716/SL1717 (Feng et al., 2006b). Endogenous expression of *Lotus japonicus Ubiquitin* (*LjUbi*) was examined with *LjUbif/R* as a control of cDNA quantity across the samples (Feng et al., 2006b). Gene specific semi-quantitative reverse transcriptase (RT-PCR) was performed on two *CYCLOIDEA* homologues, *LjCYC2* using a new primer LC2.1F (5' TCCCTTTCAGCTCAAGCCCTTACCC 3') and LEGCYCR (5' TCCCTTGCTCTTGCTCTTGC 3'), and *LjCYC3* using LEGCYCF1 (5' TCAGGGSYTGAGGGACCG 3') and CYC3.1R (5'

CCTGCTTCCTTATTAGGGATTGC 3') using 95°C for 2 min, 30 cycles of 94°C 45 s, 50-57°C for 1 min and 72°C for 2 min with a final step of 72°C for 5 min. All primers for these *CYCLOIDEA* homologues were designed in this study, except LEGCYCF and LEGCYCR which have been used previously (Citerne et al., 2003; Citerne et al., 2006; Ree et al., 2004).

6.3 Results

6.3.1 Petal micromorphology of flowers at anthesis within Loteae and the lateralization of petals in section *Rhyncholotus* and *Pedrosia*

I recorded three major epidermal types within Loteae.Their distribution is mainly restricted to specific petals (Table 6.2). The dorsal petal is characterized by papillose conical cells with striations (PCS). The lateral petal is mainly characterized by tabular rugose cells with striations (TRS) or by a combination of TRS and PCS. In general TRS is located at the base of the petal and PCS, when present, is situated at the tip of the petal and usually on the exposed side (abaxial side) of the petal. Tabular flat cells with striations (TFS) characterize the ventral petal. No PCS were recorded on the ventral petal in any of the species analyzed in this study (Fig. 6.4A-F). The majority of the species analyzed in the Loteae and specifically in the section *Pedrosia* have this distribution of epidermal types (Table 6.2).

The four bird-pollinated species have in general an increase in area covered by TRS in the dorsal and ventral petals. The dorsal petal completely lacks papillose conical cells (PCS) and only a small section of the abaxial side of the lateral petal has PCS (Fig. 6.4G-M). The abaxial side of the dorsal petal has elongated non-differentiated cells (ND) which are characteristic of early developmental stages before differentiation. These four species also have trichomes on the abaxial side of the dorsal and lateral petals (Fig. 6.4G). This epidermal distribution suggests a lateralization (increased amount of TRS and absence of PCS) in the dorsal and ventral petals.

I also observed a lateralization of the abaxial side of the dorsal petal in species closely related to the bird-pollinated clade, but all these species still have PCS on the adaxial side in the dorsal petal. Six of these species also have trichomes but only on the abaxial side of the dorsal petal (Table 6.3).

6.3.2 Expression patterns of *CYCLOIDEA* homologues during flower development

All three *CYCLOIDEA* homologues analyzed are expressed asymmetrically within a dorsiventral axis in the flower (dorsal and lateral petals) in all five species studied. *LjCYC2* is expressed early during flower development in three species, *L. japonicus*, *L. filicaulis* and *L. sessilifolius*, with a similar flower morphology and similar epidermal types on each petal. The expression of this gene is still observed at late developmental stages but it tends to reduce as the flower completes its development. On the other hand, *LjCYC2* expression was not detected in early developmental stages of the two bird-pollinated species analyzed. The earliest expression of *LjCYC2* in these two species was observed at stage 10.

Expression of *LjCYC3* was not detected in the early stage of development in any of the species from section *Lotus* (*L. japonicus* and *L. filicaulis*). The earliest expression of this gene was not detected until stage 10 in *L. japonicus* and later on *L. filicaulis* (stage 13). Contrarily, the expression of this gene was detected early on the two bird-pollinated species and also in its closely relative from section *Pedrosia*, *L sessilifolius*. The expression continued more or less similar at late develomental stages in all three species (Fig. 6.5). No major changes on *LjCYC1* expression were observed between the bee and bird-pollinated species, and only minor discrepancies within each pollination type (Fig. 6.5).

6.4 Discussion

6.4.1 Lateralization of the dorsal petal in the bird-pollinated species and the adaptive value of papillose conical cells in melittophilous species

The evolutionary transition from melittophilous ancestors to ornithophilous species within Macaronesian *Lotus* required several floral modifications. Flower symmetry was not altered during the transition (both syndromes have zygomorphic flowers), but the size and the role of each petal in pollinator attraction and pollen placement is modified.

The SEM survey also indicates that the epidermal micromorphology in the four bird-pollinated species has been modified in comparison with the rest of the species within the tribe Loteae and also in comparison with its closely related species within *Pedrosia* (Fig. 6.4). These results indicate that the dorsal and ventral petals in the ornithophilous species have suffered a lateralization, an increase of the presence of TRS instead of the typical epidermal types that characterizes each petal (Feng et al., 2006b). This lateralization is particularly evident in the dorsal petal where papillose conical cells are absent. Most of the species from the tribe Loteae and the section *Pedrosia* analyzed in this study have a distribution of epidermal types reported before (Table 6.2). Papillose conical cells are mainly distributed in the dorsal petal and tabular flat cells with striations (TFS) are located exclusively on the ventral petal (Feng et al., 2006b; Ojeda et al., 2009).

These differences of epidermal types between the ornothophilous species and their closest melittophilous relatives within *Pedrosia*, suggest that the modification of epidermal types must have an adaptive value. Papillose conical cells have an important role in pollinator attraction by increasing the light that is reflected from the flower (Comba et al., 2000; Glover and Martin, 1998, 2002; Martin and Glover, 2007; Noda et al., 1994). Conical cells can also provide tactile cues for the pollinators (Kevan and Lane, 1985a) and it has been shown that this epidermal type

also provides an aids to the bee´s grip for flower handling, thus increasing foraging efficiency (Whitney et al., 2009a).

The above evidence together with the distribution of papillose conical cells in the adaxial side of the dorsal petal and the abaxial side at the tips of the lateral petals (Table 6.1) suggest that this epidermal type may have a dual role within the flower depending of its location, in pollinator attraction when located in the dorsal petal and in aiding petal handling and foraging efficiency when located on the abaxial side of the lateral petal (as the lateral petal in many papilionoids works as a landing platform for insects). Additionally, papillose cells tend to be mainly distributed on the exposed sides of the petals within the Leguminosae and have been reported on the ventral petal when this petal type has an exposed position within the flower (Ojeda et al., 2009).

In melittophilous species the dorsal petal has a major role in attraction, as it is the main area exposed when a pollinator approaches the flower (Fig. 6.3), while lateral petals serve as a landing platform (Proctor et al., 1996). On the other hand, due to its position in the flower and its orientation, the dorsal petal in the bird-pollinated does not have a major role on pollination attraction. It has a reduced size and it is bent backwards. As a consequence the dorsal petal is no longer the main exposed part of the flower functioning in pollinator attraction (Olesen, 1985). Its reflexed position within the flower also allows the insertion of the beak when a bird is foraging for nectar. Additionally, this petal type completely lacks PCS cells. The adaptive value of PCS in this side of the petal is reduced and this epidermal type has been replaced by TRS. Papillose conical cells were only observed in a reduced area of the lateral petal. This cell type is located in a more exposed location, perhaps to guide birds to the location of the nectar at the base of the flower (Fig. 6.4, arrows).

Loss of papillose conical cells has also been reported during a transition of pollination syndromes from insect to wind pollination in *Thalictrum*. In this group, conical cells are distributed in petaloid organs (either sepals or stamen) where these organs have a role in pollination attraction. Wind pollination is derived within this group and conical cells are absent on the sepals and stamen filaments (Di Stilio et al., 2009).

My analysis also suggests that this modification in petal micromorpholgy within *Pedrosia* s.l. is gradual. I found a slight lateralization of the dorsal petal within a group of four species (*L. sessilifolius, L. arinagensis, L. kunkelii* and *L. mascaensis*) refered to as the *L. sessilifolius* group (Sandral et al., 2006) (Table 6.3). This group belongs to same clade as the bird-pollinated species and is the most closely related group with a bee pollination syndrome. However, the lateralization is confined only to the abaxial side, the least exposed area side of the dorsal petal. This gradual evolutionary modification of epidermal types is also supported by the distribution of trichomes. Three of the above mentioned species from the *L. sessilifolius* group also have trichomes on the dorsal petal.

It is worth mentioning that this lateralization is not exlusive to this clade, as other species within the section *Pedrosia* also have a lateralization of the dorsal petal and trichomes on the abaxial side of the dorsal petal (Table 6.3).

6.4.2 Late expression of *LjCYC2* is associated with modification of epidermal types and the evolution of ornithophily

CYCLOIDEA is a transcription factor required for zygomorphy establishment in *Anthirrhinum majus* (Luo et al., 1996). Orthologues of this gene have been identified and isolated in other asterids (*Gerbera, Linaria*, Gesneriaceae, and *Senecio*) (Broholm et al., 2008; Cubas and Vincent, 1999; Kim et al., 2008a; Zhou et al., 2008) and in rosids (*Iberis, Lotus, Lupinus* and

Pisum) (Busch and Zachgo, 2007; Citerne et al., 2006; Feng et al., 2006b; Wang et al., 2008) where they play a role in determination of flower symmetry. Modifications of expression patterns in some of these orthologues have been associated with modification of flower symmetry (Broholm et al., 2008; Busch and Zachgo, 2007; Citerne et al., 2006; Zhou et al., 2008) and there is growing evidence that suggests that *CYCLOIDEA*-like genes can have several effects on flower development, depending of the timing of their expression.

Four homologues, *LjCYC1, 2, 3* and *5*, have been identified in the legume family (Citerne et al., 2003), and at least two of these genes, *LjCYC2* and *LjCYC3*, seem to play a major role in flower symmetry and petal identity in the legumes examined so far (Citerne et al., 2006; Cronk, 2006b; Feng et al., 2006b; Ree et al., 2004; Wang et al., 2008).

LjCYC2 is expressed very early during flower development in *L. japonicus* (before stage 7) and its expression specifies the dorsal-ventral symmetry of the flower. This gene is also required for dorsal petal identity and the development of papillose conical cells (PCS) in this petal. Antisense transgenic plants of *L. japonicus* lack PCS on the dorsal petal, while plants that overexpress this gene develop PCS on all three types of petals, thus dorsalizing the flower (Feng et al., 2006b). PCS are formed early during flower development and I observed early expression of this gene in all three species analyzed that have PCS on the dorsal petal (Fig. 6.5). In contrast, the two bird-pollinated species analyzed do not develop PCS on the dorsal petal and the absence of this epidermal type is associated with an absence of an early *LjCYC2* expression (Fig. 6.5).

Late expression of *CYCLOIDEA*-like genes seems to have additional effects ofnflower development. For example, a homologue of *CYCLOIDEA* is expressed late during flower development in *Iberis amara* and *Oreocharis benthamani* and this late expression represses growth on the adaxial side of the flower (dorsal petal) (Busch and Zachgo, 2007; Du and Wang, 2008). *CYCLOIDEA*-like genes can also repress or abort other organs within the flower when

expressed late during development. There is evidence that *OpdCYC1*, a *CYC*-like gene from *Opithandra ghushanensis* (Genneriaceae), is expressed early during development in all five stamen primordia, but late during development its expression is concentrated in the dorsal and ventral stamens that are reduced or aborted in this species (Song et al., 2009a).

Late expression of *LjCYC2* in the dorsal petal of the bird-pollinated species may also reduces petal growth. I observed that the growth of the dorsal petal in the ornithophilous species is reduced in comparison with the other two petals (Fig. 6.6A) and this contrasts with the growth pattern that I observed in the melittophilous species, which showed a similar rate of growth of all petals (Fig. 6.6B and C) with a tendency to have a reduction of *LjCYC2* expression in late developmental stages (Fig. 6.5). Therefore, late expression of *LjCYC2* in the bird-pollinated species may have two effects on flower development in this group; a lack of PCS differentiation, and a reduction of petal growth, both features that are flower modifications that ocurred during the evolutionary transition from mellitophily to ornitophily in this group.

6.4.3 Early expression of *LjCYC3* is associated with a lateralization of the flower and suggests a molecular pre-adaptation to bird pollination

An additional gene, *LjCYC3* specifies lateral petal identity and the formation of TRS on this petal. Functional analyses in *L. japonicus* have shown that an overexpression of *LjCYC3* increases the presence of TRS in all three types of petal and causes a lateralization, but its down regulation ventralizes the lateral petal, with an absence of TRS on this petal (Feng et al., 2006b). Here I found that *LjCYC3* expression is detected late during flower development in *L. japonicus* (Fig. 6.5), confirming previous studies that also indicate that this gene is not detected during early developmental stages (0-6) using in *situ hybridization* (Feng et al., 2006b). The early expression of this gene observed in both ornithophilous species and its closely related species *L. sessilifolius*

is associated with an increase of TRS on the dorsal petal, thus supporting the idea that all these three species have a lateralized dorsal petal (Fig. 6.5).

It has been suggested that *LjCYC2* and *LjCYC3* interact during flower development. Early expression of *LjCYC2*, combined with a lack of early expression of *LjCYC3* in *L. japonicus* are necessary for the differentiation of PCS on the dorsal and TRS on the lateral petal of this species (Da Luo, unpubl.). The modifications of the epidermal types and petal identities in the ornithophilous species seem to be explained by a combination of two heterochronic processes during the expression of *CYCLOIDEA* homologues. A delayed (or post-displacement) expression of *LjCYC2* combined with a precocious (pre-displacement) of *LjCYC3* expression during flower development. My analysis does not indicate a change of heterotopy, as reported in other flower modifications, such as that reported in *Cadia purpurea* (Citerne et al., 2006).

The distribution of epidermal types in *L sessilifolius* is also explained by these two mechanisms. This species has a slightly less lateralized dorsal petal. In constrast to the ornithophilous species, this species has an early expression of both genes, *LjCYC2* and *LjCYC3* and the lateralization of the flower is weaker in comparison with the ornithophilous species. Given that this species is the closest relative to the four bird-pollinated species (see Chapter 4), it is possible that this evolutionary change of epidermal types and flower morphology required different steps of heterochronic modifications of expression of these genes, first a precocious expression of the lateral identity gene *LjCYC3*, followed by a down regulation of the dorsal petal identity gene. Taken together my results indicate that the clade where the bird pollination syndrome evolved in *Pedrosia* may have been facilitated by a molecular pre-adaptation of these two *CYCLOIDEA* homologues.

Table 6.1 List of samples collected for the analysis of epidermal types in Loteae

Taxon	Collection information	Voucher/Herbarium
Acmispon		
Section *Anisolotus* (Benh.) D.D. Sokoloff		
A. americanus (Nutt.) Rydb.	Cult. UBC PI 215232	*Ojeda 240*/UBC
Anthyllis		
A. hermannieae L.	UBCBG, No. 035419-0389-2000	*Ojeda 138*/UBC
Coronilla		
C. valentina L.	UBCBG, without number	*Ojeda 33*/UBC
C. varia L.	UBC campus, Vancouver	*Ojeda 39*/UBC
Hosackia		
H. chihuahuana S. Watson	Cult. UBC PI 18085	*Ojeda 79*/UBC
Lotus		
Section *Bonjeana* (Rchb.) D.D. Sokoloff		
L. hirsutus L.	UBCBG, No. 032962-0447-1996	*Ojeda 58*
Section *Chamaelotus* Kramina and D.D. Sokoloff		
L. glinoides Del.	Cult. PI 246736	-
Section *Erythrolotus* Brand		
L. conimbrensis Brot.	Cult. PI 238334	-
Section *Heineckenia* Webb & Berth.		
L. arabicus L.	Cult. UBC PI 214109	*Ojeda 151*/UBC
L. gebelia Vent.	Cult. PI 464685	
Section *Krokeria* (Moench) Ser.		
L. edulis L.	Cult. UBC PI 244281	*Ojeda 152*/UBC
Section *Lotea* (Medik.) DC.		
L. halophilus Boiss. & Spruner	Cult. UBC PI 238336	*Ojeda 153*/UBC
L. weilleri Maire	Cult. UBC PI 631729	*Ojeda 154*/UBC
Section *Lotus*		
L. burttii Borsos	Cul. UBC seeds, Miyasaki University	*Ojeda 72*
L. corniculatus L.	UBC campus, Vancouver	*Ojeda 27*
L. filicaulis Durieu	Cult. UBC	*Ojeda 71*
L. japonicus Gifu B129	Cult. UBC	*Ojeda 70*
Section *Pedrosia* (Lowe) Christ		
L. arborescens Lowe ex Cout.	Cult. JCVC # 164/06	*Ojeda 180*/UBC
L. arenarius Brot.	Cult. UBC PI 631780	*Ojeda 78*/UBC
L. arinagensis Brawm.	Cult. JCVC # 49/04	*Ojeda 178*/UBC
L. argyrodes R.P Murray	Cult. JCVC # 5435/UDH/07	*Ojeda 189*/UBC
L. assakensis Brand	Voucher	*Jury & Upson 20510*/RNG
L. azoricus P. W. Ball	Voucher	ORT # 36336
*L. brunner*i Webb in Hooker	Cult. JCVC # 514B/07	*Ojeda 181*/UBC
L. callis-viridis Bramwell & D.H. Davis	Cult. JCVC # 145/04	*Ojeda 177*/UBC
L. campylocladus Webb & Berthel.	Carretera al Teide, T	*Ojeda 206*/UBC
L. creticus L.	Cult. JCVC # 64/07	*Ojeda 188*/UBC
L. dumetorum Webb ex R. P. Murray	Mirador Jardina, T Teno, T.	*Ojeda 213*/UBC *Ojeda 228*/UBC
L. emeroides R. P. Murray	Epina, G	*Ojeda 209*/UBC
L. eriosolen (Maire) Mader & Podlech	Cult.UBC # PI 631784, Tiznir, Morocco	
L. genistoides Webb & Berthel.	Cañon del Jierro, GC	*Ojeda 174*/UBC
L. glaucus Sol.	Cult. JCVC # 223/B/07	*Ojeda 187*/UBC
L. hillebrandii Christ	Cumbre nueva a Fuencaliente, P	*Ojeda 232*/UBC
L. holosericeus Webb & Berthel.	Cult. JCVC # 334/02	*Ojeda 201*/UBC
L. jacobaeus L.	Cult. JCVC 183/06	*Ojeda 179*/UBC
L. jolyi Battand.	Voucher	S.L. Jury & Upson 20503/RNG
L. kunkelii (Esteve) Bramwell & D. H. Davis	Cult. JCVC # 217/07	*Ojeda 176*/UBC

Taxon	Collection information	Voucher/Herbarium
L. lancerottensis Webb & Berthel.	Voucher	ORT # 37824
L. latifolius Brand	Cult. JCVC 159/06	*Ojeda 183*/UBC
L. leptophyllus (Lowe) K. Larsen	Puente de Silva, GC	*Ojeda 171*/UBC
L. macranthus Lowe	Voucher	ORT # 36675
L. maroccanus Ball	Voucher	*Jury 14471*/RNG
L. mascaënsis Burchard	Cult. JCVC # 442/99	*Ojeda 200*/UBC
L. pseudocreticus Maire, Weiller & Wilczek	Voucher	*Davies 53484*/RNG
L. purpureus Webb	Cult. JCVC # 167/06	*Ojeda 184*/UBC
L. salvagensis R.P. Murray	Voucher	ORT # 35128
L. sessilifolius D.C.	Guimar Poligono industrial, T	*Ojeda 224*/UBC
L. sessilifolius D.C.	H	
L. spartioides Webb & Berthel.	Tamadaba, GC	*Ojeda 217*/UBC
L. tenellus (R. Lowe) Sandral, Santos & D.D. Sokoloff	Playa de los Roques, T	*Ojeda 215*/UBC
Section *Rhyncholotus* (Monod) D.D. Sokoloff		
L. berthelotii Masf.	Cult. UBC LB-08 Cult. JCVC 235/07	*Ojeda 238*/UBC *Ojeda 185*/UBC
L. eremiticus A. Santos	Cult. JAO # 100/06	-
L. maculatus Breitf.	Cult UBC LM-03 Cult. JCVC # 187/07	*Ojeda 239*/UBC *Ojeda 186*/UBC
L. pyranthus P. Perez	Cult. JCVC # 210/99 Cult. JAO # 124-01	*Ojeda 175*/UBC *Ojeda 226*/UBC
Ornithopus		
O. compressus L.	Barranco Madera, P	*Ojeda 158*/UBC
Scopiurus		
S. sulcata L.	Valle de Masca, T	*Ojeda 160*/UBC

Table 6.2 Distribution of epidermal types on species analyzed. PCS= papillose conical cells, TRS= tabular rugose cells with striations, TFS= tabular flat cells. Epidermal types separated by a dash indicate that two epidermal types where observed on the same petal, s= stomata, t= trichomes

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial
Acmispon						
Section *Anisolotus*						
A. americanus	TRS[s]	**TRS**	**TRS**	TRS	**TFS**	TFS
Anthyllis						
A. hermannie	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
Coronilla						
C. valentine	PCS	**PCS**	**PCS**	PCS	**TRS/TFS**	TFS
C. varia	PCS	**PCS**	**PCS**	TRS	**TFS**	TFS
Hosackia						
H. chihuahuana	PCS	**PCS**	**PCS**	TRS	**TRS/TFS**	TFS
Lotus						
Section *Bonjeana*						
L. hirsutus	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
Section *Chamaelotus*						
L. glinoides	TRS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
Section *Erythrolotus*						
L. conimbrensis	PCS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
Section *Heineckenia*						
L. arabicus	TRS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
L. gebelia	PCS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
Section *Krokeria*						
L. edulis	TRS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
Section *Lotea*						
L. halophilus	TRS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
L. weilleri	PCS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
Section *Lotus*						
L. burttii	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. corniculatus	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. filicaulis	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
L. japonicus Gifu B129	PCS	**PCS**	**TRS**	TRS	**TFS**	TFS
Section *Pedrosia*						
L. arborescens	PCS	**PCS**	**PCS**	TRS	**TFS**	TFS
L. arenarius	PCS	**PCS**	**PCS**	TRS	**TFS**	TFS
L. arinagensis	TRS	**TRS**	**TRS**	TRS/PCS	**TFS**	TFS
L. argyrodes	TRS/PCS	**TRS**	**TRS/PCS**		**TFS**	TFS
L. azoricus	TRS/PCS	**PCS**	**TRS/PCS**	TRS/PCS	TFS	TFS
L. assakensis	PCS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
L. brunneri	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. callis-viridis	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. campylocladus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. creticus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. dumetorum	TRS	**PCS**	**TRS/PCS**	TRS	TFS	TFS
L. emeroides	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. eriosolen	TRS	**TRS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. glaucus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. genistoides	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. holosericeus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. hillebrandii	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. jacobaeus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS

Taxon	Dorsal petal		Lateral petal		Ventral petal	
	abaxial	**adaxial**	**abaxial**	**adaxial**	**abaxial**	**adaxial**
L. jolyi	TRS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. kunkelii	TRS^{t}	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. lancerottensis	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. latifolius	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. leptophyllus	TRS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. macranthus	TRS	**PCS**	**TRS/PCS**	TRS	**TFS**	TFS
L. maroccanus	TRS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. mascaënsis	TRS^{t}	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. pseudocreticus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. purpureus	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. salvagensis	PCS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. sessilifolius subsp. *sessilifolius*	TRS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. sessilifolius subsp. *villosissimus*	TRS^{t}	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. spartioides	TRS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
L. tenellus	TRS	**PCS**	**TRS/PCS**	TRS/PCS	**TFS**	TFS
Section *Rhyncholotus*						
L. berthelotii	ND^{t}	**TRS**	**TRS^{t}/PCS**	TRS	**TRS**	TRS
L. eremiticus	ND^{t}	**TRS**	**TRS^{t}/PCS**	TRS	**TRS**	TRS
L. maculatus	ND^{t}	**TRS**	**TRS^{t}/PCS**	TRS	**TRS**	TFS
L. pyranthus	ND^{t}	**TRS**	**TRS^{t}/PCS**	TRS	**TRS**	TRS
Ornithopus						
O. compressus	PCS	**PCS**	**PCS**	TRS/PCS	**TFS**	TFS
Scopiurus						
S. sulcata	TRS^{S}	**PCS**	**TRS**	TRS	**TFS**	TFS

Table 6.3 Classification of the levels of lateralization (meassured by the presence of TRS) observed in *Rhyncholotus* and *Pedrosia* species according with the distribution of epidermal types and trichomes. * Species reported with trichomes on the dorsal petal (Sandral et al., 2006), but not confirmed in this study. *L. chazalei* and *L. loweanus* were not analyzed. The specimen of *L. assakensis* analyzed did not have trichomes.

	Section *Rhyncholotus*	**Section *Pedrosia***		
Syndrome	**Ornithophilous species**	**Melittophilous species**		
Groups		**Closely related species of *Rhyncholotus***	**Species within the same clade of *Rhyncholotus***	**Species from a different clade of *Rhyncholotus***
Species	*L. berthelotii* *L. maculatus* *L. pyranthus* *L. eremiticus*	*L. mascaënsis* *L. kunkelii* *L. sessilifolius* subsp. *villosissimus* **Species from a different clade of *Rhyncholotus*** **L. assakensis* **L. loweanus* **L. chazalei*	*L. argyrodes* *L. azoricus* *L. arinagensis* *L. sessilifolius* subsp. *sessilifolius*	*L. spartioides* *L. tenellus* *L. leptophyllus* *L. dumetorum*
Epidermal type	-Modified TRS and increased presence of this epidermal type in dorsal and ventral petal	-Increased presence of TRS on the abaxial side of the dorsal petal	- Increased presence of TRS on the abaxial side of the dorsal petal	- Increased presence of TRS on the abaxial side of the dorsal petal
Lateralization	-Strong lateralization of dorsal and ventral petals	-Slight lateralization of dorsal petal	-Slight lateralization of dorsal petal	-Slight lateralization of dorsal petal
Trichomes	-Present on dorsal and lateral petals	-Present on dorsal petal	-No trichomes	-No trichomes
PCS	-Absent on dorsal petal	-PCS on dorsal petal	-PCS on dorsal petal	-PCS on dorsal petal

Figure 6.1 Developmental stages of flower in *Lotus japonicus*

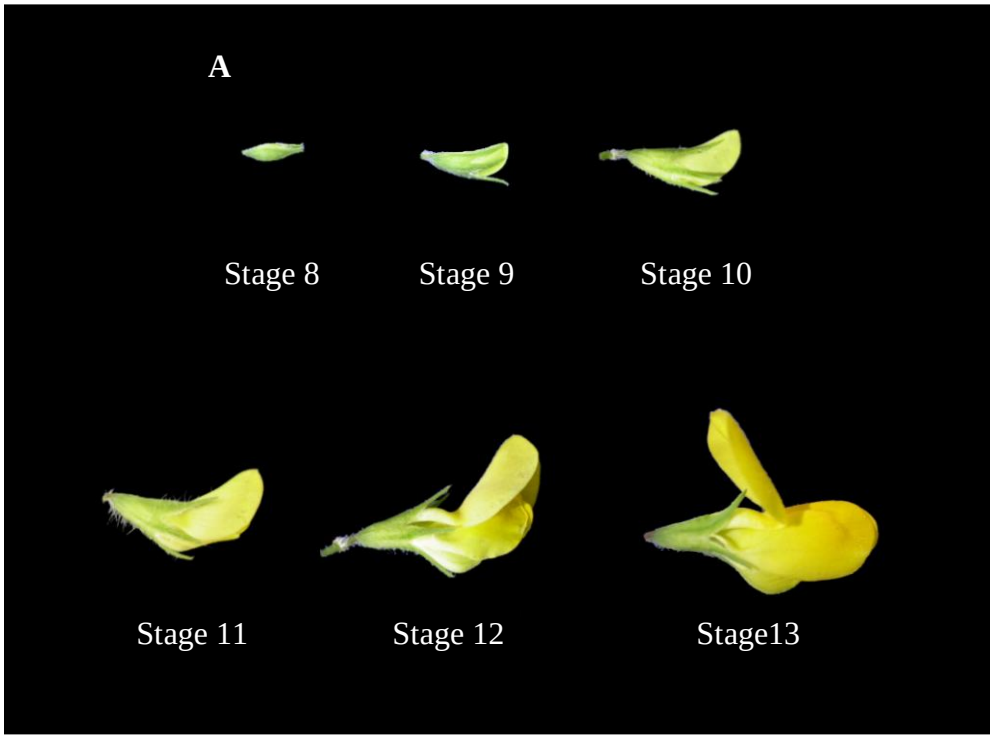


Figure 6.2 Members of *Lotus* section *Rhyncholotus* with an ornithophilous pollination syndrome. A, *Lotus maculatus*, B, *L. pyranthus*, C, *L. berthelotii*, and D, *L. eremiticus*; and four representative species of section *Pedrosia*, E, *L. arenarius*, F, *L. latifolius*, G, *L. jacobaeus* and H, *L. sessilifolius* subsp. *sessilifolius* with a bee-pollination syndrome. a=dorsal petal, b = lateral petal and c=ventral petal. Photo credits: A, C, E, H from I. Ojeda and B, D, F, and G from F. Oliva-Tejera.

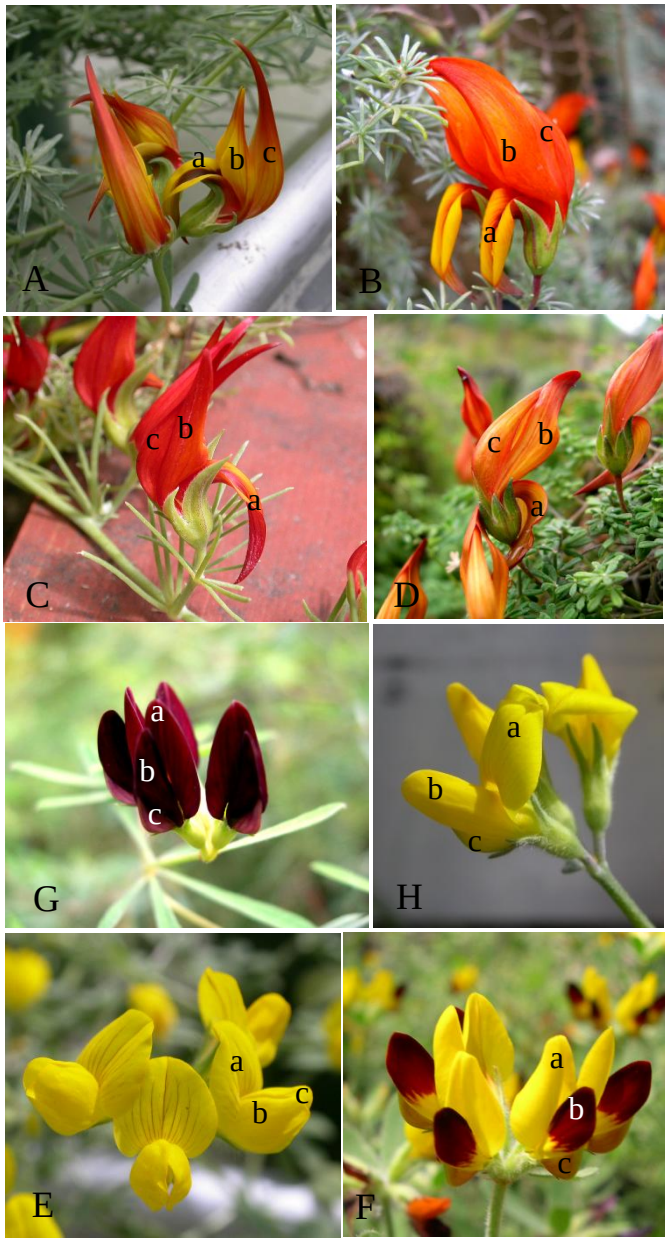


Figure 6.3 Zygomorphic flowers of A, *Lotus japonicus*. B, these flowers have three types of petals, a) dorsal petal with a bilateral symmetry, b) two asymmetrical lateral petals, and c) two asymmetrical ventral petals. All petals were separated and flattened. The base of the dorsal petals in both species was separated from the rest of the petal. C, *Lotus berthelotii* with a bird-pollination syndrome. D, size comparison between *L. berthelotii* and *L. japonicus*. E, hypothetical mechanism by which a bird seeks for nectar and pollen is placed either on the top of the head or in the throat (according to Olsen, 1985). E, *Bombus canariensis canariensis* one of the main insect pollinators in the Canary Islands foraging on *L. hillebrandii* from El Hierro.

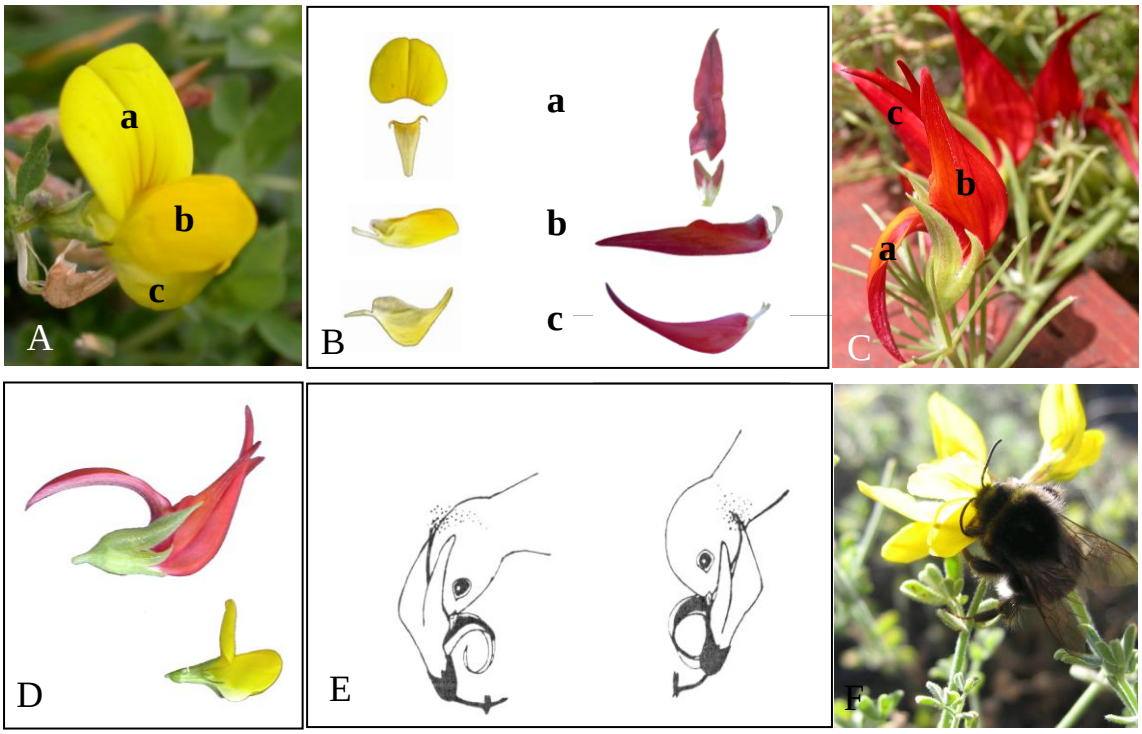


Figure 6.4 Major epidermal types recorded in Loteae. A and B, papillose conical cells (PCS), C and D, tabular rugose cells with striations (TRS) and E an F, tabular flat cells with striation (TFS) in *Lotus japonicus*. G, non-differentiated cells with trichomes, H, tabular rugose cells with striations, I, papillose conical cells with striations, H, J-M tabular rugose cells with striations in *L. berthelotii* (TRS). Arrows indicate the localization of papillose conical cells in the lateral petals. Scale bars: 100 µm, G and L. 50 µm = A, H, C, D, F, K and M. 25 µm = I and J.

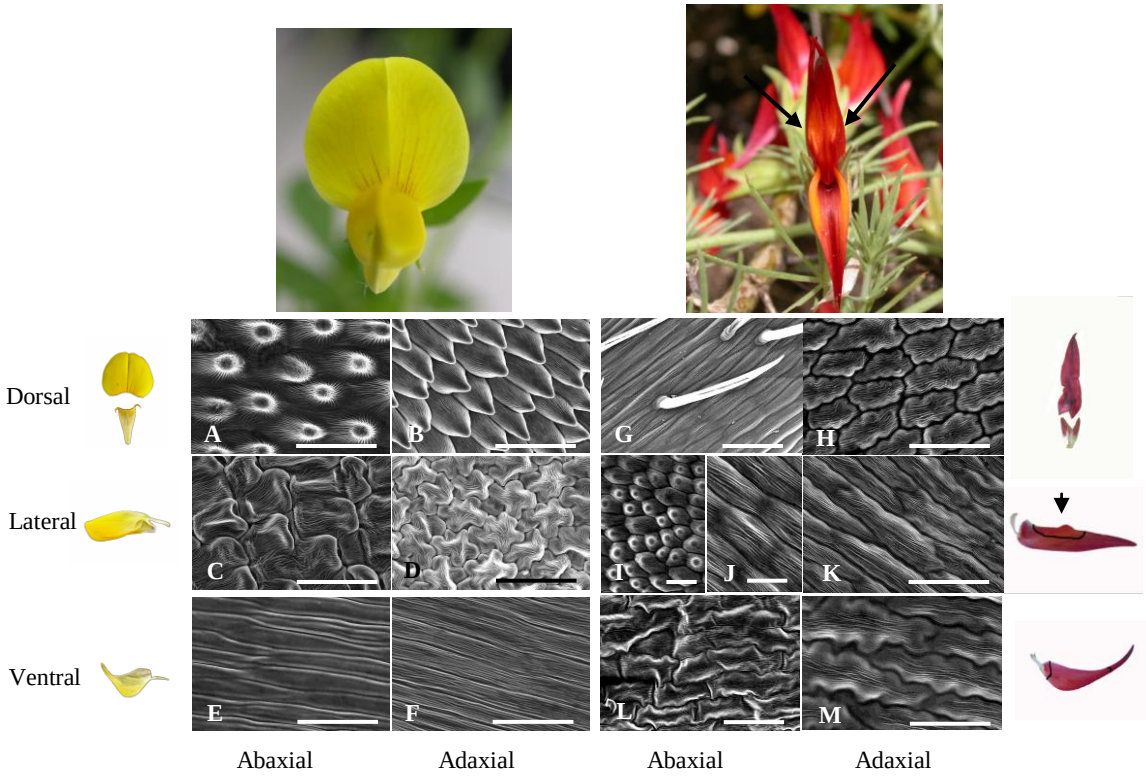


Figure 6.5 Expression patterns of *LjCYC1*, *LjCYC2* and *LjCYC3* in A) *Lotus japonicus*, B) *L. filicaulis* both from section *Lotus*, C) *L. sessilifolius* from section *Pedrosia*. All these three species have a flower morphology adapted to bee pollination and have similar petal size and shape. D) *L. berthelotii* and E) *L. maculatus* from section *Rhyncholotus* have a flower morphology adapted to ornitophily by opportunistic passerines. The base of the dorsal petal has been detached from the rest of the petal. dp= dorsal petal, lp= lateral petal and vp=ventral petal.

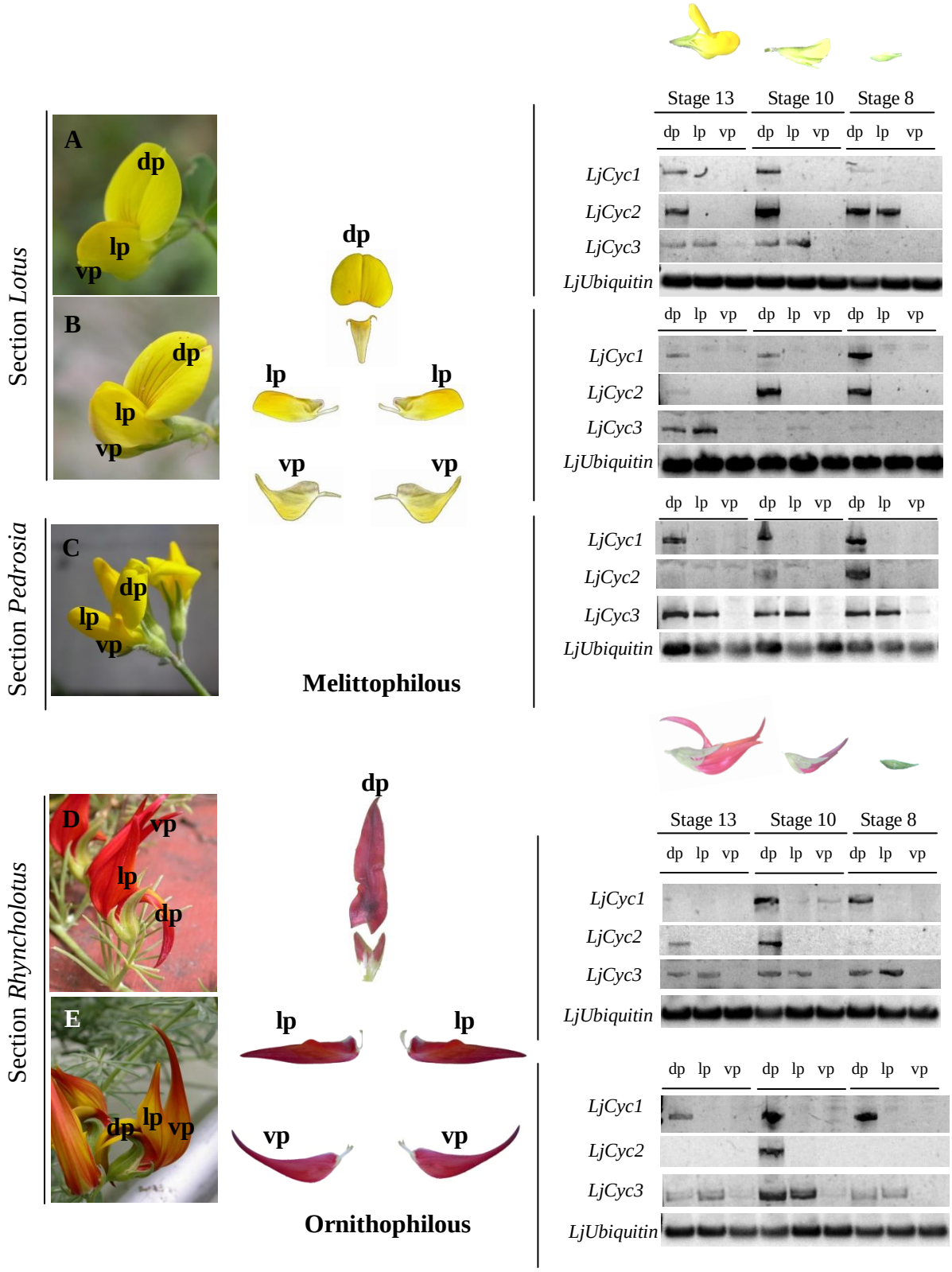
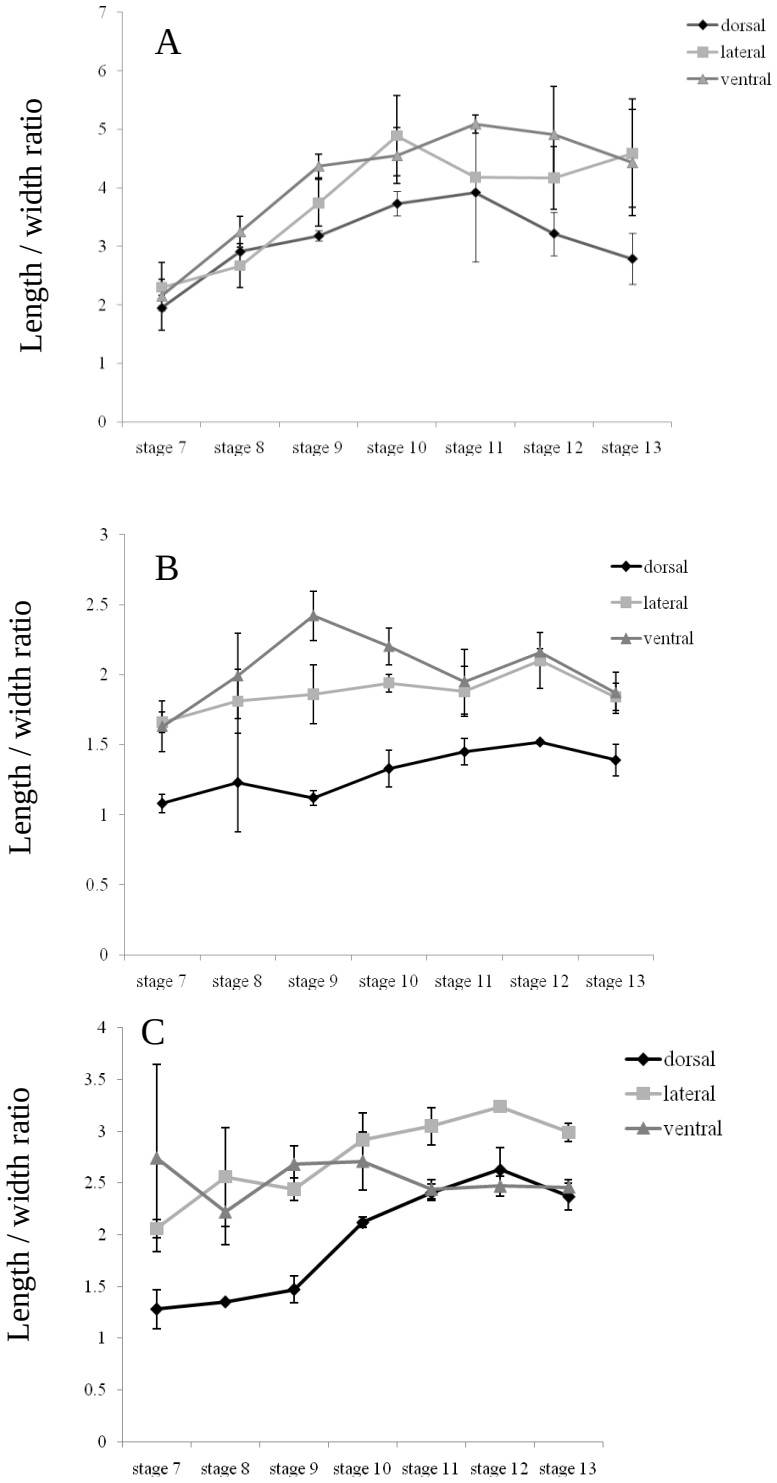

Section Lotus
Section Pedrosia
Section Rhynchotolus
A
dp
lp
vp
B
dp
lp
vp
C
dp
lp
vp
D
vp
lp
dp
E
dp
lp
vp
dp
lp
lp
vp
vp
Melittophilous
dp
lp
lp
vp
vp
Ornithophilous
Stage 13
Stage 10
Stage 8
dp lp vp dp lp vp dp lp vp
LjCyc1
LjCyc2
LjCyc3
LjUbiquitin

Figure 6.6 Petal gowth meassured as the length/width ratio during different stages of flower development in A) *L. berthelotii,* B) *L. sesilifolius* and C) *L. japonicus*. Mean values with standard deviations. (n=3-5 flowers on each developmental stage).



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7 Biochemical evolution of bird-pollinated flowers in Macaronesian *Lotus* (Leguminosae)[1]

7.1 Introduction

Flower colour depends on a combination of many factors, including epidermal surface (Noda et al., 1994), co-pigments, metal ions and pH of the vacuolar content, and the type of pigments produced (Grotewold, 2006; Tanaka et al., 2008). Flavonoids are one of the main pigments in plants and are responsible for the red to blue colours of many plant parts, including flower colours. The flavonoid biosynthetic pathway is well conserved across angiosperms, and it is the metabolic pathway responsible for the production of several of the most common types of plant pigments. Anthocyanins and flavonols are two major pigment types derived from this pathway, the former produce orange/red to violet/purple colours in flowers while the latter can produce very pale-yellow colours. Both types of pigments derive from dihydroflavonols and the type of pigment produced largely depends on the enzymes active downstream of these precursors. Flavonol synthase (FLS) is responsible for producing flavonols, while dihydroflavonol 4-reductase (DFR) is the first enzyme for commitment to anthocyanin production.

There are three main branches in the anthocyanin pathway and their colour depends mainly on the number of hydroxyl groups on the B-ring. As the number of hydroxyl groups increases, the peak of absorbance shifts from the red side to the blue side of the spectrum (Tanaka et al., 1998); the larger the number of hydroxyl groups the bluer the colour.

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[1]A version of this chapter will be submitted for publication: Ojeda, I. Santos-Guerra, A., Oliva-Tejera, F. Alfredo Valido, Jaen Molina, Xinxin, Xue, R. Caujape, J. Marrero, A. and Cronk. Q. C. B. Biochemical evolution of bird-pollinated flowers in Macaronesian *Lotus* (Leguminosae)

Pelargonidin with one hydroxyl group generally produces red colours, cyanidin derivatives with two groups typically give magenta or blue colours, and delphinidin with three hydroxyl groups is mainly responsible for blue colours (Grotewold, 2006; Tanaka et al., 2008). Red/orange flowers are usually associated with bird pollination (ornithophily) and this trait is probably one of the most conspicuous features of this syndrome (Proctor et al., 1996). It is thought that red flowers evolve either to advertise certain features, such as copious nectar, to birds (Grant, 1966), or because it is not attractive to competing insect pollinators (Cronk and Ojeda, 2008); either way, flower colour has an important role in the type of pollinators attracted.

Pelargonidin derivatives are often associated with hummingbird pollination, while cyanidin derivatives seem to be associated with pollination by perching birds (Scogin, 1988). Delphinidin derivatives are less frequently found in bird-pollinated species but are often found in blue bee-pollinated flowers. Several transitions in flower colour associated with transitions in pollination syndromes have previously been investigated at the biochemical and molecular level. Bee-bird transitions have been investigated in modifications from blue to red flowers (Rausher, 2008) and yellow to red (Taylor, 1984).

Modifications of pigments in association with blue (bee) to red (bird) transitions of flower colour have been previously explored in three genera. Red bird-pollinated species of *Ipomoea* have derivatives of the pelargonidin branch and the transition from its bee ancestor involved the inactivation of the cyanidin branch (Zufall and Rausher, 2004). Most of the red, hummingbird pollinated, species within this genus have pigments of the pelargonodin branch with no derivatives of other branches of the anthocyanin pathway (Streisfeld and Rausher, 2009b; Zufall, 2003). In *Penstemon*, another group with hummingbird pollination, red flowers have evolved independently several times and have pigments of the cyanidin or pelargonidin branch, or both (Rausher, 2008; Scogin and Freeman, 1987).

In other examples, bird-pollinated flowers have evolved from yellow flowered insect-pollinated ancestors. In some *Iochroma* species, for example, red-flowered hummingbird pollinated species, which have pelargonidin pigments, probably evolved from yellow ancestors that lacked this pigment and produced only carotenoids (Smith, 2006). In *Mimulus aurantiacus*, there are red-flowered populations associated with hummingbird pollination and yellow moth-pollinated populations. It seems that natural selection is maintaining this difference in flower colour (Streisfeld and Rausher, 2009a). Red-flowered populations are able to produce cyanidin and pelargonidin pigments, while yellow-flowered populations completely lack these two pigments (Streisfeld and Kohn, 2005). Red hummingbird pollinated flowers of *Aquilegia* also produce cyanidin and pelargonidin pigments (Taylor, 1984).

In this study my objective is to explore a transition from yellow-flowered species pollinated by insects (bumblebees) to red/orange-flowered pollinated by opportunistic passerines birds in a group of Macaronesian *Lotus* (Fig. 7.1A and B). Within Macaronesian *Lotus*, this pollination syndrome is found in a group of four species (the "rhyncholotus group"). Additional features associated with this syndrome include flower size, shape, orientation, longevity as well as nectar composition and concentration (Dupont et al., 2004; Olesen, 1985; Ollerton et al., 2009). On the other hand, their most closely related species within section *Pedrosia* (36 spp) have the standard traits associated with bee pollination found in the genus *Lotus*. These traits include a horizontal flower orientation, low nectar production (with sucrose as the major sugar) and yellow flowers at anthesis (Fig. 7.1C). Some species within this group alter flower colour after anthesis, in which case old flowers may assume brown, pink, orange, purple or red colours depending on the species (Fig. 7.1B-F).

In *Lotus* the yellow colour is known to be mainly due to carotenoids (Suzuki et al., 2007) but flavonols and anthocyanins are also known to occur (Reymaud and Lussignol, 2005; Suzuki

et al., 2008) and are likely to be important modifiers of spectral reflectance. Pigment composition in the bird-pollinated *Lotus* species has been investigated in only one species, in which a cyanidin-based pigment was identified (Beale et al., 1941). There are no previous analyses of the pigment composition in the Macaronesian group for the bee-pollinated species. Outside of Macaronesian *Lotus*, pigments have been investigated in the model legume *Lotus japonicus* (Suzuki et al., 2008).

7.1.1 Objectives of the study

My goals in this study are: 1) to identify pigment modifications (anthocyanins and flavonols) associated with the evolutionary transition from yellow bee-pollinated flowers to red/orange bird-pollinated flowers, 2) to determine the biochemical changes associated with post-anthesis flower colour modification 3) to determine floral reflectance in relation to pollinator perception in differently coloured *Lotus* flowers, and 4) to examine the molecular basis of changes in pigmentation by analyzing the relative expression patterns of three biosynthetic enzyme genes between the two pollination syndromes.

7.2 Material and methods

7.2.1 Pigment extraction and composition analysis

In order to determine the type of pigments produced in flowers of the species in this group, the petals were separated from the rest of the flower and dried. Pigments were extracted from petal samples (20 mg) with a buffer (MeOH/H_20/AcAc) and later treated with HCl 2N at 100°C for 30 min (Shimada et al., 2005). Samples were injected into a LC Agilent 1100 series light chromatograph (LC-MS) with a LC7MSD trap XCT Plus. One species (*L. sessilifolius*) was

analyzed three times to assess variation but the remaining species were analyzed only once as the variation in pigment composition was minimal.

Pigments from the samples were identified using retention times of a mixture of six anthocyanin standards (cyanidin, peonidin, pelargonidin, petunidin, delphinidin and malvidin from; Chromadex, and three flavonols, quercetin, kaempferol and isorhamnetin (from SIGMA) prepared and analyzed under similar conditions. A total of 18 species were analyzed representing all flower colours reported in this group. This sample included the four bird-pollinated species, five yellow bee-pollinated species that do not alter flower colour after anthesis and nine bee-pollinated species that change flower colour after anthesis. My sample also included two species from section *Lotus,* the model legume *L. japonicus* and *L. filicaulis,* a species that changes flower colour after anthesis from yellow to red (Table 7.1).

The relative amount of each pigment was calculated from the area under peaks in the MS spectra for each pigment identified using the LC/MSD trap software 5.2. The relative amount of each pigment was used to establish which branches of the anthocyanin pathways were active and to determine pigment pathway alterations during transitions of flower colour.

7.2.2 Measurement of petal reflectance

Petal reflectance was measured under field conditions (*in situ* in the wild and in plants under nursery conditions) with a portable spectrophotometer (Ocean Optics USB2000) at wavelengths from 300 to 700 nm. The three types of petals were measured on the side naturally exposed to pollinator vision (abaxial side of the dorsal petal and adaxial side of lateral and ventral petals). All measurements were taken five times on the same section of the petal (middle part) for each species and the averages of these measurements were used to estimate the reflectance graph for each species. In total 19 species were analyzed, including all four bird-pollinated species, five

yellow bee-pollinated species that do not change colour and 10 bee-pollinated yellow species that change colour after anthesis (measured both before and after the colour change) (Table 7.2). The species included all flower colours reported in this group, except pink and brown flowers, which were not available for this study.

Reflectance graphs of each species were later classified according to their correspondence to action spectra of the four visual receptors found in pollinating organisms (UV, blue, green and red) (Chittka et al., 1994) and used to determine perceived flower colour by pollinators.

7.2.3 Reconstruction of flower colour change evolution

Flower colour modification after anthesis was analyzed using parsimony (DELTRAN) as implemented in MacClade 4.0 (Maddison and Maddison, 2000). Ancestral state reconstruction was carried out using Mesquite (Maddison and Maddison, 2009). Flower colour change was recorded for each species based on observations in the field, from cultivated plants in botanical gardens (Jardin de Aclimatación de la Orotava, Jardin Canario Botánico Viera y Clavijo) and from plants cultivated at the University of British Columbia (UBC) glasshouses. For those species not cultivated or observed in the field, flower colour was obtained from the literature (Bramwell and Bramwell, 2001; Brochman et al., 1997; Jardim and Francisco, 2000; Mader and Podlech, 1989; Monod, 1980; Sandral et al., 2006).

Colour change was coded as a binary character (absence and presence of flower colour change) and the evolution of this trait was mapped on a phylogenetic tree of the group based on four gene regions (ITS, *Cytochrome B6*, *trnH-psbA* and *matK*) (see Chapter 4).

7.2.4 Gene expression of dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS) and O-methyl transferase (OMT)

In order to further explore the activity of the anthocyanin pathway and its different branches in this group during flower colour transitions, I analyzed the expression of three genes using reverse transcriptase PCR (RT-PCR). I extracted RNA from two developmental stages, open mature flower at anthesis (before colour change) and flowers after colour modification (post-change) in each petal separately. RNA was extracted using the Pure Link^{TM} Plant RNA Reagent from Invitrogen following the manufacturer's protocol. RNA was treated with DNAse, visualized on an agarose gel (2%) and its concentration measured using a Nanodrop spectrophotometer. RNA was converted to cDNA using the RevertAid ^{TM} H Minus First Strand cDNA Synthesis Kit from Fermentas according to the manufacturer's protocol. Genomic contamination was assessed using the intron in *Lotus japonicus CYCLOIDEA 2* (*LjCYC2*) and endogenous expression of *Lotus japonicus Ubiquitin* (*LjUbi*) was examined with *LjUbif/R* as a control of cDNA quantity across the samples (Feng et al., 2006b).

Gene expression comparisons were carried out in five species: *L. japonicus* and its close relative *L. filicaulis* (*Lotus* section *Lotus*), *L. sessilifolius* (section *Pedrosia*, closely related to the bird-pollinated group), which has a colour change with red post-change flowers, and two species from the bird-pollinated ("rhyncholotus") group, *L. berthelotii* (with red flowers) and *L. maculatus* (red-orange flowers).

Specific primers were designed for four of the DFR copies previously isolated in *L. japonicus* (Shimada et al., 2005). *DFR1* was amplified using the following primer pair: forward LjDFR1F (5´-GGATGAGACCTGCTGGGGTGACC-3´) and reverse *LjDFR1R* (5´-GATTCAGGGTGCTCGAAG-3´). DFR2 using forward LjDFR2F (5´-CGCCACTGTAAGAGACCCTG-3´) and reverse *LjDFR2R* (5´-AACATCGCTCCAGCAGCTC-

3´). DFR3 using forward LjDFR3F (5´-CTCATGGAGGGCGGCTAC-3´) and reverse LjDFR3R (5´-GATCCTTGGAATTAAAGT-3´). DFR5 using forward LjDFR5F (5´-GAGAAGGTTGGTATTCAC-3´) and reverse LjDFR5R (5´-TGATGAGTGAGAGAGCAG-3´).

A conserved region of the ANS gene was amplified using the following primer pair: LjANSF2 (5´-GCAGTGGGATACAATCTA-3´) and LjANSR1 (5´-ATGGAGAGGTCACGCTTG-3´). This primer pair was designed based on a conserved region of the ANS from three species, *L. corniculatus* (AY028931) based on a partial (Paolocci et al., 2005), *L. japonicus* (chromosome 2, Miyakogusa.jp accession No. CM0304.350.nc) and *Glycine max* mRNA complete cds (EU334548).

A specific primer pair was designed from a conserved region of OMT using the following primer pair: LjOMTF2 (5´-TCTGGAGACCAGTGTGTACC-3´) and LjMOTR2 (5´-TGAGTCTCTTGTGGTAGTTG-3´). This primer pair was designed from a conserved region of a previously sequence from *Glycine max* (TIGR accession TC190220) (Kim et al., 2006), *Medicago sativa CCMOT* (U20736) and the best hit I found from the *L. japonicus* genome for this gene (chr. 4, CMO 227.500.nc + phase). Amplification of the above mentioned genes was carried out following PCR conditions: 95 °C for 2 min, 30 cycles of 94 °C for 45 s, 50-57 °C for 1 min and 72 °C 2 min, and 72 °C for 2 min.

7.3 Results

7.3.1 Pigment flower composition in *Lotus*

The flavonoids identified (cyanidin, quercetin, petunidin, peonidin and malvidin) suggest that two branches of the anthocyanin pathway, the cyanidin and delphinidin branches, are active in mature flowers at anthesis in Macaronesian *Lotus* (section *Pedrosia*), both in the yellow flowered species and in the bird-pollinated group (Table 7.3). Additionally, I identified three

different flavonols (or their derivatives): kaempferol, quercetin, and isorhamnetin. Based on this sampling it seems that the pelargonidin branch of the anthocyanin biosynthetic pathway is not active in mature flowers in any of the sections of *Lotus* tested. Furthermore, I did not observe derivatives of the delphinidin branch in the two species tested from section *Lotus* (*L. japonicus* and *L. filicaulis*). In my sample therefore the delphinidin pathway was restricted to section *Pedrosia*, whereas the cyanidin pathway was common to all species.

Pigments in species with yellow flowers but without post-anthesis flower colour modification: Yellow flowers of these species are characterized by a mixture of flavonols (mainly isorhamnetin) and traces of anthocyanin pigments. I found aglycones of delphinidin and cyanidin in three out of five species analyzed, which suggests that these branches are active even in those species that do not change flower colour after anthesis. However, the contribution of the anthocyanin pigments to overall colour in these species is likely to be minimal compared to flavonols, which are present in large amounts, and carotenoids. Additionally, the fact that anthocyanins were found in aglycone forms indicates a lack of pathway progression down these biosynthetic branches (Fig. 7.2A) (Table 7.3).

Species that modify flower colour after anthesis: Yellow flowers (pre-change) have a similar composition of flavonols (mainly isorhamnetin) to those species that do not modify flower colour, but the relative amount of delphinidin and cyanidin aglycones is relatively greater (Fig. 7.2B). In older flowers (post-change) further anthocyanidin pathway progression occurs. In addition to delphinidin and cyanidin, I found derivatives of peonidin, malvidin and petunidin in these species. The pigment composition of these post-change flowers varied accordingly with the

colour of each species. For instance, purple and pink flowers tend to have more derivatives of the delphinidin branch while red and orange flowers have more derivatives of the cyanidin branch.

The closest related species (*L. sessilifolius* and *L. mascaensis*) of the bird-pollinated flowers have a colour change to red flowers in late anthesis. In these species, all five anthocyanins detected in the bird-pollinated species (cyanidin, delphinidin, malvidin, peonidin, petunidin) are already present in the yellow flowers but their amounts increased during flower colour modification (post-change flowers) (Fig. 7.2B and C). Flower colour change therefore appears to modify the flux into already active branches in the anthocyanin pathway; the flux of pigment production is presumably re-directed to the cyanidin and delphinidin branches instead of to flavonols, which decrease.

Red bird-pollinated flowers: these four species contain a different proportion of flavonols and anthocyanins in comparison to their relatives with yellow flowers. Bird-pollinated flowers contain mainly cyanidin derivatives and the main flavonol is quercetin (rather than isorhamnetin) (Fig. 7.2D). However, the same branches of the anthocyanin pathway are active and flavonols still contribute to flower colour in these species. However, the flux of pigment production is towards the cyanidin and delphinidin branches of the anthocyanin pathway and flavonol production is reduced, especially isorhamnetin (a derivative of quercetin). Thus, the transition from yellow bee-pollinated species to red bird-pollinated species in this group involves only a quantitative re-direction of pigment production from flavonols to anthocyanins. These results show three major modifications of anthocyanin and flavonol composition between bee and bird-pollinated flowers: 1) a down-regulation of flavonol production, with a modification of flavonol composition, and with quercetin as the main flavonol produced in bird-pollinated flowers, 2) a down-regulation of

derivatives of the delphinidin branch, and 3) an up-regulation of derivatives of the cyanidin branch (mainly cyaninidin).

7.3.2 Flower reflectance and colour change perception

Yellow flowers have at least three different major reflectance types (Table 7.4). We found that only seven species of this group have a reflectance type with a UV peak in the spectrum. The remaining species have a reflectance peak above 500 nm (Fig. 7.3A-C). For flowers that change colour, pre-change and post-change flowers within the same plant have a different reflectance, as expected. Flower colour modification after anthesis shifts the reflectance peak of the flowers towards the red spectrum (Table 7.4 and Fig. 7.3B and C). This change is expected to make post-change flowers less evident to bees (which cannot see red), as they will stand out less from a green foliage background (Table 7.4). We found that flowers with deep purple colours tend to have a reflectance peak above 600 nm while red-orange flowers have a peak above 500 nm.

Red-orange flowers of bird-pollinated species have reflectance in the green and red receptor action spectra, with the exception of *L. pyranthus* which has a UV peak in early anthesis orange flowers (older flowers turn redder). Only two of the bird-pollinated species have a reflectance peak only in the red receptor action spectrum above 600 nm and are thus completely uncoloured to bees as they lack a red-light receptor (Fig. 7.3D).

7.3.3 Gene expression comparisons during flower colour modification

Our expression analysis results suggest that all three structural enzymes of the anthocyanin pathway analyzed, DFR, ANS and OMT, are expressed at late developmental stages. Two copies of dihydroflavonol 4-reductase (DFR), DFR1 and DFR3, do not have differences in expression patterns among the species analyzed. DFR1 is expressed in all species while we did

not detect DFR3 expression in flowers of any of the species analyzed. We found differences in expression patterns in two copies of DFR. DFR2 is expressed in bee-pollinated flowers (including pre-change and post-change flowers) but it is not expressed in either of the two bird-pollinated species. The expression patterns of the four DFR copies suggest that particular copies may have specific activity on particular branches of the anthocyanin pathway. DFR5 expression was only observed in those species that produced pigments of the delphinidin branch, for which it may be specific (Fig. 7.4).

Anthocyanidin synthase (ANS) is expressed in all species analyzed at approximately the same levels, even in yellow flowers (pre-change). We only observed a slight increase in expression level in red (post-change) flowers of one species (Fig. 7.4).

O-methyltransferase (OMT) expression was detected in all bee-pollinated species (in both yellow pre-change and red post-change flowers). However, this enzyme seems to be down-regulated in the bird-pollinated species at late developmental stages (Fig. 7.4).

7.4 Discussion

7.4.1 The changing balance of anthocyanin and flavonol pigment composition in bee and bird pollination in *Lotus*

In many systems, transitions in pollination syndromes have involved the activation of previously absent branches of the anthocyanin biosynthetic pathway (Rausher, 2008; Scogin and Freeman, 1987; Streisfeld and Rausher, 2009b; Taylor, 1984; Zufall, 2003; Zufall and Rausher, 2003), (Smith, 2006). However, in contrast, the transition in *Lotus* from bee (yellow) to bird (red) flowers does not involve novel inactivation and/or activation of branches of the anthocyanin pathway. Rather, this transition involved the increasing production of anthocyanin pigments already present in small amounts, and also quantitative alterations in the proportions of flavonols,

such as quercetin, kaempferol and isorhamnetin. In the yellow-flowered species, isorhamnetin was often particularly abundant. Flavonols are significant in insect attraction, as they absorb UV light (Tanaka et al., 2008). Reflectance from these flowers (yellow to humans) therefore tends to stimulate the green receptor of the insect eye rather than the blue or UV receptors.

Both anthocyanin and flavonol pigments are produced from the same precursor (dihydroflavonols) as branches of the same flavonoid biosynthetic pathway, and the specific production of each pigment depends on the branches of the pathway that are active (Grotewold, 2006; Tanaka et al., 2008).

Most of the flux of pigment production in yellow species is towards flavonols, in particular derivatives of dihydroquercetin (DHQ), also a precursor of the cyanidin branch (Fig. 7.5). The transition in flower colour at the biochemical level therefore involves the re-direction of pigment flux production towards the cyanidin branch, which is the main anthocyanin observed in bird-pollinated flowers (Fig. 7.2d) whereas the flavonols kaempferol and isorhamnetin are greatly reduced in bird-pollinated flowers and has a minor contribution to overall pigment composition in comparison to bee-pollinated flowers (Fig. 7.2). It may be that this redirection of flux results from a small but significant shift in the competitive balance of enzymes for substrates in these branches. In lisianthus (*Eustoma grandiflorum*) and carnation (*Dianthus caryophylus*) flavonol synthase (FLS) and DFR play a critical role regulating the production of flavonols and anthocyanins, and there is a regulation to avoid competition for the substrate. In both species, the production of flavonols and anthocyanins is clearly divided, and there is no overlap in the expression of FLS with DFR (Noda et al., 2004; Stich et al., 1992; Uddin et al., 2002). In contrast to the findings for lisianthus and carnation, I did not find any clear-cut division in the production of flavonols and anthocyanins; therefore I suspect that in *Lotus* there is some level of competition

as both enzymes use the same precursor. However, the expression of FLS needs further examination in order to corroborate the findings.

7.4.2 Post-pollination floral colour change as a mechanism to promote foraging efficiency

An interesting feature of Macaronesian *Lotus* (*Lotus* section *Pedrosia*) is the ability to change colour, possibly in response to pollination. In this particular group I found that 58% of the species have the ability to modify flower colour after anthesis. Flowers at anthesis (pre-change) are always yellow in all bee-pollinated species and post-change flowers vary from red (8 spp), orange (2 spp), pink (1 sp), brown (4 spp) to purple (6 spp) (Fig. 7.1).

In all cases pre-change and post-change flowers have differences in spectral reflectance, suggesting that they are likely perceived differently by pollinating bees (Fig. 7.3B and C). This suggests that such colour change perhaps evolved as a strategy to increase foraging efficiency (Gori, 1983; Jones and Cruzan, 1982; Jones and Cruzan, 1999; Oberrath and Böhning-Gaese, 1999).

Red, late anthesis, flowers may still contribute to the overall floral display to attract distant pollinators but at short range, un-pollinated flowers (with reward) can be distinguished from visited (no reward) flowers. Pollinator activity is therefore directed efficiently to non-pollinated flowers. Empirical studies of *Syrmatium glabrum* (=*Lotus scopiarus*) support the role of flower colour modification as a strategy to increase foraging efficiency (Jones and Cruzan, 1999).

7.4.3 Late-anthesis flower colour changes as a possible preadaptation in the evolution of the bird pollination syndrome in *Lotus*

Red-orange bird-pollinated flowers evolved within a group of *Lotus* that already have a previous capacity to produce the pigments observed in bird-pollinated flowers. The five most closely related species to the bird-pollinated species have red late anthesis flowers (Fig. 7.1H). The ancestor of the bird-pollinated species was therefore likely to have had red flowers in late anthesis, a potential pre-adaptation facilitating the evolution of this pollination syndrome, as it would already have had the capacity to produce cyanidin and delphinidin derivatives. However, within *Lotus* as a whole the ability to change flower colour appears to be derived and has evolved at least three times within this group. Although it has been lost in some of the bird-pollinated species, which are always red/orange and do not modify flower colour after anthesis, it is retained in *L. pyranthus*, which starts out orange and deepens to reddish-orange (Fig. 7.6).

7.4.4 Flower colour change after anthesis as a possible facilitating factor in the evolution of bird pollination in other groups

Flower colour change is not a unique feature of *Lotus* section *Pedrosia*. It is reported at least in 20% of angiosperm families (Weiss, 1995), parallel evolution that is apparently driven by the ability of colour flower change in fully turgid flowers after anthesis to act as a signal increasing efficiency in plant-pollinator interactions. Additional floral changes (such as flower orientation, size, shape and odour production) sometimes also modify in conjunction with flower colour modifications (Eisikowitch and Rotem, 1987; Raguso, 2004; Willmer et al., 2009), and it is likely that all of these modifications are an integrated system to increase foraging efficiency in some plant species (Raguso, 2008).

The increase of red pigmentation is one of the most common modifications in these species, perhaps because red is less visible to bees, helping to direct pollinators to non-pollinated flowers. The shift from bee to bird pollination also potentially benefits from flowers becoming less visible to bees. Therefore a heterochronic modification from red colouration in late development to red colouration early in development may be a simple mechanism to initiate change in pollination syndromes.

7.4.5 Red flowers in bird-pollinated *Lotus* may avoid bee visits

For bees, *Lotus* flowers that all look the same to the human eye are surprisingly variable. Insects have three types of receptors, UV, blue and green, and they do not react to reflectance peaks above 585 nm (Chittka et al., 1994). I identified three types of reflectance pattern in yellow flowers (Table 7.4), two of which have not been reported previously in species with yellow flowers of *Lotus* (Floral Reflectance Database, FreD: http://reflectance.co.uk/new/) (Arnold et al., 2008).

In contrast, typical red-orange bird-pollinated flowers tend to have reflectance peaks above 585 nm and are considered uncoloured to bees. These bird-pollinated flowers are not invisible to insects but are much harder to distinguish from the background green foliage (Chittka and Waser, 1997). A previous study has reported the reflectance of *L. berthelotii* (Ollerton et al., 2009), which is the typical reflectance of a bird-pollinated flower (Altshuler, 2003; Chittka et al., 1994). My results indicate that only two out of the four bird-pollinated species, *L. berthelotii* and *L. eremiticus*, have this pattern, with a lack of reflectance in the UV and blue, and a peak in the red range above 585 nm. The other two bird-pollinated species, *L. maculatus* and *L. pyranthus*, have significant reflectance peaks in wavelengths perceived by green visual receptors, and are similar in that regard to the bee-pollinated red flowers after late anthesis colour change (Table

7.4). This provides a link between the two sorts of flowers which may be significant in understanding the evolution of bird pollination in the group.

7.4.6 Down-regulation of O-methyltransferase (LjOMT) in bird-pollinated *Lotus*

One of the main differences that we found between yellow bee-pollinated flowers and red bird-pollinated flowers is that the amount of isorhamnetin is reduced in the latter (Fig. 7.2). A flavonoid 3' O-methyltransferase has been previously characterized in *Glycine max* (Kim et al., 2006). Based on this sequence, I designed primers to study the expression of the ortholog, LjOMT, in *Lotus*. My expression analysis of LjOMT showed a down-regulation in the two bird-pollinated species that we examined (Fig. 7.4). Isorhamnetin, produced from quercetin, is the main flavonol in yellow species, and the evolutionary change in pollination syndrome involved a reduction in the production of this pigment (Fig. 7.5). These data suggest the possibility that changes in expression of this enzyme may be involved in the flux change of pigment production towards the cyanidin branch in these two bird-pollinated species (Fig. 7.5).

7.4.7 Possible specialization of dihydroflavonol 4-reductase (LjDFR) copies in *Lotus*

Dihydroflavonol 4-reductase (DFR) is the first enzyme in the pathway specific for anthocyanin production (Grotewold, 2006) and it catalyzes the conversion of dihydroquercetin (DHQ), dihydrokaempferol (DHK) and dihydromyricetin (DHM) to uncoloured leucocyanidin, leucopelargonidin and leucodelphinidin, respectively (Fig. 7.5). This enzyme seems to be a single copy gene in many plant species examined, including arabidopsis, grape, tomato, barley, snapdragon, rice and aquilegia (Bongue-Bartelsman et al., 1994; Chen et al., 1998; Hodges and Derieg, 2009; Holton and Cornish, 1995; Kristiansen and Rohde, 1991; Sparvoli et al., 1994; Winkel-Shirley et al., 1992). Multiple copies have been found in some groups and it seems that in

these species only one copy is active or expressed in flowers. For instance, three copies of DFR have been isolated in *Petunia hybrida*, but only *dfrA* is expressed in flowers (Forkmann and Ruhnau, 1987).

Based on substrate preferences, a region of DFR has been proposed for substrate specificity (Beld et al., 1989). An amino acid residue at position 134 of *Gerbera* DFR appears to determine substrate preference (Johnson et al., 2001) and two types of DFR have been identified, those having an asparagine (Asn) in this position (Asn-type DFR) and another with an aspartic acid (Asp) in this position (Asp-type DFR). Some plants such as *Cymbidium* (Johnson et al., 1999), *Petunia* (Forkmann and Ruhnau, 1987; Johnson et al., 2001) and *Gentiana* (Tanaka et al., 1996) have only DFRs of the Asn-type in this position and cannot produce pelargonidin-based orange-red in their flowers by converting DHK efficiently.

In the legumes examined so far, between three and five copies of DFR have been found, and both types have been reported (Shimada et al., 2005; Xie et al., 2004). The maintenance of multiple copies of both types has led to the suggestion that these copies perhaps have subfunctionalized roles. In *L. japonicus*, it has been found that the five DFR copies have a differential expression on different tissues. For instance, the DFR3 copy is not expressed in floral tissue and is only expressed in leaves and stems (Shimada et al., 2005). In our analysis we did not find DFR3 expression in any of the five species tested (Fig. 7.4), which indicates that this copy was not likely to have been involved during the transition in flower colour.

I found that DFR2 (Asn-type) is down-regulated in the two bird-pollinated species (Fig.7. 4). This copy is expressed in all three bee-pollinated species, but not across all three types of petals. In addition to flower tissue, expression of this DFR copy has been reported in pods, stems and roots of *L. japonicus* (Shimada et al., 2005). This enzyme has greater activity with

dihydrokaempferol (DHK) and dihydroquercetin (DHQ) substrates rather than with dihydromyricetin (DHM) (Shimada et al., 2005).

I also found that DFR5 (Asp-type) is only expressed in those species with delphinidin-based pigments. This copy has a higher activity towards DHQ and less to DHM, and, consistent with this, I did not observe expression of this copy in *L. japonicus* or *L. filicaulis*, both species that lack delphinidin-based pigments in petals. However, a previous study reported DFR5 expression in floral tissue of *L. japonicus* (Shimada et al., 2005), but in this case all flower parts were included and so it is not certain that the expression was in the petals only.

Table 7.1 List of species in section *Pedrosia* (including bird-pollinated members of the rhyncholotus group) analyzed for pigment composition.

Taxon	Flower colour at anthesis	Post-changed colour	Collection information	Voucher, herbarium
Lotus* section *Lotus				
Lotus filicaulis Durieu	Yellow	Red	Cult. UBC	*Ojeda 71*/UBC
Lotus japonicus Gifu-B129 (Regel) K. Larsen	Yellow	-	Cult. UBC	*Ojeda 70*/UBC
***Lotus* section *Pedrosia* (Lowe) Christ.**				
Lotus arinagensis Brawm.	Yellow	Red	Playa Arinaga, GC	
Lotus argyrodes R.P Murray	Yellow	Purple	Cult. JCVC # 5435/UDH/07 Punta de Pargo, M	*Ojeda 189*/UBC
Lotus brunneri Webb in Hooker	Yellow	-	Cult. JCVC # 514B/07 Sal, CV	*Ojeda 181*/UBC
Lotus callis-viridis Bramwell & D.H. Davis	Yellow	-	Anden Verde, GC	
Lotus campylocladus Webb & Berthel.	Yellow	-	Road Arona-Ifonche, T	
Lotus dumetorum Webb ex R. P. Murray	Yellow	-	Road Baradero, San Andres Anaga, T	
Lotus emeroides R.P. Murray	Yellow	Pink	Epina, G	
Lotus glaucus Sol.	Yellow	Orange	Cult. JCVC # 223/B/07 Porto Nurbita, M	*Ojeda 187*/UBC
Lotus jacobaeus L.	Yellow	Purple	Cult. JCVC 166/06, Cha de Calderas, CV	
Lotus latifolius Brand	Yellow	Purple	Cult. JCVC, Santo Antao CV	Marrero et al JCVC
Lotus mascaensis Burchard	Yellow	Red	Cult JCVC	Jaén 214/03 JCVC
Lotus sessilifolius D.C. subsp. *sessilifolius*	Yellow	Red	Guimar Poligono Industrial, T	
Lotus spartioides Webb & Berthel.	Yellow	-	Tamadaba, GC	
Lotus tenellus (R. Lowe) Sandral, Santos & D.D. Sokoloff	Yellow	Red	Arachico San Roque, T	
***Lotus* section *Rhyncholotus* (Monod) D.D. Sokoloff**				
Lotus berthelotii Masf.	Red	-	Cult. JAO 152-96	-
Lotus eremiticus A. Santos	Red	-	Cult. JAO 430-95	-
Lotus maculatus Breitf.	Red-orange	-	Cult. JAO 431-95	-
Lotus pyranthus P. Pérez	Orange	Red	Cult. JAO 124/01	*Ojeda 226*/UBC

Table 7.2 *Lotus* species from section *Pedrosia* (including bird-pollinated members of the rhyncholotus group) measured for petal reflectance.

Taxon	Collection information	Voucher, herbarium
Lotus* section *Pedrosia (Lowe) Christ.		
Lotus arinagensis Bramwell		
Lotus argyrodes R.P Murray	Cult. JCVC # 5435/UDH/07 Punta de Pargo, M	*Ojeda 189*/UBC
Lotus brunneri Webb in Hooker	Cult. JCVC # 514B/07 Sal, CV	*Ojeda 181*/UBC
Lotus callis-viridis Bramwell & D.H. Davis		
Lotus campylocladus Webb & Berthel.		
Lotus creticus L.	Cult. JCVC # 64/05	*Ojeda 188*/UBC
Lotus dumetorum Webb ex R. P. Murray	Teno al to, T	
Lotus glaucus Sol.	Cult. JCVC # 223/B/07 Porto Nurbita, M	*Ojeda 187*/UBC
Lotus jacobaeus L.		
Lotus kunkelii (Esteve) Bramwell & D. H. Davis	Cult. JCVC # 217/07	*Ojeda 176*/ UBC
Lotus lattifolius Brand	Cult. JCVC, Santo Antao CV	Marrero et al JCVC
Lotus mascaensis Burchard	Punta Teno, T	Jaén 214/03 JCVC
Lotus purpureus Webb		
Lotus sessilifolius D.C. subsp. *sessilifolius*	Playa San Marcos, Icod de los Vinos, T	
Lotus tenellus (R. Lowe) Sandral, Santos & D.D. Sokoloff	Risco del Fraile, Tano, T	
Lotus* section *Rhyncholotus (Monod) D.D. Sokoloff		
Lotus berthelotii Masf.	Cult. JAO 152-96	-
Lotus eremiticus A. Santos	Cult. JAO 430-95	-
Lotus maculatus Breitf.	Cult. JAO 431-95	
Lotus pyranthus P. Pérez	Cult. JAO 124/01	*Ojeda 226*/UBC

Table 7.3 Relative amounts of anthocyanidins and flavonols identified in *Lotus* section *Pedrosia* and *Rhyncholotus*. Relative amount was estimated based on the reflectance peak from each pigment. Pigment concentration for flowers before/after colour (in bold) change

	Anthocyanins					**Flavonols**		
	Cyanidin	Peonidin	Delphinidin	Malvidin	Petunidin	Quercetin	Kaempferol	Isorhamnetin
Bird-pollinated species								
L. berthelotii	22.7	0.78	6.55	0.16	0.98	14.63	1.93	0.70
L. maculatus	0.1	0	0.07	0	0	10.68	0.10	0.55
L. eremiticus	9.15	0.58	8.90	0.92	0.45	5.24	0.14	2.65
L. pyranthus	2.82/**23.4**	0/**0.18**	5.1/**28.1**	0/**0**	0/**0.72**	7.76/**12.40**	0.34/**0.62**	0.13/**0.32**
Bee-pollinated species								
With flower colour change								
L. sessilifolius	2.14/**6.07**	0.02/**1.80**	1.72/**9.31**	0/**2.90**	0/**0.87**	2.47/**3.50**	0.71/**0.87**	6.21/**14.40**
L. mascaensis	1.83/**8.10**	0/**0.90**	1.83/**10.26**	0/**3.99**	0/**2.53**	4.21/**4.36**	2.23/**2.55**	15.48/**15.68**
L. argyrodes	0/**7.97**	0/**5.84**	0/**7.06**	0/**39.2**	0/**6.98**	1.07/**1.51**	2.20/**24.35**	3.70/**15.70**
L. jacobaeus	0/**6.3**	0/**18.64**	0/**26.30**	0/**42.5**	0/**40.6**	2.97/**15.7**	2.43/**5.11**	4.37/**5.17**
L. glaucus	0/**9.5**	0/**2.69**	0/**15.92**	0/**1.97**	0/**6.58**	12.21/**23.12**	1.90/**4.53**	6.23/**15.94**
L. arinagensis	0/**4.5**	0/**0.24**	0/**11.03**	0/**1.62**	0/**3.99**	6.24/**14.32**	0.70/**4.45**	2.39/**8.25**
L. emeroides	0/**4.0**	0/**1.48**	0/**3.7**	0/**3.12**	0/**3.97**	7.79/**8.78**	1.49/**1.16**	5.20/**33.90**
L. tenellus	0.82/**5.35**	0/**0.81**	0.17/**8.96**	0/**1.75**	0/**3.73**	13.78/**11.35**	1.67/**0.87**	6.23/**2.92**
Without flower colour change								
L. spartioides	1.1	0	2.55	0	0	7.38	2.08	9.45
L. callis-viridis	0.04	0	0.04	0	0	2.2	0.84	1.97
L. dumetorum	0.08	0	0	0	0	3.69	1.67	8.08
L. brunneri	0	0	0	0	0	3.95	2.43	1.49
L. campylocladus	0.5	0	0.48	0	0	3.34	2.01	5.69

Table 7.4 Classification of flower reflectance of bee and bird-pollinated flowers according to human and bee perception.

	Anthesis (pre-changed)			After colour change (post-changed)		
Bird-pollinated species	**Human colour perception**	**Bee-flower colour**	**Reflectance**	**Human colour perception**	**Bee-flower colour**	**Reflectance**
L. berthelotii	Red	Uncoloured	u- b- g- r+	-		-
L. eremiticus	Deep red	Uncoloured	u- b- g- r+	-		-
L. maculatus	Orange	Green	u- b/ g+ r+	-		-
L. pyranthus	Orange	UV-green	u+ b- g+ r+	Red	Green	u- b- g+ r+
Bee-pollinated species						
Do not change flower colour						
L. campylocladus	Yellow	UV-green	u+ b- g+ r+	-		-
L. callis-viridis	Yellow	UV-green	u+ b- g+ r+	-		-
L. creticus	Yellow	UV-green	u+ b- g+ r+	-		-
L. dumetorum	Yellow	Green	u- b- g+ r+	-		-
L. brunneri	Yellow	Green	u- b- g+ r+	-		-
Change flower colour						
L. purpureus	Yellow	UV-green	u+ b- g+ r+	Brown	Green	u- b- g+ r+
L. latifolius	Yellow	UV-green	u+ b- g+ r+	Red	Green	u- b- g+ r+
L. glaucus	Yellow	Blue-green	u- b/ g+ r+	Orange	Green	u- b- g/ r+
L. kunkelii	Yellow	Blue-green	u- b/ g+ r+	Red	Green	u- b- g/ r+
L. sessilifolius	Yellow	Green	u- b- g+ r+	Red	Green	u- b- g/ r+
L. arinagensis	Yellow	Green	u- b- g+ r+	Red	Green	u- b- g/ r+
L. mascaensis	Yellow	Green	u- b- g+ r+	Red	Green	u- b- g/ r+
L. tenellus	Yellow	Green	u- b- g+ r+	Red	Green	u- b- g/ r+
L. argyrodes	Yellow	Green	u- b- g/ r+	Purple	Uncoloured	u- b- g- r+
L. jacobaeus	Yellow	Green	u- b- g+ r+	Purple	Uncoloured	u- b- g- r+

Figure 7.1 Red-orange flowers in the bird-pollinated species of the "rhyncholotus group", (A) *Lotus berthelotii* and (B) *L. pyranthus*. Yellow flowers of bee-pollinated species that do not modify flower colour after anthesis (C) *L. campylocladus*, and late-anthetic flowers after colour change in (D) *L. glaucus*, (E) *L. eriosolen*, (F) *L. jacobaeus*, (G) *L. emeroides* and (H) *L. sessilifolius*.

Figure 7.2 Liquid chromatography-mass spectrometry (LC-MS) of bee-pollinated flowers (A) *Lotus spartioides*, a species with yellow flowers that do not modify flower colour after anthesis, (B) pre-change yellow flowers of *L. sessilifolius*, (C) post-change red flowers of *L. sessilifolius* and (D) a bird-pollinated species, *L. berthelotii*, with red flowers.

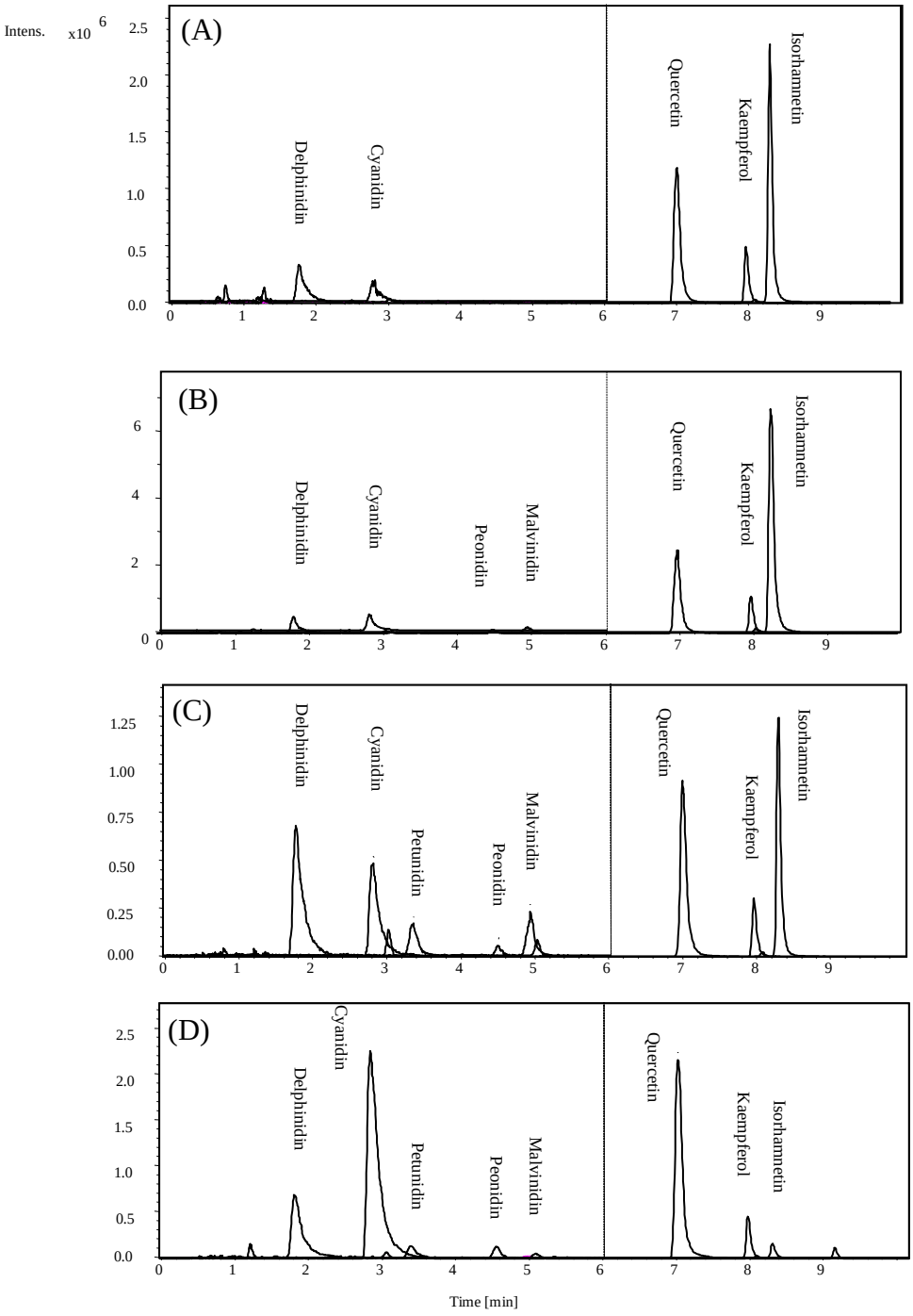


Figure 7.3 Reflectance of yellow bee-pollinated flowers that do not change flower colour (A) *Lotus callis-viridis* with a UV peak (u+ b- g+ r+), a species that modifies flower colour after anthesis (B) *L. latifolius* (yellow and red flowers) , (C) *L. sessilifolius* (yellow and red flowers), and (D) reflectance of *L. berthelotii*, a bird-pollinated species with red flowers.

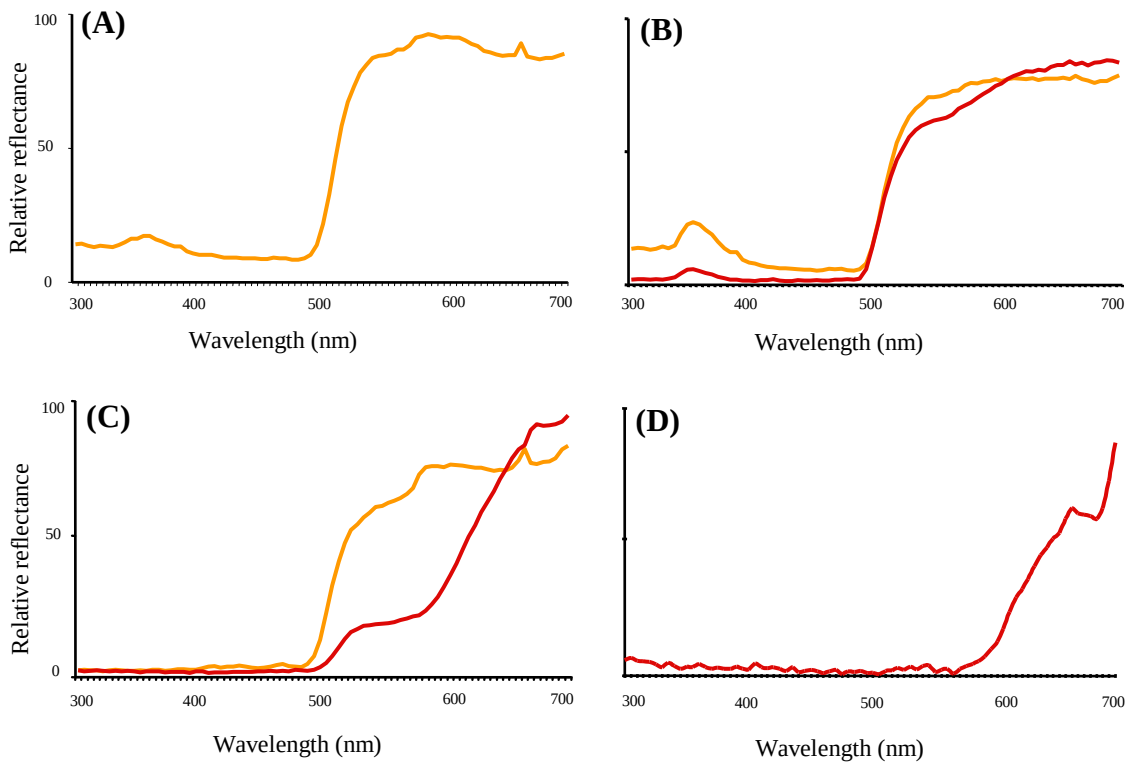


Figure 7.4 Gene expression comparisons of three structural genes of the anthocyanin pathway, *dihydroflavonol-4-reductase* (*LjDFR1, 2, 3* and *5*), *anthocyanin synthase* (*LjANS*) and *O-methyl transferase* (*LjOMT*) at mature stages of flower development. *Ubiquitin* was used as an internal control. Bee-pollinated species of *L. japonicus, L. filicaulis* and *L. sessilifolius*, the two latter with pre-change yellow flowers and post-change red flowers. Bird-pollinated species from the rhyncholotus group with red-orange flowers that do not change colour after anthesis.

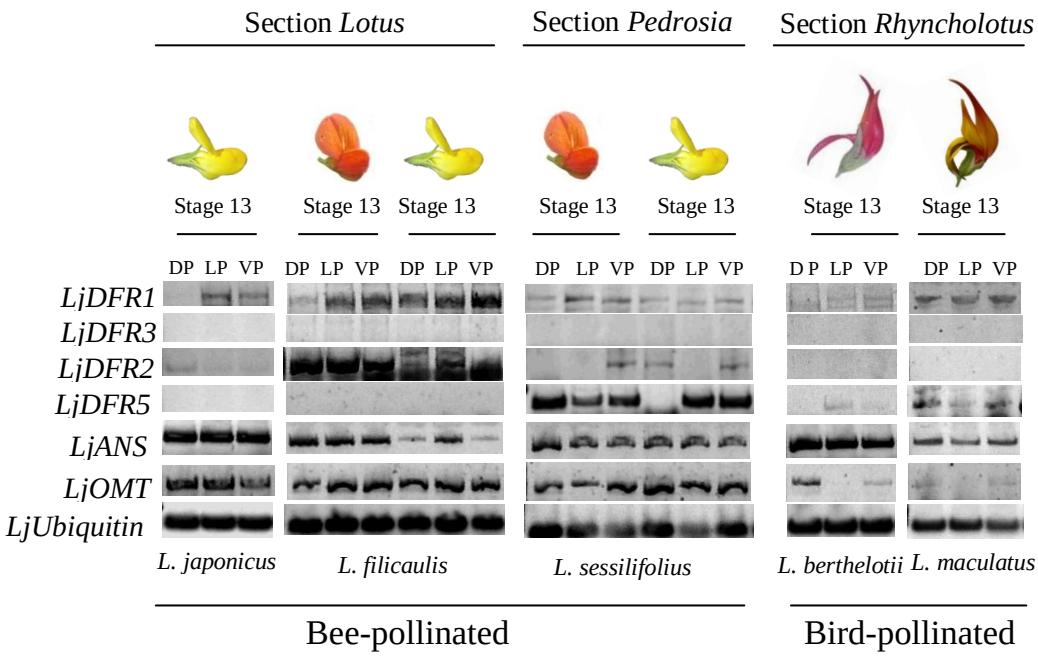


Figure 7.5 Schematic representation of the anthocyanin pathway and the major modifications during the evolutionary transition from yellow flowers (bee pollination) to red flowers (bird pollination) in *Lotus*. Colours at the end of the major pigments indicate the colours produced for each pigment. Bold arrows indicate the pathways active in the bird-pollinated species. The pelargonidin branch is inactive in the three types of petals in this group. Major transitions in pigment composition in bird-pollinated species (1) up-regulation of the cyanidin branch, (2) down regulation of the delphinidin branch and (3) down regulation of flavonol production with a sub sequent modification of flavonol composition. Gray square indicates the branch of the anthocyanin pathway with main pigment production in bird-pollinated species.

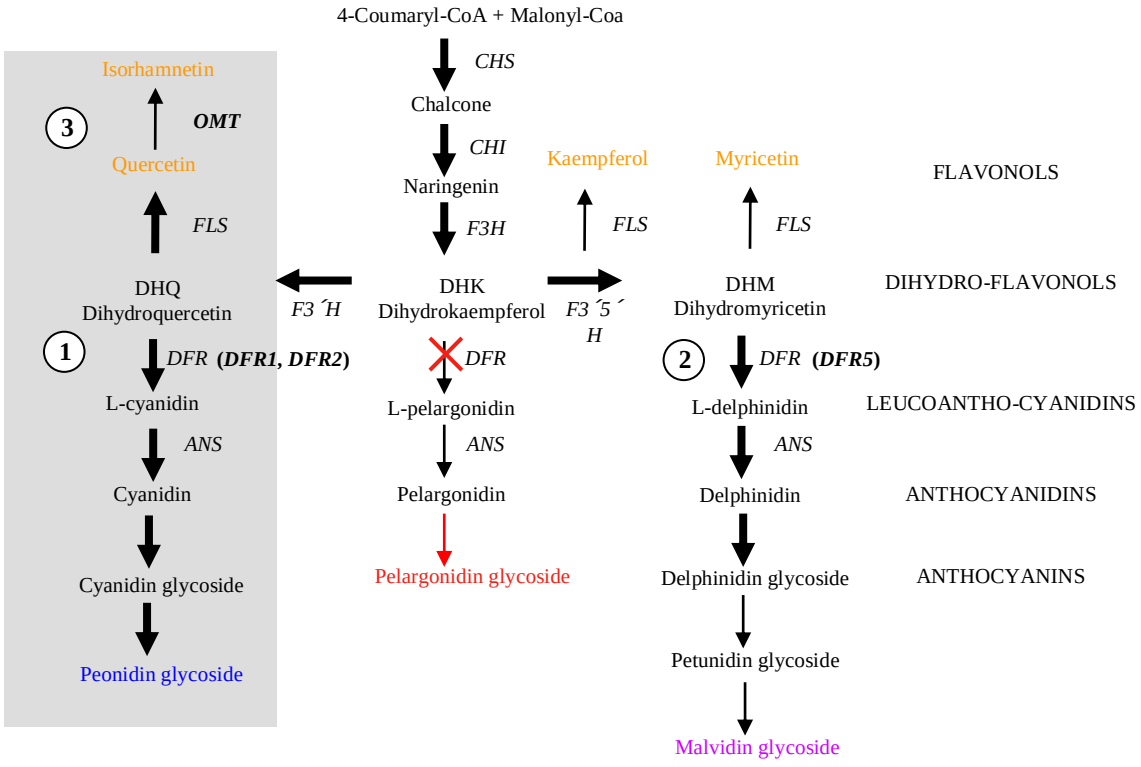
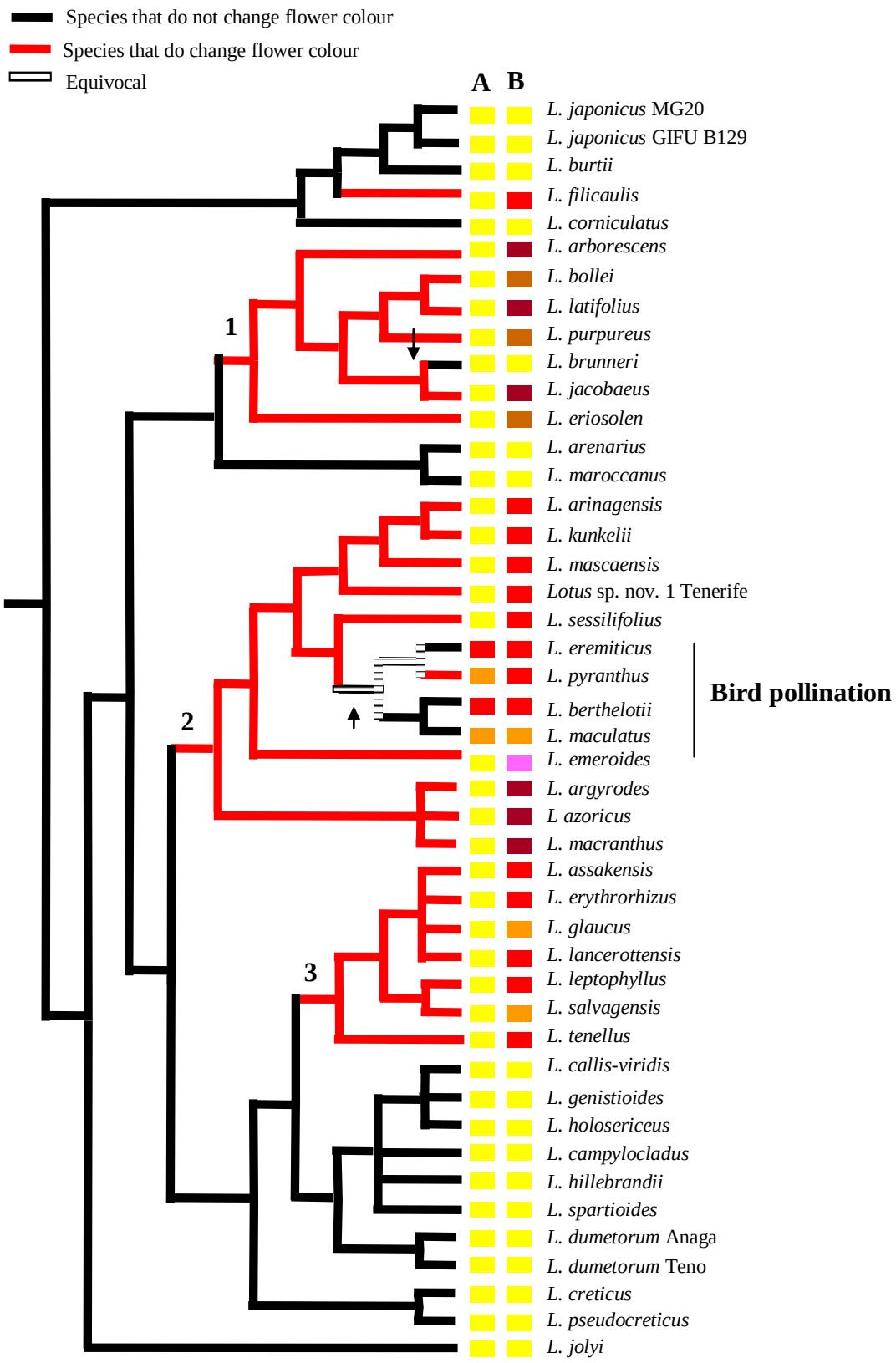


Figure 7.6 Molecular tree based on one nuclear (ITS) and three plastid regions (*CYB6, trnH-psbA* and *matK*). Character mapping of the trait flower colour change after anthesis in *Lotus* sections *Pedrosia* and *Rhyncholotus*. Red branches show clades where this trait has evolved and the numbers on the tree the times this trait evolved within this group (1-3). Arrows indicate the numbers of reversals, one of which occurred in three species of the rhyncholotus group. (A) represents flower colour at anthesis (pre-change) and (B) indicates flower colour after change (post-change).


Species that do not change flower colour
Species that do change flower colour
Equivocal
A
B
L. japonicus MG20
L. japonicus GIFU B129
L. burtii
L. filicaulis
L. corniculatus
L. arborescens
L. bollei
L. latifolius
L. purpureus
L. brunneri
L. jacobaeus
L. eriosolen
L. arenarius
L. maroccanus
L. arinagensis
L. kunkelii
L. mascaensis
Lotus sp. nov. 1 Tenerife
L. sessilifolius
L. eremiticus
L. pyranthus
L. berthelotii
L. maculatus
L. emeroides
L. argyrodes
L azoricus
L. macranthus
L. assakensis
L. erythrorhizus
L. glaucus
L. lancerottensis
L. leptophyllus
L. salvagensis
L. tenellus
L. callis-viridis
L. genistioides
L. holosericeus
L. campylocladus
L. hillebrandii
L. spartioides
L. dumetorum Anaga
L. dumetorum Teno
L. creticus
L. pseudocreticus
L. jolyi
Bird pollination
1
2
3

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8. Conclusion

8.1 Conclusion and future directions

The study of transitions in pollination syndromes has been addressed in closely related species with contrasting flower features(see revison in Thomson and Wilson, 2008). To date several plant groups have been studied using this approach, but only a few flower features have been dissected at the genetic and molecular level (Cronk and Ojeda, 2008; Galliot et al., 2006b). The particular transition from bee (melittophily) to bird pollination (ornithophily) has been studied in at least six genera (*Ipomoea, Aquilegia*, *Mimulus, Penstemon, Silene* and *Costus*), all species adapted to hummingbird pollination (Thomson and Wilson, 2008).

Chapter 2 is an overview of the major trends of floral features associated with bird pollination. I highlighted that some models systems are currently been used to understand the transition in pollinators (from bee to bird pollination). Of all the floral features that are modified during the this transition, flower colour is perhaps the best characterized so far (Rausher, 2008). Three genera, *Ipomoea, Mimulus* and *Aquilegia*, in particular have been used as model systems for the study of the evolution of flower colour and other features (e.g., spur length) during pollination shifts (Bradshaw and Schemske, 2003; Des Marais and Rausher, 2010; Hodges and Derieg, 2009; Rausher, 2008; Streisfeld and Rausher, 2009b). *Petunia* is also a promising system to study pollination transitions. To date, this system has been used to dissect the transition from diurnal bees (*P. integrifolia*) to nocturnal hawkmoths (*P. axilaris*) (Galliot et al., 2006b; Hoballah et al., 2007; Stuurman et al., 2004). *Petunia exserta*, a closely related species of *P. axilaris*, has red flowers that lack scent production and is hummingbird pollinated. This is also a particular interesting species pair currently under investigation (Hermann and Kuhlemeier, 2011).

However, it is worth noting that all the systems mentioned above are hummingbird-pollinated; therefore these systems include only modification from insect to hummingbirds. Bird

pollination has evolved several times under the selective pressure of different types of birds (e.g., honeyeaters in Australia or sunbirds in South Africa), and therefore additional groups that have evolved similar floral features under the selection of different birds are of particular interest to further understand the evolution of ornithophily.

Here, I explored the transition from melittophily to ornithophily in a group of four species of *Lotus* within the Canary Islands. This group of species is putatively pollinated by opportunistic passerines nectar feeders and represents a different system. This group is less studied that the other plant groups mentioned above, but it has some characteristics (easy to propagate in the nursery, short growing season and genomic resources from *Lotus japonicus*) that can contribute to the understanding of pollinations shifts. Most importantly, it is a system that evolved under the selection pressure other than hummingbirds, and therefore could potentially provide new insights about the evolution of bird pollination.

Additional systems from other areas are needed to better understand the evolution of bird pollination. For example, it is estimated that at least 15% of the flora of southwestern Australia is bird pollinated (Keighery, 1982), and about 3-4 % (600-800 spp.) of the flora of South Africa has evolved to this syndrome (Steve Johnson, pers. comm.), yet there are no any single system under investigation under the perspective mentioned in Chapter 2.

In this thesis, I particularly addressed modifications in two floral traits during the transition for bee to bird pollination: petal epidermal micromorphology and flower colour.

My goal in Chapter 3 was to characterize the major epidermal types within the Leguminosae. Before my analysis, the epidermal types reported in this large family were reduced to a few species and without a phylogenetic or evolutionary context. My results provided a general overview of these epidermal types and some evolutionary trends of these epidermal types. Further analyses are needed in particular groups, especially those where I identified transitions of

epidermal types or the loss of characteristic epidermal types in papilionoids. More intensive sampling is also required in particular groups within this family, in particular basal papilionoids and mimosoids. Finally, modifications of epidermal types can be further analyzed in terms of transitions of pollination syndromes (e.g., in *Erythrina*).

Chapter 4 is a combined molecular phylogeny of the Macaronesian *Lotus* (*Pedrosia* and the rhyncholotus group). My goals were to identify the closest relatives of the bird-pollinated species and to estimate the time when this syndrome evolved. I found that bird pollination evolved recently in Tenerife and La Palma, about 1.2 Ma. These four species shared a MRCA about 2 Ma with L. *sessilifolius*. Therefore, these results suggest that ornithophily evolved de novo in this archipelago, likely under the selection of these opportunistic passerine birds. Bird pollination has evolved in at least other five genera within Macaronesia (Olesen, 1985; Valido et al., 2004), and among these groups, it seems that bird pollination in *Lotus* evolved at the same period as in the *Echium* lineage (García-Maroto et al., 2009). The evolutionary transition of pollination syndromes in *Lotus* seems to be associated with the availability of niches within the last 2 Ma, in conjunction with the increase of volcanic activity in Tenerife (ca. 3 Ma) and the emergence of La Palma (1.77 Ma) and El Hierro (1.12 Ma).

I also identified *L. sessilifolius* as the closest bee-pollinated relative of this group. *Lotus sessilifolius* is distributed in four islands (Hierro, La Palma, La Gomera and Tenerife) within this archipelago. However, I was unable to determine if bird pollination evolved once or twice in the rhyncholotus group or the closely related *L. sessilifolius* population within this species complex. More variable regions, such as ISSR, are required to fully understand the evolution of this syndrome in this group.

The low levels of variability observed in this group became more evident when I tested the applicability of six gene regions (*rbcL*, *rpoB*, *trnH-psbA*, *rpoC1*, *matK* and ITS) as barcodes in a

group of 38 species. In Chapter 5, I described my findings on the applicability of these gene regions on species discrimination and conservation in this group. My goal in this chapter was only to test these regions and not to provide a set of regions that could be used in this group or in other recent island radiations. The gene regions *matK* + *rbcL* have recently been suggested for use in combination for species identification (DNA barcoding) (CBOL, 2009).

The Macaronesian *Lotus* group represents a recent island radiation and it provides a particularly difficult challenge for barcoding. Some of these species (10) are of conservation concern and therefore barcoding has practical applications in conservation management. The intergenic region *trnH-psbA* was the most variable and had greatest discriminatory power (18%) of the plastid regions when analyzed alone, but was also the one that showed the most intraspecific variation, a problem that has been suggested before in other plant groups (Edwards et al., 2008). ITS was the best region of all when analyzed alone with a discriminatory power of 26% of the species discrimination. The *matK* region performed poorly in terms of PCR and sequencing success. The recommended combination, *matK*+ *rbcL* identifies to species level only 18% of the species and only one of the 10 endangered species. When combined in pairs, four plastid pair combinations showed slightly better discriminatory power than the recommended combination. The inclusion of ITS increased the number of species identified when combined with each of the chloroplast regions in pairs. When all regions were combined, I identified 52% of the species and 40% of the endangered or threatened species. These results indicate that novel approaches to barcoding will be needed in recently evolved groups such as those of recent island radiations.

Chapter 6 explored modifications in petal epidermal surface between the two contrasting flower morphologies. Bee-pollinated species are characterized by papillose conical cells (PCS) in the exposed areas of the flower. It has been reported before that this epidermal type is widespread

in angiosperms (Kay et al., 1981), but a previous survey (Christensen and Hansen, 1998) was unequivocal in indicating whether bird-pollinated flowers are characterized by PCS. My goals in this chapter were to determine the petal epidermal modifications between bee- and bird-pollinated species in Macaronesian *Lotus* and associate this shift in pollination syndrome with the expression of three petal identity genes. I found that bird-pollinated *Lotus* species (rhyncholotus) have lost PCS in dorsal petals and most of the surface of the three petals is covered by tabular rugose cells (TRS). These species only have PCS in a highly localized area of the lateral petals, which is exposed to birds. The complete lack of PCS in the dorsal petal in the rhyncholotus group is associated with a down regulation of *LjCYC2* (dorsal identity gene). All bee-pollinated species I analyzed have an early expression of this identity gene. My results also indicate that the closest relative of the rhyncholotus group might have a molecular pre-adaptation. *LjCYC3*, the lateral identity gene, is expressed early in both *L. sessilifolius* and the two rhyncholotus species I tested, but not in the other bee-pollinated species of *Lotus*. This strong early expression of the lateral petal identity gene is also associated with an increase of TRS in the rhyncholotus and its closely related species, *L. sessilifolius.*

Therefore while a heterochronic contraction of *LjCYC2* expression is directly associated with the evolution of bird pollination, a heterochronic expansion of *LjCYC3* in the clade in which bird pollination evolved may have been a facilitating molecular pre-adaptation. Additional species within the *L. sessilifolius* group (*L. arinagensis, L. mascaensis, L. emeroides* and *L. kunkelii*) can also be analyzed to corroborate these findings. Besides this study, there are no other similar analyses that compared closely related species with contrasting morphologies; therefore more examples are needed to determine if transitions from bee to bird pollination are characterized by a lack of PCS. The five species of *Erythrina* I analyzed in Chapter 3 have papillose conical cells (either PKR or PCS) on all three types of petals. However, *Erythrina* is

completely pollinated by birds, either hummingbirds or passerine birds (Bruneau, 1997), therefore more detailed analyses from sister species with contrasting pollination syndromes are further required.

Species pairs within *Ipomoea*, *Aquilegia*, *Penstemon*, *Mimulus, Silene*, *Salvia* and *Costus* where several transitions have occurred within each genus are of potential interest. Additionally, a more widespread analysis, including species from several regions can also provide a more complete picture of the trends in petal epidermal micromorphology modifications during pollinator transitions.

Additional genes known to be involved in PCS differentiation (such as *MIXTA* in snapdragon) (Comba et al., 2000) and trichome differentiation (e.g., *GLABRA1*) deserve further examination within this group. Homologues copies of *MIXTA* have been isolated in Legumes (Beverly Glover, pers. comm.) and could provide a better understanding of the transition of epidermal types in Macaronesian *Lotus*.

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Finally, in Chapter 7 I studied modifications in flower color between the two syndromes. Red flowers are one of the main floral traits usually associated with bird pollination. In Macaronesian *Lotus*, bird-pollinated species are red-orange while bee-pollinated are yellow, at least before modifying flower color after anthesis. I found that 58% of the species within *Pedrosia* have the ability to modify flower color after anthesis. Yellow flowers can change to red, purple, brown, orange and pink post-anthesis colours, likely as a strategy to increase foraging efficiency of insect pollinators, as shown in other plant groups (Eisikowitch and Rotem, 1987; Gori, 1983; Jones and Cruzan, 1982; Oberrath and Böhning-Gaese, 1999; Willmer et al., 2009). My objectives in this chapter were: (1) to determine if the modification in flower colour is perceived differently by pollinators, (2) to establish the pigments modifications during the

transition to bird pollination, and (3) to determine whether genes in the biosynthetic pathway have differential expression patterns during the transition.

My reflectance analyses from yellow and post-anthesis flower colors within the same species showed that the two colour types are perceived differently by bees, and likely this affects flower visitation. The red-orange bird-pollinated flowers have the typical reflectance reported for other bird-pollinated species, which suggests that bees will have difficulty to distinguish them from the green background (Arnold et al., 2008; Arnold et al., 2010; Chittka et al., 1994; Chittka and Waser, 1997; Ollerton et al., 2009). This evidence suggests that the red/orange colours of these bird-pollinated flowers may have evolved as a deterrent (anti-bee) trait, rather to attract birds.

From my results of the mapping of the evolution of flower colour, it seems that the clade in which bird pollination evolved had a pre-adaptation to modify flower color. I found that the pigments involved in the modification of these colours are already present in the yellow flowers before the modification of flower colour. The modification of flower colour within the bee-pollinated species is due to an increase of anthocyanidin production with no modification in flavonol composition.

In contrast, the transition from bee to bird pollination required the redirection of anthocyanidin production (especially the cyanidin branch) together with a modification of flavonol composition (the main flavonol in bee-pollinated species is isorhamnetin); bird-pollinated flowers have more quercetin than isorhamnetin derivatives. Therefore, the rhyncholotus group evolved the red /orange flowers maintaining the same pathways active but modifying the flux and composition between anthocyanidins and flavonols. Thus, the evolution of red/orange flowers in bird-pollinated species appears to be a heterochronic modification of

pigment production. Bird-pollinated species have red flowers from early developmental stages, instead of a late expression due to modification of flower color after anthesis.

On the other hand, transitions from blue (bee-pollinated) to red (bird-pollinated) in species of *Ipomoea*, *Mimulus* and *Penstemon* (Rausher, 2008) required the inactivation/activation of different branches of the anthocyanidin pathway. In *Ipomoea*, for instance, the cyanidin branch (responsible for blue pigments) is inactivated and the down regulated *f3h* gene has apparently accumulated structural mutation, while *DFR* has evolved a greater specificity to the precursor leading to pelargonidin (responsible for red colors) (Zufall and Rausher, 2004).

In the particular case of Macaronesian *Lotus*, the expression patterns of *ANS* and *DFR* are in agreement with the pigments I identified in the petals, providing further evidence that in this particular group only two branches, the cyanidin and delphinidin are active in this group. I also found evidence that might suggest that *LjOMT*, responsible to convert quercetin to isorhamnetin, is down regulated in the two bird-pollinated species. Finally, it seems that there is a specialization in some of the different copies of *DFR* in *Lotus*. I found that *DFR2* is down regulated in the two bird-pollinated species and might indicate that this copy has more specificity to the delphinidin branch.

The expression patterns reported here should be considered as preliminary results and further analyses are required to fully understand this colour modification. One future avenue of further research could be the inclusion of *FLS*, responsible for flavonol production, as well as the further characterization of *ANS* in *Lotus*. Only *DFR* has been fully characterized in *Lotus japonicus* (Shimada et al., 2005), however, it is not known yet the number of copies in *LjOMT* and *ANS*..

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