Analysis of DNA Variations in Genes under Selection on the Mountain Pine Beetle (MPB) Fungal Associates

By

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Abstract:

The Mountain Pine Beetle (MPB), *Dendroctonus ponderosa* Hopkins, is a small bark beetle that has affected over 13 million hectares of forests in western Canada and the USA since 1990, and more recently has affected forests at higher elevations and more northern latitudes (Logan et al. 2001). The MPB attacks the trees alone; it has a mutualistic association with fungal species that are mainly in the Ophiostomoid family of the Ascomycota phylum, which provides many biological benefits (Lee et al. 2006a). To develop a better understanding of the biology and to provide information for the development of MPB growth models, single-nucleotide polymorphisms (SNPs) were identified from multiple candidate *Grosmannia clavigera* DNA samples. *G. clavigera* is one of the mutualistic fungal species, and DNA samples were used from various populations in western North America. PCR using DNA oligonucleotides was the main method to obtain the amplified genes for sequencing. After sequencing at Laval University, Geneious software was used to contig, edit, and align sequences. Using concatenated sequences, SNPs that were found within the adaptive genes were used to construct three different phylogenetic diagrams. The geographical locations of the populations tested in the phylogenetic analysis were compared to the genetic distance of the different samples. The data shows that there is a trend where populations of *G. clavigera* that are further apart geographically have a greater genetic distance, and populations that are geographically closer together share similar SNP variations.

Keywords:
Fungal phylogenetic, phylogeography, Mountain Pine Beetle (*Dendroctonus ponderosae*), Fungal symbionts, *Grosmannia clavigera*

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1. Introduction

1.1 The Recent Mountain Pine Beetle Epidemic

The Mountain Pine Beetle (MPB), *Dendroctonus ponderosa* Hopkins, is a small bark beetle that has affected greater than 13 million hectares of forests in western Canada and the USA since 1990, and more recently has affected forests at higher elevations and more northern latitudes (Logan et al. 2001). The MPB has in the most recent outbreak crossed the Rocky Mountain barrier, and has thus started to invade hybrid lodge pole-jack pine hybrid (*Pinus contorta* var. *latifolia-Pinus banksiana*) stands in forests that are largely a part of the boreal jack pine forests that span across the entire continent east of the Rocky Mountains (Safranyik and Carroll 2006). This insect has highly influenced, and will most likely continue to influence, the forest management practices used in the industry.

1.2 Mountain Pine Beetle and Fungal Symbiont Biology

The MPB does not enter the tree alone; it has a mutualistic association with fungal species that are mainly in the Ophiostomoid family of the Ascomycota phylum, largely in part because this type of organism provides many biological benefits (Lee et al. 2006). This tiny bark beetle species carries parts of blue stain fungi such as the spores within its mycangia, which are like sacs that reside on the maxillary cardines of both the male and female beetles (Whitney and Farris 1970). The three main species of the fungal associates of the MPB are: *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wingfled; *Leptographium longiclavatum* Lee, Kim and Breuil; and *Ophiostoma montium* (Rumbold) von Arx (Lee et al. 2005).

When the adults are able to enter the phloem tissue successfully, the females create brood galleries in the phloem tissue and inoculate the cambium with the blue-stain fungi spores (Six and Klepzig 2004). As the blue-stain fungus mycelium grows and spreads through the sapwood, it can be used as both a food source for the larvae (Six and Paine 1998) and can also aid the beetles in restricting the tree's defenses, which help the beetle to weaken the host tree (Raffa and Berryman 1983).

1.3 Phylogeography of *Grosmannia clavigera*

There is a significant correlation in the population structure between the MPB and its fungal symbionts, which reveals that there is an intimate relationship between the two (Tsui et al. 2014). This type of intimate relationship can be described as obligate since the beetle is the only vector by which the fungus can move across the landscape and into host trees, so it has an obligate dependency on the beetle for movement (Roe et al. 2011). Within this circumstance the phylogeographic structure of the fungi ends up reflecting the northern and southern populations in its range and shows congruent patterns of genetic diversity (Roe et al. 2011). It has also been determined that over a broad scale genetic analysis that there were populations structured congruently across the three previously described species of blue-stain fungi (Roe et al. 2011).

When the population structure was determined for *Grosmannia clavierga* by Tsui et al. (2012) the presence of repeated multilocus haplotypes in the regional populations and the linkage disequilibria in six of the regional populations indicated that clonal propagation is the most likely form of reproduction (Tsui et al. 2012), even though it was determined that this species is capable of sexual reproduction

(Tsui et al. 2009). Sexual recombination of alleles may be an important contributor of the high genetic diversity that has been observed in the *Grossmannia clavigera* populations (Tsui et al. 2012). By knowing that this species is capable of sexual recombination of genes, there is an inherent potential for adaption.

1.4 Analysis of Single-nucleotide Polymorphisms within Selected Genes

The study of single-nucleotide polymorphisms (SNPs) can be very useful for identifying DNA markers for evolutionary studies, especially for non-model organisms (Ojeda et al. 2014). Specially designed primers for *L. longiclavatum* were previously made and used to target SNPs in potentially adaptive genes that may play a role in pathogenicity towards the host (Ojeda et al. 2014). In my study, after selecting one individual to represent each distinct population of *Grosmannia claviegera*, I used the primers from Ojeda et al. (2014) to test for SNP genetic variation within specifically selected genes. By comparing inter-population variation of single-nucleotide polymorphisms it can be determined if the geographical distance between populations is also matched by the amount of relative genetic variation.

2. Materials and Methods

2.1 DNA samples used in the analysis

I obtained 13 specific DNA gene sequences from 12 individual samples of previously extracted DNA. Each sample used represents a different population of the species *G. clavigera.* The DNA samples that I amplified are described in the following table.

Table 1. DNA samples selected to be amplified and used for study

Species	Country, Province/State, Locality	Host/Substrate	Internal Code/DNA code	Date Collected
Gc	Canada/BC, Sparwood	*Pinus contorta*/Larvae	M002-12-03-06-UC30DL32/ss500	12-April-08
Gc	Canada/BC, Fairview	*P. contorta*/larvae	M001-13-01-06-UC09DL35/ss235	03-April-07
Gc	Canada/Bc, Willmore-Kakwa	*P. contorta,* wood	M002-02-02-03-UC11G1/4ss433	26-March-08
Gc	Canada/BC, Tumbler Rdidge	Not Available	M024-08-01-06/TRIA295	30-June-2010
Gc	Canada/BC, Smithers	Beetle Larvae	M024-01-01-01/TRIA35	18-May-2010
Gc	USA/Utah, Logan Canyon	*Pinus flexilis*	M033-04-03-01/TRIA655	14-June-2011
Gc	USA/Idaho, Boise	*P. contorta,* Ploeam B	M033-10-01-03/TRIA783	19-July-2011
Gc	Canada/BC, Kootenay/Yoho	*P. contorta,* wood	M002-16-01-05-UC32G34/ss484	05-December-2007
Gc	USA/Montanan, Greenough	*Pinus ponderosa*	M033-05-02-06/TRIA944	24-October-2011
Gc	USA/South Dakota	*P. ponderosa*	M033-01-01-15/TRIA500	28-June-2011
Gc	USA/Utah, Unitas	*P. ponderosa*	M033-04-02-03/TRIA 667	9-June-2011
Gc	USA/Washington, Okanogan-Wenatchee	*P. albicaulis*	M033-11-01-04/TRIA838	26-July-2011

The remaining set of data used in the phylogeographic analysis were obtained from the lab that made used the *G. calvigera* DNA samples to test the primers that were used to find prove the presence of SNPs (Ojeda et al. 2014).

Table 2. DNA oligonucleotides targeting 10 genes of the MPB fungal associates. The genes were selected based on their expression during growth on host tissue

Contig in *G. clavigera* reference genome	*Leptographium* genome reference	Primer Sequence (5'-3')	Sequence description	Product size (bp)
GLEAN_6904	39304_66970_4784777.g2 1	6904F TGAAAGGACAGCAGGACAGTGGAA 6904R TCGCCATGCTCATCCTTGCTATCT	Short-cahin dehydrogenase reductase	416
GLEAN_2132	39304_66970_4784777.g1 6	2132F TATGTCCGGCCTATGTCTTTGCGT 2132R GCGGGTTAGCATTTAGCAGCACAA	Methyl transferase type 12	764
GLEAN_7264	38895_58241_3874774.g1	7264F ACAACGATTTCTGTGACCCTCGGA 7264R ACGAGTCATTCTGCTCGTCCGAAA	C2 h2 transcription factor	528
GLEAN_8030	36886_27385_1704050.g8	8030F AACCTCGTCATCTTCCTTGTGCTT 8030R TGTCACCAACAACAAAGTCGGCAC	ABC transporter	718
GLEAN_6897	39304_66970_4784777.g1	6897F AAGCACACAGTCCACAAACACAGC 6897R TGACTTCGTACTCGCTCAACAGCA	Fungal-specific transcription factor domain protein	581
GLEAN_7953	39012_19536_1576951.g6	7953F TCTCGACGCAGATGATCTTTGGCT 7953R AGCGCTGGAAGGAATAACTCACCA	Cytochrome P450 momooxygenase	795
GLEAN_5485	38547_30050_2163587.g3	5485F CAATTTCTTCTACGCTGGACGCGAT 5485R TGGCGCAGTAGATGGTACATGACA	Cytochrome P450 monooxygenase	477
GLEAN_2141	39304_66970_4784777.g3	2141F TCAAGGACAAGACCAAGGTGCTCA 2141R TTTCGTGTACGTCGGGATGTCGTT	FAD-binding protein	503
GLEAN_684	36332_15963_1175472.g5	684F CTTTGAAACCTECGCGTGTGCATGA 684R TTGCACAATGGCAACAGGTGTCTC	Lignostilbene diooxigenase	499

Another gene that was amplified from each sequence was GLEAN_8030F1 made by Ojeda et al. at the University of British Columbia. The other 4 sets of DNA oligonucleotides that were used were alpha tubulin, BT/BT12 (beta tubulin), UMF1, and EF (elongation factor) from Ojeda et al. group. These four genes are considered to be the 'housekeeping' genes, and they occur with a high frequency within the genome of *G. clavigera* (Ojeda et al. 2014). Each PCR reaction that was done to amplify the genes was done so with all of the DNA samples and one set of primers at a time in this study.

2.2 Method used to amplify the DNA

PCR Protocol:

For the primers T2/BT12, alpha tubulin, and GLEAN_6904, the protocol that successfully amplified all of the different DNA samples was 95 °C for 3 minutes, (95°C for 30 seconds, 57 °C for 1 minute, 72°C for 1 minute) x30 cycles, 72°C for 5 minutes. The resting temperature was set at 4°C. This protocol did not work successfully for the other primers on any of the DNA samples, so to trouble shoot the PCR protocol the RT PCR machine with a gradient setting was used. The TB/BT12 primer (with samples ss500, TRIA655, and TRIA783), the alpha tubulin primer (with TRIA500 and TRIA677), and primer 6904 (with TRIA500) at the annealing temperatures varying in the PCR machine settings. One lane was 55°C, 57°C, 60°C, 61°C, 62°C, 63°C. The annealing temperature that lead to the most product being produced was 61°C, so it was used for the rest of the PCR runs.

PCR machine and master mixes:

Using a Bio-Rad PCR machine, the master mix I used for each primer was:

(each unit is multiplied by the number of samples, plus one extra unit, for each primer)15 µl water, 2.5µl buffer, 0.75 µl dNTPs, 0.5 µl MgCl, 2.5 µl forward primer, 2.5 µl reverse primer, and 0.2 µl Taq polymerase. For each sample 3 µl DNA with 22 µl of the master mix. The positive control that was used was the DNA sample TRIA 409.

Genetic Analysis:

The software used to edit the DNA sequences and create the phylogenetic trees was Geneious. The genetic analysis was done first by creating consensus sequences and then creating nucleotide alignments for each set of samples for each gene. The DNA gene sequences were then concatenated for each sample. The concatenated nucleotide alignment was used to create the phylogenetic trees. The genetic distance model for creating the phylogenetic diagram was the Tamura-Nei model, the tree build method selected was the neighbor-joining method, and there was no outgroup selected for these particular trees.

3. Results

3.1 DNA that was successfully sequenced

Majority of the genes were sequenced from each sample. Using these sequences, three different concatenated sequences were made for each sample: all genes, 'housekeeping' genes, and GLEAN genes. The following table lists the two main groups of genes and the samples that were used to create the phylogenetic diagrams.

Table 3. DNA samples that were sequenced and used to make concatenated sequences

GLEAN genes	'Housekeeping' genes
SS500 Canada/BC, Sparwood SS235 Canada/BC, Fairview TRIA 35 Canada/BC, Smithers SS433 Canada/AB, Kakwa TRIA 295 Canada/BC, Tumbler Ridge TRIA 655 USA/Utah, Logan Canyon TRIA 783 USA/Idaho, Boise TRIA 838 USA/Washington, Okanagan-Wenatchee SS484 Canada/BC, Yoho TRIA 944 USA/Montana, Greenough TRIA 667 USA/Utah, Unitas	TRIA 500 USA/South Dakota, Black Hills TRIA 655 USA/Utah, Logan Canyon SS235 Canada/BC, Fairview TRIA 667 USA/Utah, Unitas TRIA 783 USA/Idaho, Boise TRIA 838 USA/Washington, Okanagan-Wenatchee SS484 Canada/BC, Yoho TRIA 35 Canada/BC, Smithers TRIA 944 USA/Montana, Greenough SS500 Canada/BC, Sparwood SS433 Canada/AB, Kakwa TRIA 295 Canada/BC, Tumbler Ridge

When all genes were concatenated together the size of each gene and the number of SNPs were recorded, as seen in the following table.

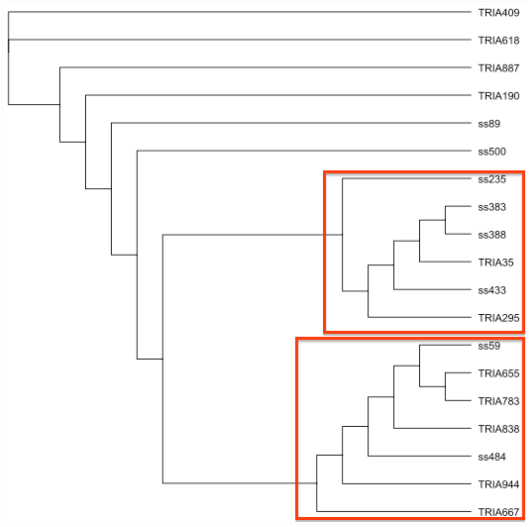
Table 4. Gene size and number of SNPs in Concatenated data for all genes for *G. clavigera* samples

Gene Name	Size (bp)	Number of SNPs
GLEAN_2132	726	3
GLEAN_2141	427	0
GLEAN_5485	452	0
GLEAN_6897	552	1
GLEAN_6904	353	1
GLEAN_7264	489	0
GLEAN_7953	721	0
GLEAN_8030	791	0
GLEAN_8030F1	685	0
Alpha tubulin	751	6
Beta tubulin	1116	>10
EF	667	>10
UMF1	443	5

All of the GLEAN_684 sequences that were retrieved had very poor quality and thus they were eliminated from the analysis at this stage, and were not included in the final phylogenetic diagrams.

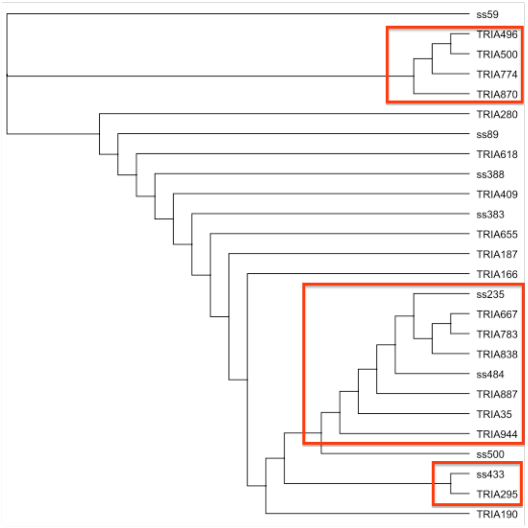
3.2 Phylogenetic diagrams

Three phylogenetic diagrams were created using the concatenated gene sequence data: all genes, 'housekeeping' genes, and GLEAN genes. Each tree contains certain regions that show specific individuals from different populations that have short genetic distance between the two of them compared to the genetic distance of neighboring sequences in the phylogenetic tree.



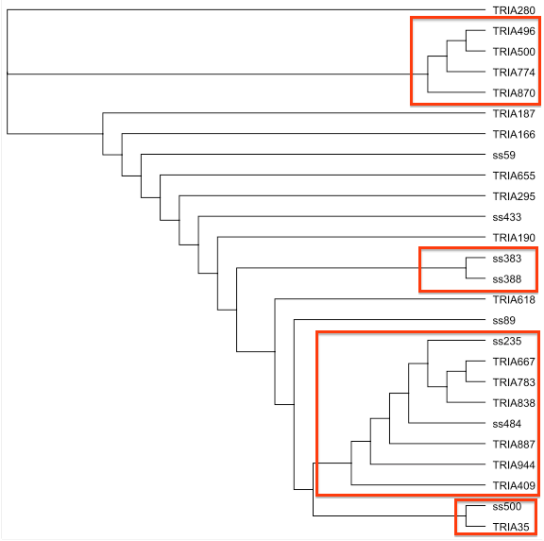
TRIA 409 USA/Colorado, Fort Collins
TRIA 618 USA/Wyoming, Bridger
TRIA 887 USA/Nevada, Rose Mount ski resort
TRIA 190 Canada/BC, Terrace
ss89 Canada/AB, Crowsnest Pass
ss500 Canada/BC, Sparwood *
ss235 Canada/BC, Fairview *
ss383 Canada/BC, Valemount
ss388 Canada/BC, Golden
TRIA 35 Canada/BC, Smithers *
ss433 Canada/AB, Kakwa *
TRIA 295 Canada/BC, Tumbler Ridge *
ss59 Canada/AB, Grande Prairie
TRIA 655 USA/Utah, Logan Canyon *
TRIA 783 USA/Idaho, Boise *
TRIA 838 USA/Washington, Okanagan-Wenatchee *
ss484 Canada/BC, Yoho *
TRIA 944 USA/Montana, Greenough *
TRIA 667 USA/Utah, Unitas *

Figure 1. Phylogenetic tree showing relatedness between concatenated data of GLEAN gene sequences, and the locality of each sequence. Asterisks (*) denote samples that were sequenced during the time of this study.



ss59 Canada/AB, Grande Prairie
TRIA 496 USA/South Dakota, Black Hills
TRIA 500 USA/South Dakota, Black Hills *
TRIA 774 USA/California, Moldoc NF
TRIA 870 USA/Wyoming, Bridger NF
TRIA 280 Canada/BC, Terrace
ss89 Canada/AB, Crowsnest Pass
TRIA 618 USA/Wyoming, Bridger NF
ss383 Canada/BC, Valemount
TRIA 655 USA/Utah, Logan Canyon *
TRIA 187 Canada/BC, Peachland
TRIA 166 Canada/BC, Peachland
ss235 Canada/BC, Fairview *
TRIA 667 USA/Utah, Unitas *
TRIA 783 USA/Idaho, Boise *
TRIA 838 USA/Washington, Okanagan-Wenatchee *
ss484 Canada/BC, Yoho *
TRIA 887 USA/Nevada, Mount Rose Ski Resort
TRIA 35 Canada/BC, Smithers *
TRIA 944 USA/Montana, Greenough *
ss500 Canada/BC, Sparwood *
ss433 Canada/AB, Kakwa *
TRIA 295 Canada/BC, Tumbler Ridge *
TRIA 190 Canada, BC/Terrace

Figure 2. Phylogenetic tree showing genetic relatedness between concatenated data of 'housekeeping' gene sequences, and the locality of each sequence. Asterisks (*) denote samples that were sequenced during the course of this study.



TRIA 280 Canada/BC, Terrace
TRIA 496 USA/South Dakota, Black Hills
TRIA 500 USA/South Dakota, Black Hills *
TRIA 774 USA/California, Moldoc
TRIA 870 USA/Wyoming, Bridger
TRIA 187 Canada/BC, Peachland
TRIA 166 Canada/BC, Peachland
ss59 Canada/AB, Grande Prairie
TRIA 655 USA/Utah, Logan Canyon *
TRIA 295 Canada/BC, Tumbler Ridge *
ss433 Canada/AB, Kakwa *
TRIA 190 Canada/BC, Terrace
ss383 Canada/BC, Valemount
ss388 Canada/BC, Golden
TRIA 618 USA/Wyoming, Bridger
ss89 Canada/AB, Crowsnest Pass
ss235 Canada/BC, Fairview *
TRIA 667 USA/Utah, Unitas *
TRIA 783 USA/Idaho, Boise *
TRIA 838 USA/Washington, Okanagan-Wenatchee *
ss484 Canada/BC, Yoho *
TRIA 887 USA/Nevada, Mount Rose Ski Resort
TRIA 944 USA/Montana, Greenough *
TRIA 409 USA/Colorado, Fort Collins
ss500 Canada/BC, Sparwood *
TRIA 35 Canada/BC, Smithers *

Figure 3. Phylogenetic tree showing genetic relatedness between concatenated data of all gene sequences, and the locality of each sequence. Asterisks (*) denote samples that were sequenced during the course of this study.

The red squares on all three of the phylogenetic trees show areas where the sequences underwent the most evolutionarily recent changes. Some figures show areas where genetic distance is significantly shorter then the others relative to the outgroup individual. In Figure 1, the top square contains six individuals, five from British Columbia and 1 from Alberta. Most of these samples are from localities that are within close proximity to the Rocky Mountain Range. The shortest genetic distance is seen between the Valemount, BC and the Golden, BC individuals, with other individuals from BC showing some genetic relatedness. In the bottom square the majority of the individuals that show recent genetic changes are from the USA.

In Figure 2, the top square has two individuals from Black Hills, South Dakota that have the most recent genetic change, and the next closest individuals are also from localities within the USA. The middle square on the phylogenetic tree has a mixture of localities present, but the shortest genetic distance can be seen between two American samples, one from Unitas, Utah and the other from Boise, Idaho. The bottom square in Figure 2 has two more northern localities that are more genetically distinct from the outgroup, Tumbler Ridge, BC and Kakwa, AB.

In Figure 3, when all of the genes are considered, some of the genetic distances change. For example, ss500 and TRIA 35 had larger genetic distances in Figure 1 and Figure 2, but in Figure 3 the two stem from a node that is representative of a recent genetic change. Other patterns that exist from Figure 1 and Figure 2 do show up again in Figure 3, such as the clad containing ss383 and ss388.

Overall in all three of the figures the longer branches that are outside of the red boxes tend to belong to genetic populations that are associated with geographical locations that are more distant from the geographical location of the genetic populations that are within the red boxes.

4. Discussion

While microsatellite markers have been used very effectively in the past, and continue to be effective, the identification of SNPs is increasingly becoming the marker of choice for large-scale population studies (Ojeda et al. 2014). The study of single-nucleotide polymorphisms (SNPs) can be very useful for identifying DNA markers for evolutionary studies, especially for non-model organisms (Ojeda et al. 2014). For non-model organisms that have a significant impact on both the ecological and economic sustainable of forest ecosystem, such as the MPB, it is important to conduct studies that can aid in the development of models to best predict future scenarios.

Specially designed primers for *L. longiclavatum* were previously made and used to target SNPs in potentially adaptive genes that may play a role in pathogenicity towards the host (Ojeda et al. 2014). In this study these primers were used to sequence genes from individuals representing various *G. clavigera* populations to identify if any SNPs were present and if they exhibited a geographic pattern. It was discovered that some SNPs, which are the result of recent evolutionary changes in the genetic structure, are shared between different populations that are geographically close together.

There are some theories as to how genetic variation is influenced in the *G. clavigera* species. Sexual reproduction of fungal species is a main source of genetic recombination, with asexual propagation spreading the new recombinants through via clones (Roe et al. 2011). While sexual recombination could potentially be responsible for the establishment of the SNP pattern across the species range it could also be responsible for a decrease in genetic variation if the MPB is preferentially selecting spores to carry, which would be a form of non-random selection (Roe et al. 2011). Studies to determine sexual and asexual reproduction regimes are needed to provide information for the mechanisms of genetic variation and pattern of distribution within a species.

While it is too soon to suggest ways that the SNP variation discovered was caused, the variation still remains. Populations that are close in proximity tend to share similar SNPs from evolutionary events in recent history. Since these polymorphisms reside in functional genes, the potential for functional adaption to different environments, competition, or hosts is possible.

5. Conclusion

Single-nucleotide polymorphisms are shared in common between individuals of different genetic populations that are from areas that are geographically close. To further the assessment of SNP variation between populations of *G. clavigera,* it is suggested that multiple individuals from a population are used rather then just one single individual. While the single individuals were chosen at random, using multiple samples can provide a better statistical analysis to annotate the

phylogenetic diagrams with, and to provide a quantitative amount of genetic relatedness.

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