MOLECULAR PHYLOGENETICS OF THE HAWAIIAN GERANIUMS

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ABSTRACT

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Pax *et al.* (1997) successfully applied molecular phylogenetics to confirm the monophyly of the Hawaiian *Geranium* lineage and identified an American origin for the lineage. However, this data from *rbc*L (a conserved, slowly evolving chloroplast gene) was insufficient for resolution of the pattern of radiation. The objective of this study was to determine the phylogeny of the Hawaiian *Geraniums* using internal transcribed spacer (ITS) regions of nuclear ribosomal DNA, the noncoding chloroplast (cpDNA) *trn*L-F region, and inter-simple sequence repeats (ISSR), which are more rapidly evolving molecular markers, in order to understand the pattern of speciation within the group. In this study of the evolution of the lineage, the following hypotheses were tested:

1) G. arboreum is the basal member of the lineage. This hypothesis is based on leaf morphology. Features such as trichome structure, size of lamia, and dentate toothing along the entire edge suggest a more basal position than any other species.

2) The three bog species (*G. hanaense, G. hillebrandii,* and *G. kauaiense*) represent a single radiation into a common habitat type on three islands. This has been suggested by morphological analysis (Funk and Wagner 1995).

3) *G. cuneatum* ssp. *tridens*, the only subspecies occurring on Maui, is genetically divergent enough to elevate it to specific status as suggested by Funk and Wagner (1995).

4) Maui, where the most taxa occur, is the island of the primary colonization event despite it being the second youngest island. The *Geraniums* radiated from Maui to

Hawai'i and Kaua'i, representing at least one colonization event from a younger, eastern island to an older, western island.

Results indicated that a clade comprised of *G. arboreum* and *G. c. hypoleucum* occupied the basal position in trees produced by the combined ITS and *trn*L-F data as well as ISSR data. *G. c. tridens* sequences did not provide enough evidence to suggest it should be elevated to specific level. Sequence data do not even strongly support the designation of the *cuneatums* as subspecies. None of the analyses placed all three bog species in a clade to suggest they underwent a single radiation into this habitat. Therefore, the bog species appear to have undergone convergent evolution of morphological traits that enable them to adapt to flooded conditions. The frequently occurring "conveyer belt" mode of colonization and radiation from oldest island to newest island is not supported. *G. kauaiense*, the only species occurring on Kaua'i, is definitely nested and not basal. The data suggests that Maui, Hawai'i, or an older now-submerged island is the origin of the radiation.

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Thanks to my Mom and Dad, *być dumnym z twój córka*.

Thanks to my husband Scott, *l'amo per sempre*.

Thomas Huxley's reaction to reading *Origin of Species* was

"How stupid of me not to have thought of that."

"Science is a wonderful thing if one does not have to earn one's living at it."

- Albert Einstein

"Evolution is cleverer than you are."

- Francis Crick

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INTRODUCTION

The genus *Geranium* comprises about 300 species and has a generally cosmopolitan distribution (Áedo *et al.* 1998). Genus *Geranium* is divided into three subgenera: *Eroideae*, *Robertanium*, and *Geranium*, distinguished by fruit dispersal (Cronquist 1981, 1988; Yeo 1984). The majority of Geraniums are herbaceous perennials with highly divided palmately lobed or cleft leaves. In contrast, the Hawaiian *Geraniums* (subgenus *Geranium,* section *Neurophyllodes*) have several unusual morphological features such as shrubby habit, stamens distinct at the base, unlobed leaves with pronounced parallel major veins and apically toothed or serrate margins, and one or both of the leaf surfaces are extremely hirsute (Hillebrand 1888; Wagner *et al.* 1990). The Hawaiian *Geraniums* vary in stature from a diffuse tree (*G. arboreum*) to decumbent shrubs with adventitious nodal rooting (*G. kauaiense, G. hillebrandii,* and *G. hanaense*). The section *Neurophyllodes* was described by Asa Gray (1854) and contains only those *Geraniums* found on Hawai'i. Although collected as early as 1793 by Archibald Menzies, the Hawaiian *Geraniums* remain rather obscure taxa since some of its species are rare plants that are poorly represented in herbarium collections. *G. arboretum* and *G. multiflorum* are already federally Endangered (US Fish and Wildlife Service 1992), while *G. hanaense* (1999), *G. kauaiense* (1997), and G. *hillebrandii* (1990) are candidates for listing.

Morphology has been used to examine relationships among the Hawaiian *Geraniums* (Wagner *et al*. 1990), using traits such as leaf margins, size of lamina, and trichome structure. Wagner *et al.* (1990) recognized nine taxa of Hawaiian *Geraniums*: *G. arboreum, G. multiflorum, G. hillebrandii, G. hanaense, G. kauaiense, G. cuneatum*

ssp. cuneatum, G. c. ssp. hololeucum, G. c. hypoleucum, and *G. c. ssp. tridens*. Phylogenetic relationships of the Hawaiian *Geraniums* have always been unclear. An age estimate for the most recent common ancestor of the Hawaiian *Geraniums* performed by Price and Clague (2002) suggests that the divergence of historically known *Geranium* taxa occurred around two million years ago. Carlquist and Bissing (1976) and Funk and Wagner (1995) suggested that *G. arboreum* and second, *G. multiflorum*, be considered the most basal members of the phylogeny based on features, such as leaf margins, that are less specialized than the other species (Figure 2). Having bright red zygomorphic flowers, *G. arboreum* is the only Hawaiian species to be bird-pollinated, while all other Hawaiian *Geraniums* are insect-pollinated (Carlquist 1980). Although vegetatively less specialized, Medeiros and St. John (1988) ventured that *G. arboreum* is an unlikely direct ancestor to the other Hawaiian *Geraniums*. Instead, they hypothesized that a species similar to *G. multiflorum* evolved in two divergent directions: bird-pollinated species (*G. arboreum*) and insect-pollinated species (all other Hawaiian *Geraniums*). Carlquist and Bissing (1976) first suggested that *G. multiflorum* is the ancestor of the *G. cuneatum* group. *G. cuneatum ssp. tridens* is thought to be the progenitor of *G. hanaense* based on characteristics such as pubescence on both leaf faces, reduced serrations on the leaf margin, and altitude of the habitats of both taxa (Medeiros and St. John 1988). Funk and Wagner (1995) elevated *G. cuneatum ssp. tridens* to specific status and suggested that *G. humile* (*G. hillebrandii*) followed by *G. kauaiense* are the most derived of the Hawaiian *Geraniums* (Figure 2).

The origin and evolution of oceanic island biota have long interested evolutionary biologists. Recent phylogenetic studies of several groups of native Hawaiian vascular

plants have led to significant insights into the origin and evolution of the Hawaiian angiosperms, 89% of which are endemic (Sakai *et al*. 1995; Wagner *et al*. 1990). The extreme isolation of the Hawaiian Islands permits profuse diversification of species from a single colonist. The extensive range of environmental diversity allows for tremendous selection pressures within short distances of each other. Unfortunately, factors such as non-native plants (banana poka, blackberry, strawberry guava), non-native animals (pigs, goats, mongoose), introduced diseases (avian malaria, avian pox), changes in natural processes (fire/lava flow suppression), habitat fragmentation, and increases in human populations are driving native habitants to extinction (Carlquist 1980; Loope *et al.* 1988). Hawai'i has only two native mammals: the hoary bat *(Lasiurus cinerus hawaiiensis)* and the monk seal *(Monachus schauinslandi)*, and no native reptiles. The lack of native mammals and reptiles leaves Hawaiian ecosystems at risk since native species have not evolved defenses to the predators and herbivores that have been introduced in the last 300-1,000 years by Polynesian and Western visitors.

Besides being actively introduced, there are many passive ways for species to become established on island ecosystems. Plumose hairs on achenes catch gusts of wind. Coconuts float for thousands of miles across the ocean. Seeds of another plant could "raft" on a coconut or driftwood. External bird dispersal (epizoochory) adaptations such as sticky seeds or barbs to enable them to attach to feet or feathers, and internal bird dispersal (dyszoochory) such as seeds having fleshy fruits or accessory tissues to entice ingestion are hypothesized to be the most common mode of dispersal that allowed plants to colonize Hawai'i (Carlquist 1974; Price and Wagner 2004; Sakai *et al.* 1995). The Hawaiian *Geraniums* produce neither plumose achenes, sticky burrs, nor appetizing fruit

to suggest an obvious method for seed dispersal. The "ballistic seed-ejection" mechanisms these *Geraniums* employ rarely manage to project seeds farther than three meters from the parent plant (Yeo 1984). The Pacific Golden Plover (*Pluvialis fulva*) is a likely vector of a number of native plant introductions into the Hawaiian Islands. It is a frequent visitor to a variety of upland sites, including montane bogs, and is therefore a possible accidental carrier of *Geranium* seed (Carlquist 1974, 1980).

Once a colonist arrives on Hawai'i, the volcanic islands offer virgin habitats. Each colonist has to contend with a partially or entirely new set of conditions, such as competitors, pathogens, and environmental differences, guiding natural selection. Every colonist has the potential to become a new species as it is isolated from the parent population. The geographical arrangement of the Hawaiian *Geraniums* is an interesting aspect of the group. Each species/subspecies only inhabits one island, species do not occur on every Hawaiian island, and the four subspecies of *G. cuneatum* have a disconnected distribution over two islands (Figure 1). Each species has a particular habitat and elevation to which it is adapted (Table 2). The question of which island was colonized first remains unanswered. The most common pattern of colonization and speciation in the Hawaiian archipelago has been from older to younger islands (Carson and Kaneshiro 1976; Crawford *et al.* 1987; Funk and Wagner 1995). Carlquist and Bissing (1976) and Funk and Wagner (1995) propose phylogenies in which the basal members (*G. arboreum* and *G. multiflorum*) are endemic to East Maui. The *Geraniums* radiating from East Maui to West Maui and Kaua'i suggests at least one colonization event from a younger, eastern island to an older, western island. If the Hawaiian *Geraniums* radiated from East Maui, their evolution must have proceeded rapidly.

Haleakala volcano, East Maui, is less than one million years old, while West Maui is less than two million years old (MacDonald *et al.* 1983). Given the morphological features of the Hawaiian *Geraniums* that so clearly set them apart from the rest of the genus, a relatively recent arrival to East Maui is unlikely. A more likely explanation is that the progenitor of the Hawaiian *Geraniums* first colonized an older, now-submerged island and dispersed to East Maui (Funk and Wagner 1995).

Although most species on Hawai'i can be traced to Polynesia, an estimated 18% of Hawaiian species have an American origin (Fosberg 1948; Wagner *et al.* 1990). The Silversword alliance (Asteraceae) (Baldwin *et al*. 1991; Baldwin and Wessa 2000), Hawaiian mints (*Stachys*, Lamiaceae) (Lindqvist and Albert 2002), woody Hawaiian violets (*Viola*, Violaceae) (Ballard and Sytsma 2000), and Hawaiian sanicles (*Sanicula*, Apiaceae) (Vargas *et al.* 1998) are examples of endemic Hawaiian groups having American ancestors.

Species-level systematics provides a framework for studying evolutionary patterns and processes. The first to study the molecular phylogeny of the Hawaiian *Geraniums* was Pax *et al.* (1997), using sequence analysis of PCR amplified fragments of the chloroplast gene *rbc*L to compare five Hawaiian species to 18 outgroup species in the genus. *Rbc*L gene sequences from the Hawaiian *Geraniums* were compared with those of a range of taxa from Australia, North America, Mexico, and India in a cladistic analysis in order to clarify its phylogenetic relationship. Pax *et al*. (1997) found that (1) the Hawaiian *Geraniums* are strongly supported as being monophyletic; (2) American representatives from Mexico and the western United States (*G. vulcanicola*, *G. subulatostipulatum,* and *G. richardsonii*), are the most similar to the Hawaiian

Geraniums; (3) *G. arboreum* is the basal member of the clade (as suggested by morphology); (4) *G. kauaiense*, a bog species occurring on the island of Kaua'i, is nested among species from Maui and Hawai'i, suggesting at least one colonization from a younger, eastern island to an older, western island. However, this data from *rbc*L (a conserved, slowing evolving chloroplast gene) was insufficient for resolution of the pattern of radiation. Molecular markers commonly used for lower-level phylogenetic analysis in plants are the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA, the noncoding chloroplast (cpDNA) *trn*L-F region, and inter-simple sequence repeats (ISSR). There is a wide acceptance of combination and simultaneous analysis of all available data sets (Bakker *et al.* 2004; Olmstead and Palmer 1994; Selvi *et al.* 2004; Small *et al*. 2004; Yockteng *et al.* 2003).

The internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA are well established as being useful in systematics. Their small size (500-700 bp) and high copy number allows for direct sequencing of PCR products and also facilitates the use of dried herbarium specimens and very old material. (Álvarez and Wendel 2003; Baldwin *et al*. 1995; Small *et al.* 2004). ITS regions have rates of substitution that are useful for evaluating generic and species level relationships (Baldwin *et al*. 1995; Gemmill *et al.* 2002; Yockteng *et al.* 2003). White *et al.* (1990) described a list of "universal" eukaryotic primers that are useful for amplifying ITS sequences from most plant and fungal phyla, removing the need for previous sequence information or custom primer design.

Chloroplast DNA (cpDNA) is the most widely used source of data in plant molecular phylogenetic analyses. The chloroplast genome contains both coding and non-

coding sequences and is found in multiple copies per chloroplast. Coding cpDNA molecules are highly conserved, which has lead to the design of "universal" PCR primers published by Taberlet *et al.* (1991). The analysis of cpDNA has been of particular interest because it is very informative over a wide range of taxonomic levels. The non-coding cpDNA regions have been used to define phylogenetic relationships among genera, among species, and within species (Baker *et al.* 1999; Jung *et al*. 2003; Olmstead and Palmer 1994; Small *et al*. 2004).

ISSR techniques are nearly identical to RAPD techniques except that ISSR primer sequences are designed from microsatellite regions and the annealing temperatures used are higher than those used for RAPD markers. These markers are derived from primers that anchor within the elements themselves, rather than in flanking regions. ISSR primers generate the variation in a given DNA sample by including one of these highly variable microsatellite sequences and an arbitrary pair of bases at the 3' end. ISSR markers are inherited in a dominant or codominant Mendalian fashion (Gupta *et al.* 1994; Zietkiewicz *et al*. 1994). The absence of a band is interpreted as primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement (Wolfe and Liston 1998). They are highly variable and more robust than RAPDs due to the use of longer anchored primer sequences. Only small amounts of fresh or preserved DNA and small reaction volumes for PCR are required. (Bussell *et al.* 2004; Wolfe *et al.* 1998). ISSR markers have been mostly used to assess genetic diversity among populations (Camacho and Liston 2001; Esselman *et al.* 1999; Maunder *et al.* 1999) but have also been used to assess the genetic relatedness of cultivars (Martins *et al.* 2003;

Arnau *et al.* 2002), as well as inter- and intra-species variations (Sudupak 2004; Yockteng *et al.* 2003).

MATERIALS AND METHODS

Plant Materials and DNA Extraction: The scientific names of the Hawaiian *Geranium* species were referred to the taxonomic system of Wagner *et al.* (1990) (Appendix A). Tissue from *G. c. cuneatum* was unavailable at the time of the study and was not included. Phylogenetic studies within the genus (Pax *et al.* 1997) show clearly that the Hawaiian *Geraniums* are monophyletic and identified a North American origin for the lineage. Species in the genus *Geranium* subgenus *Geranium* were used as outgroups: *G. richardsonii* (North America), *G. subulatostipulatum* (Mexico), and *G. vulcanicola* (Mexico). They were the most closely related species to the Hawaiian *Geraniums* as found by Pax *et al.* (1997). *G. grandiflorum* (Himalayas) sequences were obtained in this study and used as an outgroup species, as well as sequences from GenBank for the ITS1, 5.8S ribosomal RNA gene, and ITS2 regions of *G. solanderi*, *G. homeanum*, *G. sessiliflorum*, and *G. retrorsum* (New Zealand and Australia) (Gardner *et al.* unpublished) and in the subgenus *Robertium*, the *trnL-trnF* intergenic spacer regions of *G. robertianum* and *G. pusilum* (Europe, Asia, North Africa, North America) (Bakker *et al.* 2000). Plant material was collected from natural populations and preserved in silica gel. The accessions, their GenBank accession numbers, and their sources are given in Table 1. At least five DNA isolations from unique individuals were performed per Hawaiian species. Total DNA was extracted by following a modified CTAB method of Doyle and Doyle (1987) and was then purified using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

PCR Amplifications and Purification of ITS and trnL-F PCR Products: Each PCR reaction was 50µl in volume. The PCR reaction mix was prepared before aliquoting it to each tube containing the DNA template. The PCR reaction mix included: 5 µl 10X $MgCl_2$-free PCR buffer (Promega, Madison, WI), 8 µl dNTP mix (1.25 mM each dNTP - Promega), 1 µl each Primer (10 µM - Sigma-Genosys, The Woodlands, TX), 5 µl $MgCl_2$ (25 mM - Promega), 0.5 µl Taq DNA polymerase (2.5 Units – Promega), 28.5 µl H_2O, 1 µl template. The ITS and *trn*L-F regions were amplified using primers reported as universal primers by White *et al.* (1990) and Taberlet *et al.* (1991), respectively, for flowering plants (see Table 3). The sequences of the primers used are as follows: *ITS1* – TCCGTAGGTGAACCTGCGG; *ITS4* – TCCTCCGCTTATTGATATGC; *TABC* – CGAAATCGGTAGACGCTACG; *TABF* – ATTTGAACTGGTGACACGAG. Amplifications were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following amplification profile: 3 minutes at 95°C; 35 cycles of 1 minute at 95°C, 1 minute at 54°C, and 2 minutes at 72°C; and extra extension for 8 minutes at 72°C. Thirteen µl of each double-stranded DNA PCR product were resolved by electrophoresis in 1% agarose gel using 1x TBE as the gel buffer. Successful PCR resulted in a single band of ethidium bromide-incorporated DNA when viewed under ultraviolet (UV) light. PCR products were precipitated with EtOH and sodium acetate before direct sequencing.

Sequencing of PCR Products: Forward and reverse DNA sequences were obtained using the same primers as for PCR reactions in conjunction with a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Each reaction was 10 µl in volume and contained 3 µl of sterile distilled water, 2 µl BigDye® 5x Sequencing Buffer,

1 µl of primer (10 mM), 1 µl of ethanol-purified PCR product, and 2 µl of BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix. The sequencing reaction was performed in a PTC-100 thermal cycler (MJ Research) under the following amplification profile: 3 minutes at 95°C; 25 cycles of 15 seconds at 95°C, 4 minutes at 60°C, and 2 minutes at 72°C. Cycle sequencing reaction products were precipitated with EtOH and sodium acetate and suspended in 12 µl of template suppression reagent (TSR; Applied Biosystems) or ABI Hi-Di Formamide (Applied Biosystems) before automated cycle sequencing. The majority of the *trn*L-F sequences were run on an Applied BioSystems 310 automated DNA sequencer at Bowling Green State University. The majority of the ITS sequences were run on an ABI Prism 3700 DNA Analyzer by GeneGateway, LLC (Hayward, CA). All sequences were verified by comparison of their forward and reverse sequences.

PCR Amplifications of ISSR: Each PCR amplification reaction was 25µl in volume. The PCR reaction mix was prepared before aliquoting it to each tube containing the DNA template. The PCR reaction mix included: 2.5 µl 10X $MgCl_2$-free PCR buffer (Promega), 2.5 µl BSA (Bovine Serum Albumin, 4 mg/ml - Promega), 4 µl dNTP mix (1.25 mM each dNTP - Promega), 2 µl each Primer (10 µM - Sigma-Genosys), 2-3 µl $MgCl_2$ (25 mM - Promega), 0.1 µl Taq DNA polymerase (0.5 Units – Promega), and 2 µl template. The regions were amplified using primers reported for flowering plants (UBC primers nos. 807 $((AG)_8T)$ and 810 $((GA)_8T)$, from the Biotechnology Laboratory, University of British Columbia, Canada, and "Manny," $((CAC)_4RC)$ Wolfe and Liston 1998) (Table 3). Amplification was performed in a PTC-100 thermal cycler (MJ

Research) under the following amplification profile: 2 minutes at 94°C; 40 cycles of 40 seconds at 94°C, 45 seconds at 44°C, and 90 seconds at 72°C; and extra extension for 5 minutes at 72°C.

Data Analysis of ITS and trnL-F Sequences: The sequence boundary of the *trnL-trnF* intergenic spacer region was determined by comparison with published sequences (Baker *et al.* 1999). The sequences were aligned using the program Clustal-X (Thompson *et al.* 1997) with the default settings. Among the Hawaiian taxa's combined sequences, there were seven gaps: five in *G. kauaiense* and two in *G. c. tridens*. There were no other insertions or deletions among the Hawaiian sequences. Gaps were treated as missing data. A maximum parsimony analysis (Swofford *et al.* 1996) was performed using PAUP* 4.0b10 under the branch-and-bound search algorithm. A neighbor-joining analysis was additionally performed on the combination of ITS and *trn*L-F data. Relative support of various clades revealed in the maximally parsimonious trees (MPTs) was examined with the bootstrap method (Felsenstein 1985) using PAUP* 4.0b10. Bootstrap values were calculated from 1000 replicates (365 replicates for *trn*L-F sequences) with branch-and-bound search options. Characters were assigned equal weights at all nucleotide positions. The data from ITS and *trn*L-F were analyzed separately before doing a combined analysis.

Data Analysis of ISSR: The entire PCR product was resolved by electrophoresis in 1.5% agarose gel using 1x TBE as the gel buffer. Successful PCR resulted in multiple bands which were visualized using SYBR® Green I (Molecular Probes, Eugene, OR) nucleic

acid gel stain. ISSR profiles were captured digitally with a STORM 860 system at 450nm excitation. Duplicate reactions were run for all ISSR analyses to ensure the reproducibility of banding patterns. Fourteen primers were initially screened and ISSR data from three primers (UBC 807, 810, and "Manny") were obtained for eight Hawaiian *Geranium* taxa, *G. richardsonii*, and *G. vulcanicola*. ISSR data from "Manny" proved unreliable and was, therefore, excluded from analysis. Fragment sizes were estimated based on 1000kb GeneChoice Ladder II (GeneChoice, Frederick, MD). Each unique fragment size was considered a locus and was scored as diallelic (present=1 or absent=0). Bands of identical size were assumed homologous across species samples. PAUP* 4.0b10 was used to perform a maximum parsimony analysis (Swofford *et al.* 1996) by using the branch-and-bound algorithm as well as a distance analysis using UPGMA search algorithm. Relative support of various clades revealed in the maximally parsimonious trees (MPTs) was examined with the bootstrap method (Felsenstein 1985).

RESULTS

ITS: The total length of aligned ITS sequences was 667 base pairs, 574 (86%) of which were invariant, 52 (8%) were parsimony- informative, and 41 (6%) were parsimony-uninformative. The following species were designated as outgroups: *G. grandiflorum, G. solanderi*, *G. homeanum*, *G. sessiliflorum*, and *G. retrorsum*. A branch-and-bound search using all default settings resulted in two equally-parsimonious trees, each with a length of 116 mutational events (Consistency Index (CI) of 0.853, Retention Index (RI) of 0.893). The 50% majority-rule consensus tree (Figure 6) showed 1) the monophyly of the Hawaiian Geraniums supported by a 100% bootstrap and 2) a basal polytomy with the Hawaiian lineage as five poorly-resolved clades. Some further groupings emerged: *G. multiflorum, G. hillebrandii,* and *G. hanaense* were placed in an unresolved clade supported by a 86% bootstrap. *G. arboreum* and *G. c. hypoleucum* were grouped together in a second strongly supported clade (84% bootstrap) placed as sister to that of the previous clade. The node that links the Hawaiian taxa to the N. American taxon *G. richardsonii* was supported by a 71% bootstrap and the node that links the Hawaiian taxa to the Mexican taxa was strongly supported by a 99% bootstrap value.

trnL-F: Compared to ITS, the *trn*L-F region was less variable. The total length of aligned *trn*L-F sequences was 869 base pairs, 768 (88%) of which were constant, 19 (2%) were parsimony-informative, and 82 (9%) of which were parsimony-uninformative. The following species were designated as outgroups: *G. grandiflorum, G. robertianum* and *G. pusilum.* A branch-and-bound search using all default settings resulted in 300 equally-parsimonious trees, each with a length of 106 mutational events (CI of 0.962, RI of

0.895). The 50% majority-rule consensus tree (Figure 7) placed *G. multiflorum, G. hillebrandii, G. c. tridens, G. hanaense*, and G. *hololeucum* within an unresolved clade moderately supported by a 65% bootstrap. The placement of *G. kauaiense* as sister to that of the previous clade was supported by a 75% bootstrap. *G. arboreum* and *G. c. hypoleucum* had unresolved basal positioning similar to that in the ITS tree. Bootstrap analysis did not provide support for the monophyly of the Hawaiian *Geraniums*, as was depicted by the 50% majority-rule consensus tree. The node that links the Hawaiian taxa to the American taxa was strongly supported by a 98% bootstrap.

Combined analysis: In the individual analyses of ITS and *trn*L-F, the American taxa (*G. richardsonii, G. vulcanicola,* and *G. subulatostipulatum*) consistently place more closely related to the Hawaiian clade. For the combined analysis, these American taxa were designated as outgroups and the other taxa (*G. grandiflorum*, *G. solanderi*, *G. homeanum*, *G. sessiliflorum*, *G. retrorsum, G. robertianum* and *G. pusilum*) were not included. The trnL-F sequences were reduced to a length of 848 base pairs and the ITS sequences were reduced to a length of 570 base pairs after the removal of taxa not to be included in the combined analysis. The total length of aligned combined sequences was 1497 base pairs, 1419 (95%) of which were invariable, 30 (2%) were parsimony-informative, and 48 (3%) were parsimony-uninformative. The following species were designated as outgroups: *G. richardsonii, G. subulatostipulatum,* and *G. vulcanicola.* A branch-and-bound search using all default settings found three equally-parsimonious trees, each with a length of 87 mutational events (CI of 0.936, RI of 0.926). The 50% majority-rule consensus tree (Figure 8) placed *G. multiflorum, G. hillebrandii,* and *G. hanaense* together in a clade

supported by a 91% bootstrap. *G. c. hololeucum* and *G. c. tridens* are placed along with the previous clade forming an unresolved group supported by a 58% bootstrap. *G. arboreum* and *G. c. hypoleucum* were placed at a basal position supported by a 66% bootstrap. A branch supported by an 83% bootstrap value places *G. kauaiense,* nested, between the *G. arboreum/G. c .hypoleucum* clade and the unresolved *G. c. hololeucum/tridens* group (Figure 8). Eleven mutational events pair *G. arboreum* and *G. c. hypoleucum* and 15 mutational events unite the rest of the Hawaiian taxa. Three shared mutations unite *G. hillebrandii*, *G. hanaense*, and *G. multiflorum* (Figure 9). An identical tree with nearly identical bootstrap values was produced by a neighbor-joining analysis (tree length = 62, CI of 0.936, RI of 0.926) with all default options (Figure 8).

ISSR analysis: A total of 37 fragments were scored, two (5%) of which were constant, 20 (54%) were parsimony-informative, and 15 (41%) were parsimony-uninformative. 70% of the 37 characters consisted of bands shared by at least two species. Due to inconsistencies in amplification, some bands in *G. arboreum* and *G. c. tridens* were not scorable. These loci were treated as "missing information" in PAUP* as opposed to the "absence" of a band. *G. richardsonii* and *G. vulcanicola* were designated as outgroups. A branch-and-bound search using all default settings resulted in 48 equally-parsimonious trees, each with a length of 52 mutational events (CI of 0.547, RI of 0.147). The 50% majority-rule consensus tree showed a basal polytomy of *G. kauaiense* and *G. richardsonii* and placed the rest of the taxa in an unresolved clade supported by a 66% bootstrap. The 50% majority-rule consensus tree from a distance analysis using the UPGMA search algorithm (Figure 10) with all default settings showed a basal polytomy

of *G. multiflorum, G. hanaense, G. hillebrandii, G. kauaiense,* and *G. vulcanicola*. *G. arboreum* and *G. hypoleucum* were grouped together as sister to the polytomy, supported by a 62% bootstrap. This arrangement is consistent with the grouping of these two species in the ITS and combined sequences analysis trees. Finally, *G. c. tridens* and *G. c. hololeucum* were grouped as sister to the basal polytomy, weakly supported by a 54% bootstrap.

DISCUSSION

Phylogenetic analysis of the ITS and *trn*L-F regions and ISSR data has revealed several new pieces of information that prompt an revision of the phylogenetic tree of the Hawaiian *Geraniums* from trees previously suggested based on morphological data or *rbc*L data.

G. arboreum is a basal species, even while it is grouped with *G. c. hypoleucum*. All four analyses suggest this (Figures 8-10). It is surprising that *G. c. hypoleucum* was considered basal and it was at first believed possibly to be an artifact of very invariant *trn*L-F sequences. After the analysis of ITS data, the combined sequence analysis and even the ISSR data placed *G. c. hypoleucum* with *G. arboreum* with moderately- to strongly-supported bootstrap values (62-84%), it becomes apparent that this is a grouping that may very well be real. In all four analyses (ITS, *trn*L-F, combined sequences, and ISSR), *G. arboreum* and *G. c. hypoleucum* were consistently placed together. In the three sequence analyses (ITS, *trn*L-F, and combined sequences, Figures 8,9), *G. multiflorum, G. hillebrandii,* and *G. hanaense* were grouped together, showing more resolution than analysis of *rbc*L data in Pax *et al.* (1997). Although all three species occur on Maui, *G. hillebrandii* and *G. hanaense* are bog species, while *G. multiflorum* is an erect shrub found in upper forest ecotones. *Rbc*L data similarly grouped *G. hanaense* and *G. multiflorum* together in a weakly supported, most-derived clade (Pax *et al*. 1997). This pattern of speciation (Figure 11) is more complicated than phylogenies formed by analyses of morphological characteristics by Funk and Wagner (1995).

The monophyly of the Hawaiian Geraniums is strongly supported (Figure 8) which not surprising considering the distance the first colonizer had to travel and the fact

that the *Geraniums* offer no reward to anything that might carry it; it's a wonder even one colonist reached Hawai'i, let alone multiple colonists at different times.

Another interesting result of this study is that the molecular data obtained does not support the suggestion of Funk and Wagner (1995) to elevate *G. cuneatum* ssp. *tridens*, the only subspecies occurring on Maui, to specific status. That is not to say that it definitely should not be elevated, rather, the level of divergence in the sequences among all the Hawaiian *Geraniums* does not even support the designation of subspecies within *cuneatum* at all. A pairwise distance matrix created from the combined ITS and *trn*L-F data in PAUP* (Table 6) displays that each *cuneatum* subspecies is just as distant (if not more distant) from other *cuneatum* subspecies as it is from any other species. If anything can be gleaned from this matrix at all, it might be that *G. c. tridens* is more similar to *G. c. hololeucum* than any other species, which does not support the selective elevation of *G. c. tridens* to specific level. Granted, the greatest pairwise distance among the Hawaiian taxa is merely 0.00539 (*G. hanaense* and *G. c. hypoleucum*) and its significance is questionable. Sequence invariability is not uncommon among Hawaiian taxa (Ballard and Sytsma 2000; Baldwin *et al.* 1995; Ganders *et al.* 2000; Gemmill *et al*. 2002). Perhaps a focused study using ISSR markers that includes all four subspecies of *cuneatum* will provide the resolution needed to define how they should be classified. If similar amounts of divergence are found both among the *cuneatums* as well as among each of the *cuneatums* and other Hawaiian *Geraniums* (as was found in this study), it could be suggested that all the *cuneatums* should be elevated to specific status, eliminating the subspecies designation.

The bog species (*G. hanaense, G. hillebrandii,* and *G. kauaiense*) were not grouped together as Funk and Wagner (1995) suggested in their study of morphological traits, which include bog adaptations such as adventitious nodal rooting as characters for phylogenetic analysis. The molecular data (Figure 11) does not support the hypothesis that the bog species represent a single evolution of bog adaptations followed by radiation into this habitat type on East Maui, West Maui, and Kaua'i. A possible explanation for this incongruence between phylogenies based on morphology versus molecular data is the possibility of convergent evolution of traits such as adventitious nodal rooting (found in *G. kauaiense, G. hillebrandii,* and *G. hanaense*). Adventitious nodal rooting is a common trait associated with plants growing in bogs and has even appeared in introduced forest species that do not form such roots in their native habitats (Lanner 1964).

Kaua'i, the oldest island, is not the island of initial colonization. The frequently occurring "conveyer belt" mode of colonization and radiation from oldest island to newest island is not supported. *G. kauaiense*, the only species occurring on Kaua'i, is clearly nested and not basal (Figure 8). The data suggests that Maui, Hawai'i, or an older now-submerged island is the center of the radiation. This is consistent with hypotheses proposed by Carlquist and Bissing (1976), Medeiros and St. John (1988), and Funk and Wagner (1995) based on morphological features as well as phylogenetic trees produced by the study of the *rbc*L gene (Pax *et al.* 1997). This implies an uncommon back-dispersal to Kaua'i. Some of the other Hawaiian lineages that share this exceptional pattern of radiation include *Tetramolopium* (Asteraceae) (Lowrey 1995), *Schiedea* (Caryophyllaceae) (Wagner *et al*. 1995), and *Psychotria* (Rubiaceae) (Nepokroeff *et al.* 2003). When a back-dispersal is found in a phylogeny, it is usually after an initial

colonization on and radiation from the oldest island of Kaua'i. In the case of the Hawaiian *Geraniums*, the back-dispersal is suggested to come after an initial colonization of one of the two youngest islands. Using GIS analyses of changes in geological features, an age estimate for the most recent common ancestor of the Hawaiian Geraniums performed by Price and Clague (2002) suggests that the divergence of historically known *Geranium* taxa occurred around two million years ago, well after the formation of Kaua'i. The exact pattern of speciation may be related to accidental bird dispersal and/or steered by the volatile volcanic habitat in which these organisms live.

Considering their morphological diversity, the sequences of both the ITS and *trn*L-F regions among the Hawaiian *Geraniums* were remarkably invariant, which was surprising considering the successful use of these regions in other infra-generic phylogenetic studies. Such sequence invariability is not all that uncommon among Hawaiian taxa (Ballard and Sytsma 2000; Baldwin *et al.* 1995; Ganders *et al.* 2000; Gemmill *et al*. 2002; Lindqvist and Albert 2002). Lack of resolution may be indicative of a relatively recent origin for the Hawaiian *Geraniums*. Perhaps in the case of the Hawaiian *Geraniums*, adaptive radiation involved selection for morphological differences controlled by relatively few genes of large effect similar to that of the Hawaiian Silversword alliance, in which rapid morphological diversification has been accompanied by accelerated evolution of genes that regulate developmental processes (Barrier *et al.* 2001).

The adaptive radiation of the Hawaiian *Geraniums* into many different habitats despite little sequence variation is common occurrence among Hawaiian taxa. The Hawaiian Silversword alliance descended from a member of the Asteraceae family

similar to Muir's Tarweed (from California) and is comprised of 30 species in three genera. Plants of the Silversword group occupy every terrestrial habitat in Hawai'i from wet forests to dry forests and from near sea level to alpine shrublands. Although these plants are still closely related, they often look extremely different from one another (Baldwin *et al*. 1991; Baldwin and Wessa 2000; Barrier *et al.* 2001). In the case of the Hawaiian lobeliads (Campanulaceae), which also seem to have arisen from a single colonization, there are more than 110 recognized species which inhabit nearly every habitat in Hawai'i. The lobeliads' habits include alpine bog rosettes, seacliff succulents, and trees, treelets, and shrubs of mesic and wet forest edges and interiors (Givnish *et al.* 2004). The Hawaiian mints (Lamiaceae) comprise a total of 58 species in three genera and the endemic Hawaiian *Bidens* consist of 27 species. These two groups represent another example in which broad morphological and ecological variation is maintained in contrast to a strikingly low level of DNA sequence divergence (Ganders et al. 2000; Lindqvist and Albert 2002; Lindqvist *et al*. 2003). Other examples of extensive adaptive radiation include the Drepanidae (honeycreepers) among birds (James 2004); Drosophilidae (Hawaiian drosophila), Megalagrion (damselflies), and Laupala (crickets) among the insects (Carson and Kaneshiro 1976; Jordan et al. 2003; Shaw 2002); and Tetragnatha ("long-jawed" spiders) among arachnids (Gillespie 2002).

In contrast with the majority of organisms on Hawai'i, this study showed that the Hawaiian *Geraniums* are strongly affiliated with species found in North America which is remarkable, considering the distance between Hawai'i and North America (3,500 km) and that there is no geological evidence for any now-extinct islands which could have served as stepping-stones to the Hawaiian islands. These results are consistent with the

results obtained by Pax *et al* (1997). The Hawaiian *Geraniums* are in the company of the Silversword alliance (Asteraceae) (Baldwin *et al*. 1991; Baldwin and Wessa 2000), Hawaiian mints (*Stachys*, Lamiaceae) (Lindqvist and Albert 2002), woody Hawaiian violets (*Viola*, Violaceae) (Ballard and Sytsma 2000), and Hawaiian sanicles (*Sanicula*, Apiaceae) (Vargas *et al.* 1998), other endemic Hawaiian groups having American ancestors.

In this study, ISSR data was employed as an alternative source of data once it was discovered that the ITS and *trn*L-F regions were relatively invariant. This is not an uncommon strategy (Mort *et al.* 2003; Yockteng *et al*. 2003). The limited utility of the ISSR data in this study arises from amplification difficulties across primers and templates. The data the ISSR analysis yielded confirmed the controversial and unexpected results produced by the more analyses of ITS and *trn*L-F. This demonstrates the potential this method has to assess phylogenetic relationships at the sectional level. A more dedicated study that included more samples per species, as well as more than two primers, would certainly yield more data suitable for phylogenetic analyses. In addition, a study that included a more diverse sampling of outgroups, specifically those from South America, may provide evidence for a South American origin for the Hawaiian *Geraniums*, as opposed to North American.

The new information revealed in this study can be used to amend the current phylogenetic tree of the Hawaiian *Geraniums*. This study has shown that the Hawaiian *Geraniums* are an unusual group that needs to be studied further. Not only are the Hawaiian *Geraniums* important as a part of an island ecosystem, but the *Geraniums* are atypical among Hawaiian taxa in that the initial colonization event did not occur on

Kaua'i, a back-dispersal occurred in the radiation, and they are affiliated with species found in North America. Due to the precarious situation of the endangered *Geraniums*, further studies need to be done without delay, before any species of the Hawaiian *Geraniums* are lost.

Table 1: Taxa used for analysis. Sequences newly obtained in this study are indicated by an asterisk.

Species	***Collection data/source***	***Sequenced by***	***Regions sequenced***	***Accession #***
*G. arboreum**	Poli Poli Springs, Maui, *Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. multiflorum**	Maui, *Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. hanaense**	Maui, *Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. hillebrandii**	Pu'u Kukui bog, Maui, *Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. kauaiense**	Alaka'i Swamp, Kaua'i, *Perlman*	S. Kidd	ITS[1]/*trn*L-F	
*G. c. tridens**	Maui, *Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. c. hololeucum**	Mauna Kea, Hawaii, *Pax & Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. c. hypoleucum**	Mauna Loa, Hawaii, *Pax & Michaels*	S. Kidd	ITS[1]/*trn*L-F	
*G. richardsonii**	Gallatin, CO, MT	S. Kidd	ITS[1]/*trn*L-F	
*G. subulatostipulatum**	Veracruz, Mexico, *Marquez & Utrera*	S. Kidd	ITS[1]/*trn*L-F	
*G. vulcanicola**	Veracruz, Mexico, *Marquez & Utrera*	S. Kidd	ITS[1]/*trn*L-F	
*G. grandiflorum**	Price and Palmer 1993	S. Kidd	ITS[1]/*trn*L-F	
G. homeanum	*unpublished*	Gardner *et al.* 2004	ITS[1]	AY752471.1
G. solanderi	*unpublished*	Gardner *et al.* 2004	ITS[1]	AY752467.1
G. sessiliflorum	*unpublished*	Gardner *et al.* 2004	ITS[1]	AY752469.1
G. retrorsum	*unpublished*	Gardner *et al.* 2004	ITS[1]	AY752473.1
G. robertianum	Reading, UK	Bakker *et al.* 2000	*trn*L-F	AF167152.1
G. pusilum	Reading, UK	Bakker *et al.* 2000	*trn*L-F	AF167151.1

[1]internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2

Table 2. The endemic Hawaiian *Geraniums*. Information derived from Medeiros and St. John (1988) and Wagner *et al.* (1990).

Taxon	Distribution	Elevation (m)	Habitat	Max. stature/habit
G. arboreum	Maui	1520-2150	Upper forest ecotone	205 m diffuse tree
G. cuneatum ssp. cuneatum	Hawai'i	1550-1830	Subalpine scrub	0.7 m, erect shrub
G. c. hololeucum	Hawai'i	1850-3050	Alpine scrub	0.7 m, erect shrub
G. c. hypoleucum	Hawai'i	1480-2440	Alpine scrub	0.7 m, erect shrub
G. c. tridens	East Maui	2300-3250	Alpine scrub	1.5 m, erect shrub
G. hanaense	East Maui	1679-1680	Montane bog	1.5 m, descumbant shrub
G. hillebrandii	West Maui	1490-1770	Montane bog	0.3 m, erect subshrub
G. kauaiense	Kaua'i	1220-1250	Montane bog	0.3 m, descumbant subshrub
G. multiflorum	Maui	1580-2450	Upper forest ecotone	2.5 m, erect shrub

Table 3. Primers used in this study.

Primer Name	Primer Sequences (5'-3')	Primer Source
TABC	CGAAATCGGTAGACGCTACG	Taberlet *et al.,* 1991
TABF	ATTTGAACTGGTGACACGAG	Taberlet *et al.,* 1991
ITS1	TCCGTAGGTGAACCTGCGG	White *et al.,* 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al.,* 1990
UBS807	AGAGAGAGAGAGAGAGT	UBS set no. 9
UBS810	GAGAGAGAGAGAGAGAT	UBS set no. 9
ISSR Manny	CACCACCACCACRC	Wolfe and Liston 1998

Table 4. ISSR data from primer 807. Bands were scored as diallelic (1=present, 0=absent).

Band	*G. arboreum*	*G. multiflorum*	*G. hanaense*	*G. hillebrandii*	*G. kauaiense*	*G. c. tridens*	*G. c. hololeucum*	*G. c. hypoleucum*	*G. richardsonii*	*G. vulcanicola*
A	*0*	*0*	*0*	*1*	*0*	*0*	*1*	*0*	*0*	*1*
B	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*1*	*0*
C	*1*	*1*	*0*	*0*	*1*	*1*	*1*	*0*	*0*	*0*
D	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
E	*1*	*1*	*1*	*1*	*1*	*0*	*0*	*1*	*1*	*1*
F	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*0*	*1*
G	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
H	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*
I	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*0*	*1*
J	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*1*	*0*
K	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*
L	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	*1*	*0*
M	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*
N	*0*	*1*	*1*	*1*	*0*	*0*	*1*	*0*	*0*	*1*
O	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*
P	*0*	*1*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Q	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*

Table 5. ISSR data from primer 810. Bands were scored as diallelic (1=present, 0=absent). Question marks indicate missing data.

Band	*G. arboreum*	*G. multiflorum*	*G. hanaense*	*G. hillebrandii*	*G. kauaiense*	*G. c. tridens*	*G. c. hololeucum*	*G. c. hypoleucum*	*G. richardsonii*	*G. vulcanicola*
A	*1*	*0*	*0*	*0*	*0*	*?*	*0*	*0*	*0*	*1*
B	*?*	*0*	*1*	*0*	*0*	*?*	*1*	*0*	*0*	*0*
C	*?*	*0*	*0*	*1*	*0*	*?*	*0*	*0*	*0*	*0*
D	*?*	*1*	*1*	*1*	*0*	*?*	*1*	*1*	*0*	*1*
E	*?*	*0*	*0*	*1*	*0*	*?*	*1*	*1*	*0*	*0*
F	*1*	*0*	*1*	*1*	*0*	*?*	*0*	*1*	*0*	*0*
G	*1*	*0*	*1*	*1*	*1*	*?*	*1*	*1*	*1*	*1*
H	*?*	*1*	*1*	*1*	*1*	*?*	*1*	*1*	*1*	*0*
I	*?*	*1*	*1*	*1*	*1*	*?*	*1*	*1*	*1*	*0*
J	*?*	*0*	*0*	*0*	*0*	*?*	*0*	*1*	*0*	*0*
K	*1*	*0*	*0*	*0*	*0*	*?*	*0*	*1*	*0*	*0*
L	*?*	*0*	*0*	*1*	*0*	*?*	*0*	*0*	*0*	*0*
M	*?*	*0*	*1*	*1*	*0*	*?*	*0*	*0*	*0*	*0*
N	*?*	*1*	*0*	*0*	*1*	*1*	*1*	*0*	*0*	*1*
O	*?*	*0*	*0*	*0*	*1*	*?*	*0*	*0*	*1*	*0*
P	*?*	*0*	*0*	*0*	*0*	*?*	*0*	*0*	*1*	*0*
Q	*?*	*0*	*0*	*0*	*0*	*?*	*0*	*0*	*0*	*1*
R	*?*	*0*	*0*	*0*	*0*	*?*	*0*	*0*	*0*	*1*
S	*?*	*0*	*0*	*0*	*0*	*?*	*0*	*1*	*1*	*1*
T	*?*	*0*	*0*	*0*	*0*	*?*	*0*	*0*	*1*	*1*

Table 6. A pairwise distance matrix created from the combined ITS and *trn*L-F data.

	G. arboreum	*G. c. hypoleucum*	*G. hillebrandii*	*G. hanaense*	*G. multiflorum*	*G. c. hololeucum*	*G. c. tridens*	*G. kauaiense*	*G. richardsonii*	*G. subulato-stipulatum*
G. arboreum	*									
G. c. hypoleucum	0.00202	*								
G. hillebrandii	0.00471	0.00538	*							
G. hanaense	0.00472	0.00539	0.00000	*						
G. multiflorum	0.00471	0.00538	0.00000	0.00000	*					
G. c. hololeucum	0.00538	0.00472	0.00268	0.00268	0.00268	*				
G. c. tridens	0.00539	0.00472	0.00335	0.00336	0.00335	0.00201	*			
G. kauaiense	0.00405	0.00338	0.00337	0.00337	0.00337	0.00270	0.00203	*		
G. richardsonii	0.01545	0.01480	0.01742	0.01743	0.01740	0.01807	0.01812	0.01687	*	
G. subulatostipulatum	0.02086	0.02023	0.02149	0.02150	0.02147	0.02214	0.02220	0.02097	0.01943	*
G. vulcanicola	0.02019	0.01955	0.02215	0.02216	0.02213	0.02280	0.02286	0.02164	0.01741	0.00804

Figure 1. Island distribution of the endemic Hawaiian *Geranium* taxa.

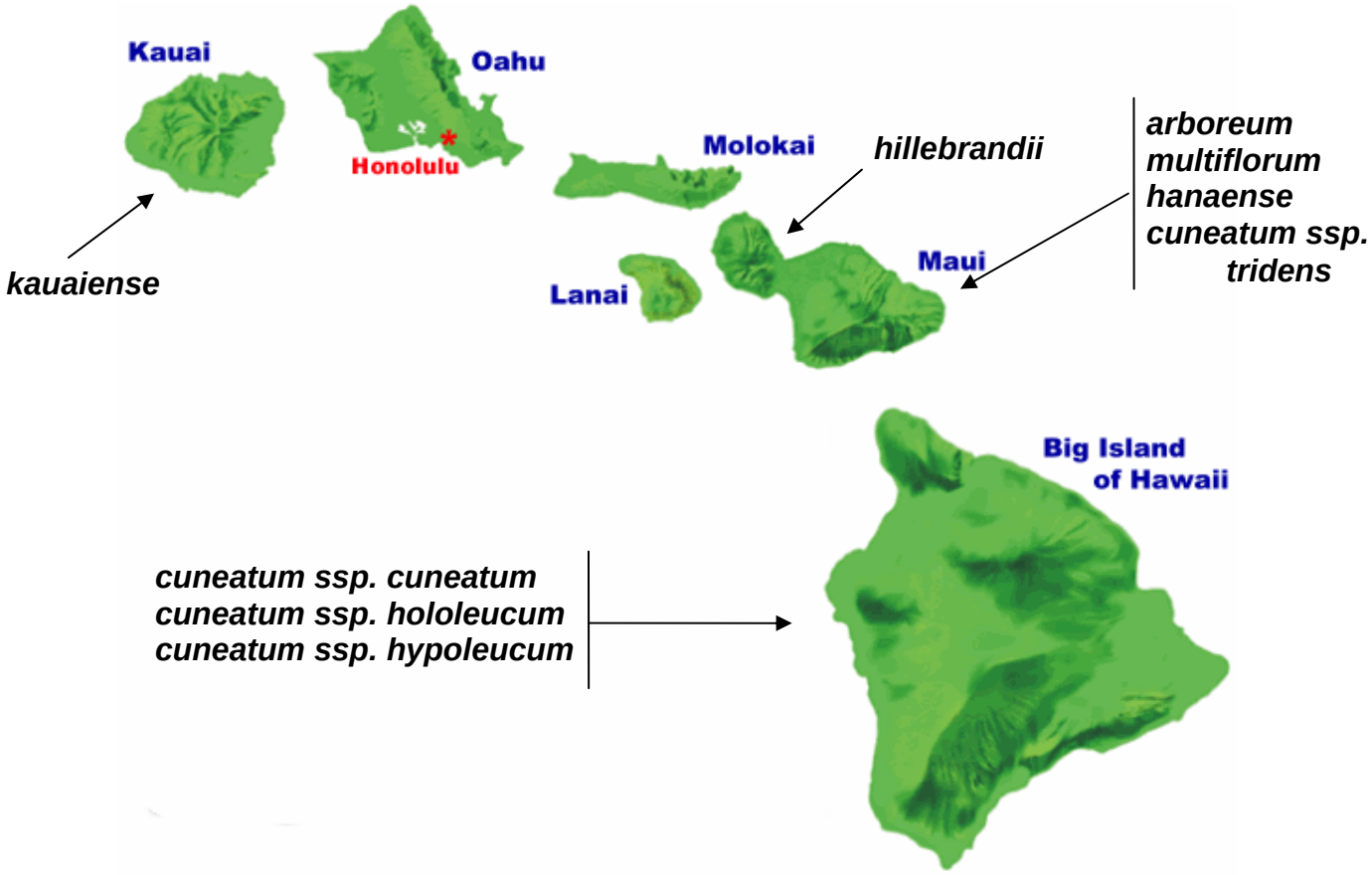


Figure 2. Phylogeny of the Hawaiian *Geraniums* based on morphological characteristics as proposed by Funk and Wagner (1995).

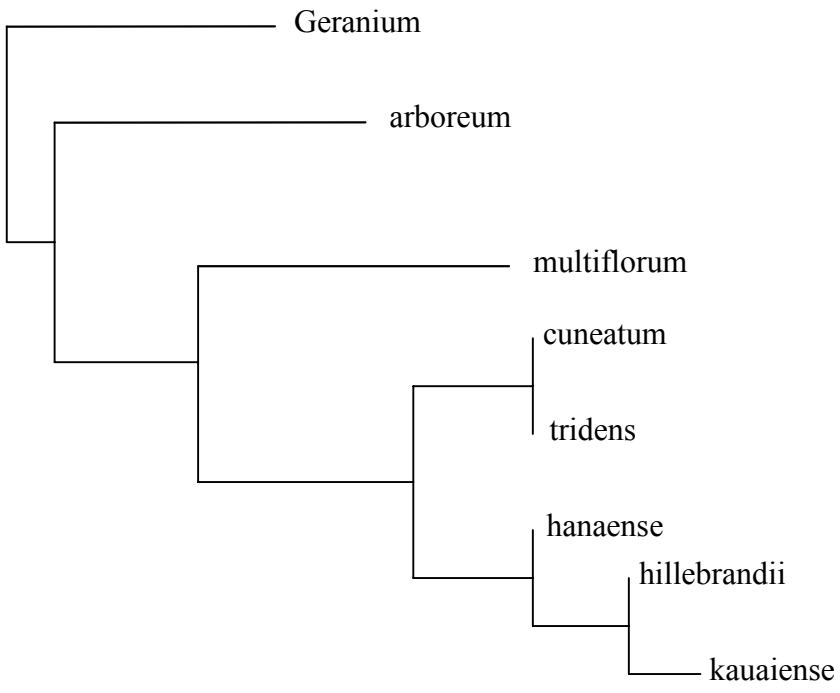


Figure 3. PCR product from the successful amplification of the ITS region of several Hawaiian taxa.

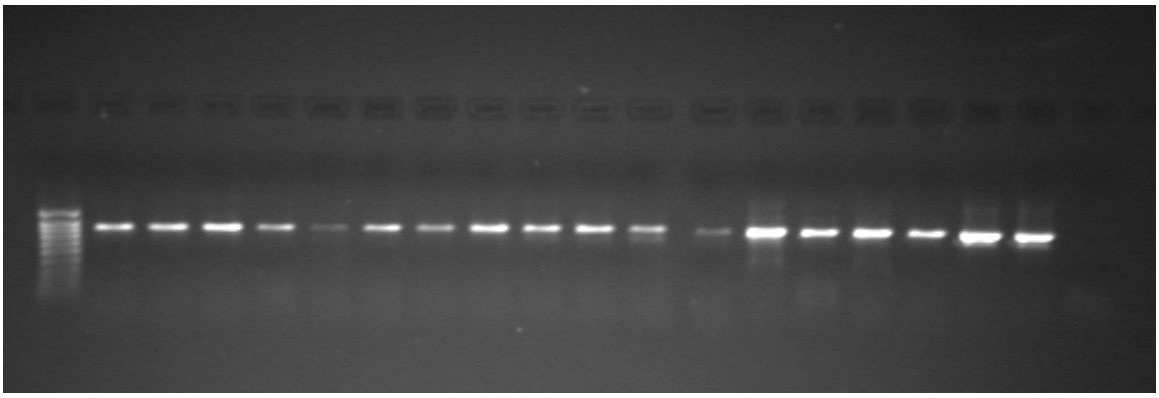


Figure 4. PCR product from the successful amplification of the *trn*L-F region of several Hawaiian taxa.

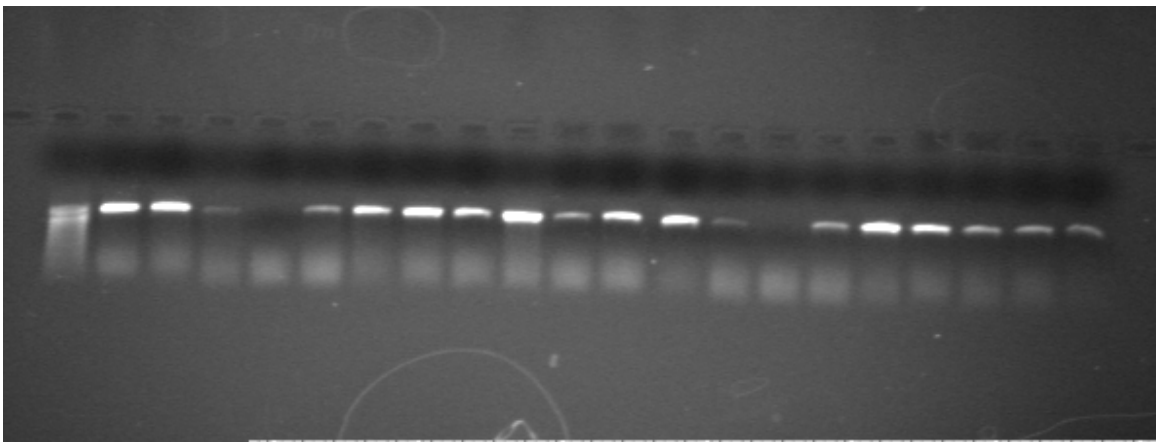


Figure 5. PCR product from the successful amplification of ISSR Primer 807 of several Hawaiian taxa and relatives in the Geraniaceae family.

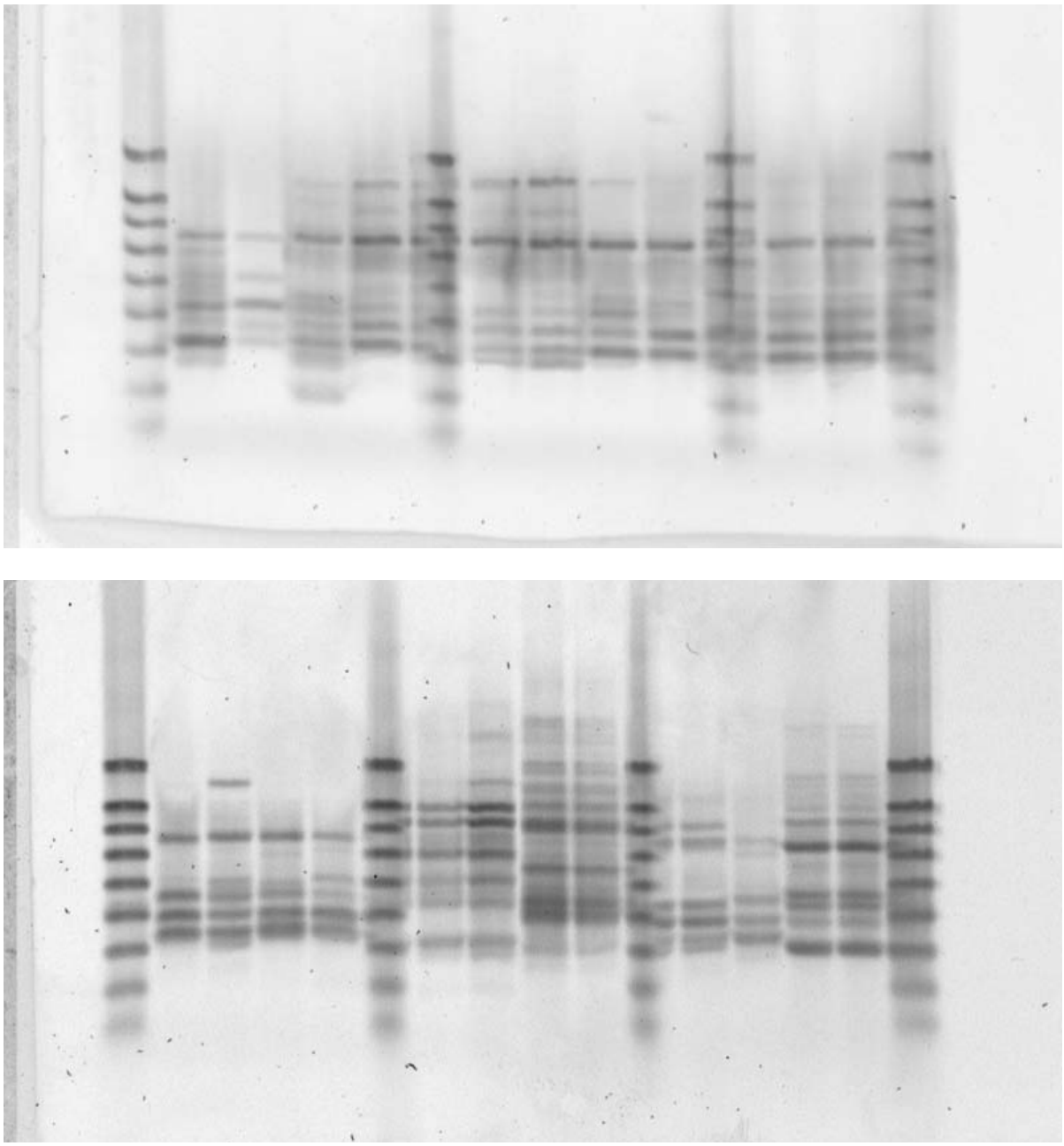


Figure 6. Phylogenetic tree (50% majority rule) from a parsimony analysis using branch-and-bound search of ITS sequences of the 8 Hawaiian *Geraniums* and relatives in the Geraniaceae family, based on 52 parsimony-informative characters. Bootstrap values (1000 replicates) are found above the branches (CI = 0.853, RI = 0.893).

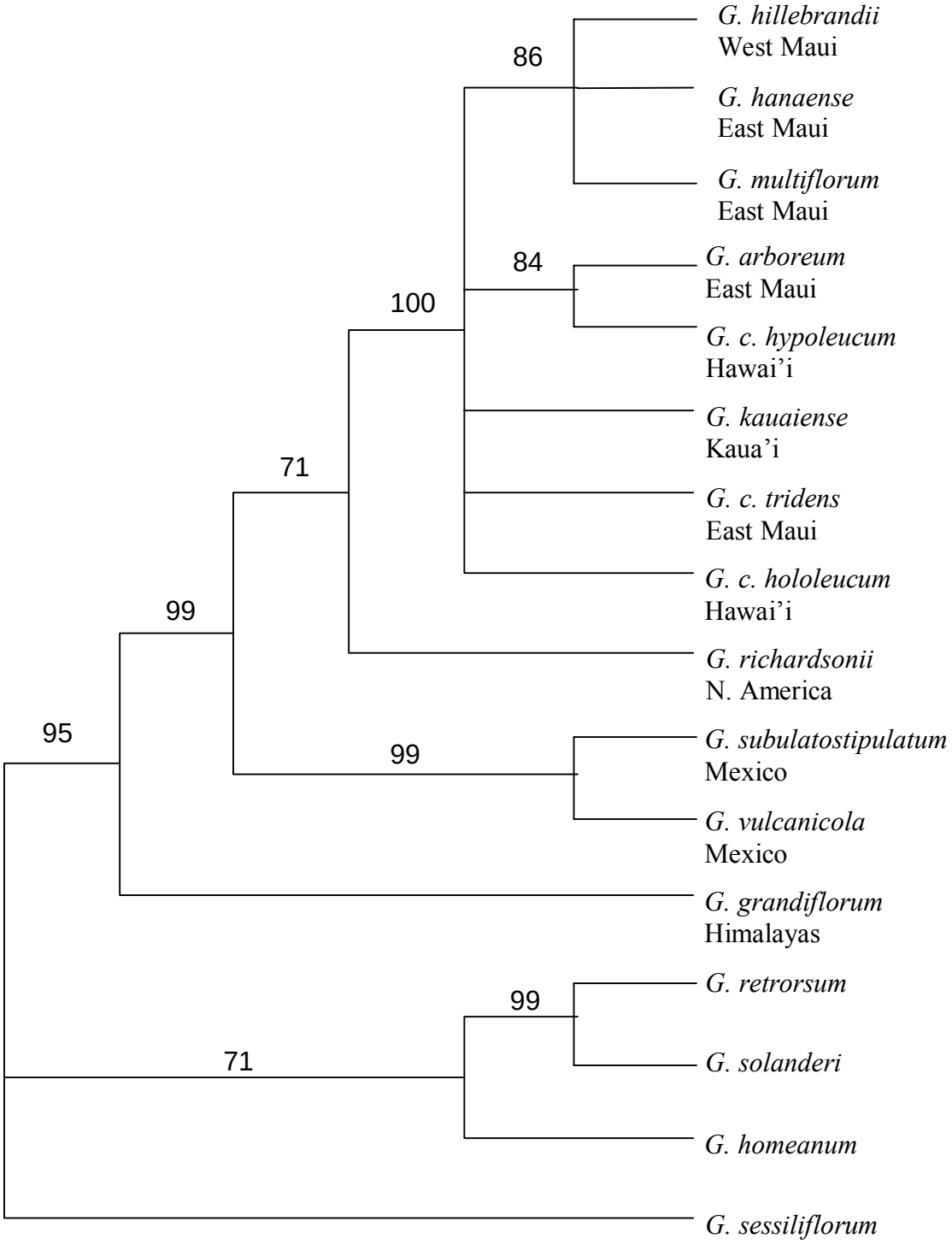


Figure 7. Phylogenetic tree (50% majority-rule) from a parsimony analysis using branch-and-bound search of *trn*L-F sequences of the 8 Hawaiian *Geraniums* and relatives in the Geraniaceae family, based on 19 parsimony-informative characters. Bootstrap values (362 replicates) are found above the branches (CI =0.962, RI = 0.895).

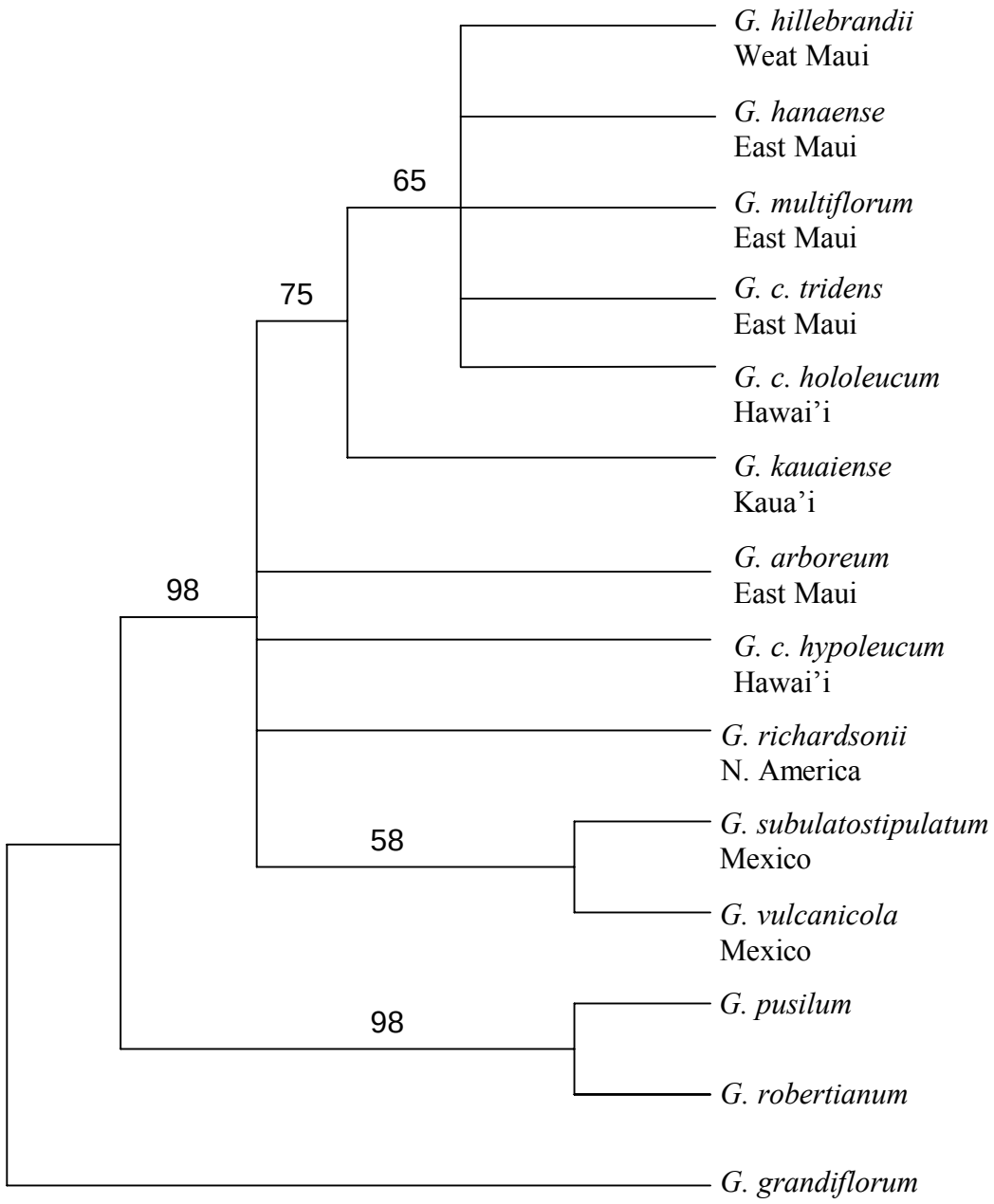


Figure 8. Phylogenetic tree (50% majority-rule) from a parsimony analysis of the combined ITS and *trn*L-F sequences of the 8 Hawaiian *Geraniums* and relatives in the Geraniaceae family, based on 30 parsimony-informative characters. Bootstrap values (1000 replicates) from a branch-and-bound search of the 50% majority-rule tree (CI = 0.936, RI = 0.926) are found above the branches.
Below the branches are the bootstrap values (1000 replicates) from a distance analysis using neighbor-joining search.

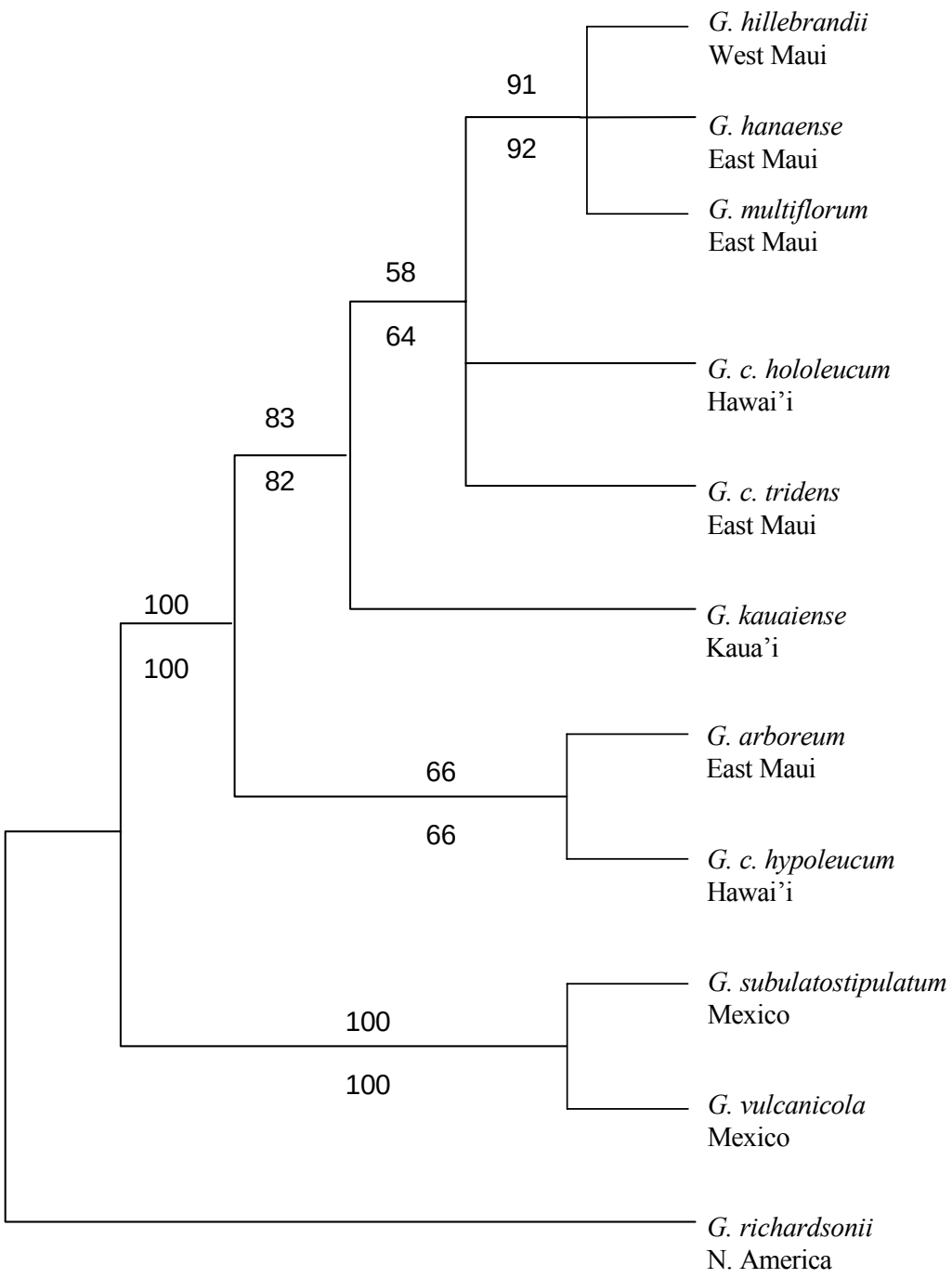


Figure 9. Phylogram of one of three equally-parsimonious trees from a parsimony analysis of the combined ITS and *trn*L-F sequences of the 8 Hawaiian *Geraniums* and relatives in the Geraniaceae family, based on 30 parsimony-informative characters, obtained from a branch-and-bound search (CI = 0.936, RI = 0.926). Numbers above the branches indicate the number of nucleotide substitutions.

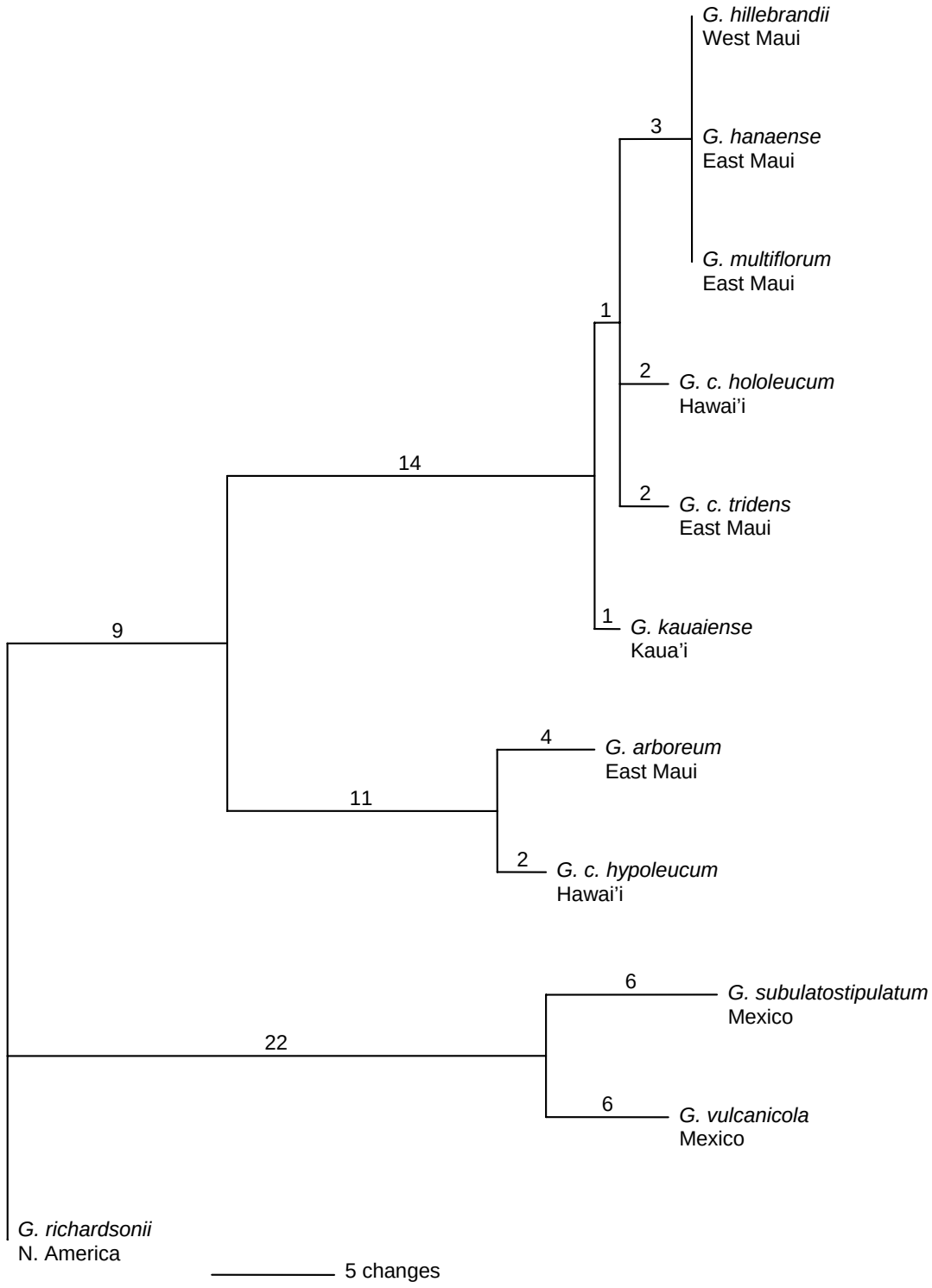


Figure 10. Phylogenetic tree (50% majority-rule) from a distance analysis using UPGMA search of ISSR data matrix of the 8 Hawaiian *Geraniums* and relatives in the Geraniaceae family, based on 20 parsimony-informative characters Bootstrap values (1000 replicates) are found above the branches (CI = 0.547, RI = 0.147).

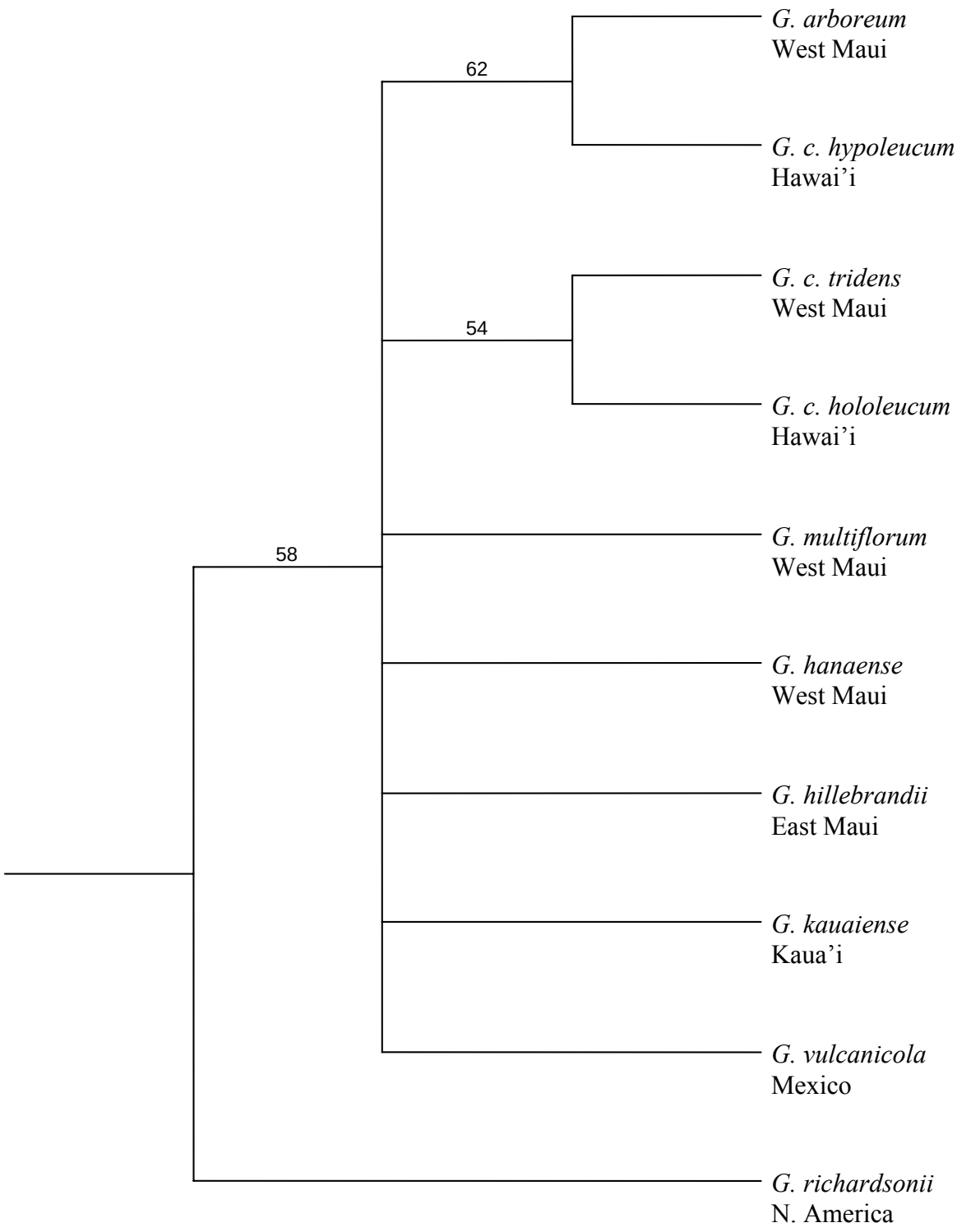
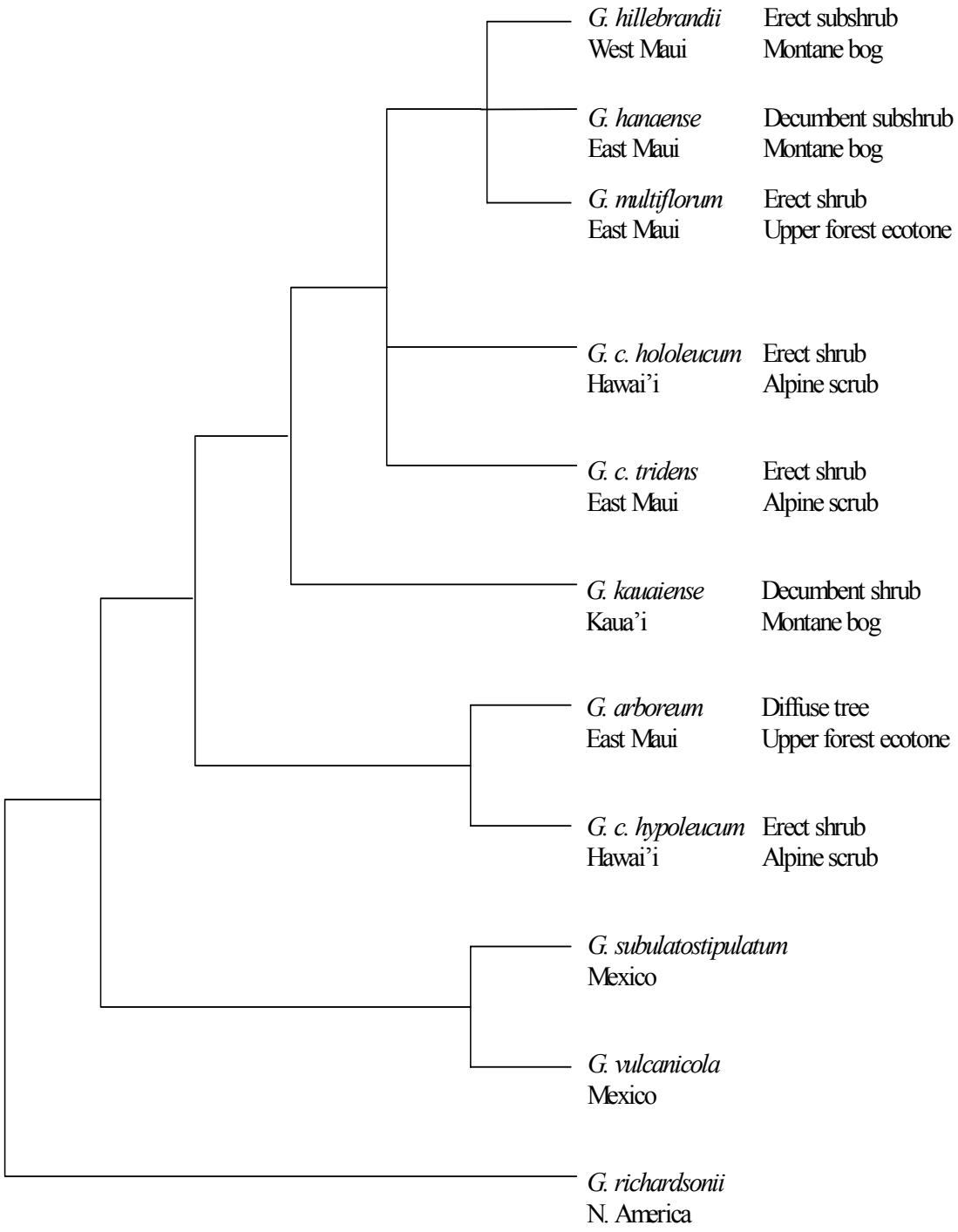


Figure 11. Habit/habitat information included on the phylogenetic tree based on the combined ITS and *trn*L-F data.



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APPENDICES

Appendix A – Nomenclature of the Hawaiian *Geraniums*

An agreement on exactly how many species and subspecies there are among the Hawaiian *Geraniums* is something to be desired. By W.J. Hooker in 1937, *G. cuneatum* was the first Hawaiian *Geranium* species to be described. Fosberg (1936) recognized *G. arboreum*, two varieties of *G. multiflorum* (*canum* and *typicum*), four varieties of *G. cuneatum* (*tridens*, *hololeucum*, *hypoleucum*, and *Menziesii*), and two varieties of *G. humile* (*mauiensis* and *kauaiensis*). Carlquist and Bissing (1976) recognized *G. arboreum*, two subspecies of *G. multiflorum* (*multiflorum* and *ovatifolium*), four subspecies of *G. cuneatum* (*cuneatum*, *hololeucum*, *hypoleucum*, and *tridens*), and two subspecies of *G. humile* (*humile* and *kauaiense*). Medeiros and St. John (1988) described a new species, *G. hanaense*. In 1990 Wagner *et al.* recognized *G. arboreum, G. multiflorum, G. hanaense, G. humile, G. kauaiense*, and the previously mentioned subspecies of *G. cuneatum*. In 1995 Funk and Wagner elevated the subspecies *G. c. tridens* to specific level. In 1997 Áedo and Garmendia acknowledged the name change of *G. humile* to *G. hillebrandii* and made a special note that *G. hillebrandii* and *G. kauaiense* are indeed separate species. For the sake of this research, nine taxa are recognized: *G. arboreum, G. multiflorum, G. hillebrandii, G. hanaense, G. kauaiense, G. cuneatum ssp. cuneatum, G. c. ssp. hololeucum, G. c. hypoleucum,* and *G. c. ssp. tridens*.

Appendix B – Detailed Protocols

CTAB Isolation

Use the IEC clinical centrifuge at room temperature.

Hot Grind: Pre-warm mortar, pestles, and 2x CTAB buffer at 65°C. Grind 0.15 g dried tissue with a pinch of sterile sand in 15 ml 2x CTAB buffer. Transfer to 50 ml centrifuge tube. Rinse mortar and pestle with 5 ml 2x CTAB buffer and add the rinse solution to the tube.

Cold grind: Put mortars and pestles in the freezer to chill. Pre-warm 2x CTAB buffer at 65°C. Grind about 1 gram frozen tissue in chilled mortar with a small amount of liquid nitrogen. Once the nitrogen boils away, scoop ground powder into 50 ml centrifuge tube. Rinse mortar and pestle with 20 ml 2x CTAB buffer and pour solution into the tube.

After grinding, incubate/shake slowly at 65°C for 40 minutes.

Extract with 20 ml SEVAG (24:1 chloroform: isoamyl alcohol) in hood. Mix gently.

Burp before centrifuging (IEC clinical centrifuge) at level 7 for 4 minutes.

Remove aqueous phase with nipped plastic transfer pipette. Put the aqueous phase in a new 50 ml centrifuge tube. Add 2/3 volume -20°C isopropanol (e.g. 11 ml isopropanol to 16 ml sample). Put in -20°C freezer overnight.

Centrifuge at level 5 for 6 minutes. Pour off supernatant (watch for sliding pellet). Add 5 ml wash buffer (10mM NH_4OAc, 76% EtOH), dislodge pellet, and let it sit for 15 minutes.

Spin down DNA at level 3 for 5 minutes; pour off liquid. Prop at an angle down to air dry and remove alcohol. Let it set for 30-60 minutes. Put in vacuum-oven with no heat for about 30 minutes. Re-suspend in TE (100-400 µl, depending on the pellet).

Qiagen Mini Spin Columns

Use the CLP Silent Spin centrifuge at room temperature.

Before starting, prepare a 65°C water bath, a bucket of ice, and six 1.5 ml tubes for each sample (3 tubes will need to have the caps nipped off). Transfer genomic DNA sample to 1.5 ml tube. Raise volume to 400 µl using Buffer AP1. Add 4 µl RNAse A. Incubate in water bath at 65°C for 10 minutes, mix (invert tube) 2-3 times during incubation. Add 130 µl Buffer AP2. Incubate on ice for 5 minutes. The solution will get very cloudy. Centrifuge at 14K rpm for 5 minutes. Pipette supernatant to lilac QiaShredder column. Centrifuge at 14K rpm for 2 minutes. Transfer the flow-through to a new tube (measure how much there is). Add 1.5 volumes (of the flow-through) of Buffer AP3 and mix well. Transfer 650 µl of the solution to a mini column. Centrifuge at 8K rpm for 1 minute. Apply the rest of the solution to the mini column and centrifuge again. If there is gelatinous material in the column, centrifuge it a little longer. Add 500 µl Buffer AW, centrifuge at 8K rpm for 1 minute. Pour off flow-through. Add 500 µl more Buffer AW, centrifuge for 2 minutes at 14K rpm. Throw away flow-through collection tube. Put column in a new 1.5 ml tube. Add 100 µl Buffer AE. Incubate at room temperature for 5 minutes. Centrifuge at 8K rpm for 1 minute to elute (Elution A). Add 100 µl more Buffer AE, incubate, and centrifuge for Elution B.

PCR – ITS, trnL-F

(50 µl reaction)

5 µl 10x $MgCl_2$–free buffer
8 µl dNTP mix (1.25 mM each dNTP – 62.5 µl each 10mM dNTP plus 250 µl water)
1 µl Primer 1 (10 µM)
1 µl Primer 2 (10 µM)
5 µl $MgCl_2$ (25 mM)
0.5 µl taq (2.5 Units)
1 µl template
28.5 µl dH_2O

Thermocycler profile ("SARAH")

1) 95°C for 00:03:00
2) 95°C for 00:01:00
3) 54°C for 00:01:00
4) 72°C for 00:02:00
5) 34 times to (2)
6) 72°C for 00:08:00
7) hold at 4°C

Primer Name	**Primer Sequences (5'-3')**
TABC	CGAAATCGGTAGACGCTACG
TABF	ATTTGAACTGGTGACACGAG
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

PCR – ISSR

(25 µl reaction)

2.5 µl 10x $MgCl_2$–free buffer
2.5 µl BSA (4 mg/ml)
4 µl dNTP mix (1.25 mM each dNTP – 62.5 µl each 10mM dNTP plus 250 µl water)
2 µl Primer (10 µM)
1.5-3 µl $MgCl_2$ (25 mM)
0.1 µl taq (2.5 Units)
2 µl template

Thermocycler profile ("ISSR")

1) 94°C for 00:02:00
2) 94°C for 00:0:40
3) 44°C for 00:00:45
4) 72°C for 00:01:30
5) 39 times to (2)
6) 72°C for 00:05:00
7) hold at 4°C

Primer Name	**Primer Sequences (5'-3')**
UBS807	AGAGAGAGAGAGAGAGT
UBS810	GAGAGAGAGAGAGAGAT
ISSR Manny	CACCACCACCACRC

Ethanol Precipitation of PCR Product with Sodium Acetate

Use the CLP Silent Spin centrifuge at 4°C with strip tube adapter.

Bring each sample up to 50 µl by adding dH2O. Add 1/10 volume of 3 M NaOAC pH 5.2 (1/10 of 50 µl = 5 µl). Add two volumes (2 x 50 µl = 100 µl) of 100% EtOH. Gently mix and refrigerate at -20°C for at least 20 minutes (or overnight). Centrifuge at 14K rpm for 10 minutes. Carefully pipette off ethanol. Add two volumes (2 x 50 µl = 100 µl) 70% EtOH and centrifuge again for 10 minutes. Pour off ethanol. Let dry in vacuum oven (no heat) for 25 minutes. Re-suspend in dH_2O.

Sequencing Reaction

10 µl reaction

2 µl BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix
2 µl BigDye® 5x Sequencing Buffer
2 µl Primer (10 µM)
1 µl template
3 µl dH_20

Thermocycler Profile ("BIGSARAH")

1) 95°C for 00:03:00
2) 95°C for 00:00:15
3) 60°C for 00:04:00
4) 72°C for 00:02:00
5) 24 times to (2)
6) hold at 4°C

Primer Name	**Primer Sequences (5'-3')**
TABC	CGAAATCGGTAGACGCTACG
TABF	ATTTGAACTGGTGACACGAG
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

Ethanol Precipitation of Sequencing Reaction with Sodium Acetate

Use the CLP Silent Spin centrifuge at 4°C with strip tube adapter.

Add 1/10 volume of 3 M NaOAC pH 5.2 (1/10 of 10 µl = 1 µl). Add two volumes (2 x 10 = 20 µl) of 100% EtOH. Gently mix and refrigerate at -20°C for at least 20 minutes (or overnight). Centrifuge at 14K rpm for 10 minutes. Carefully pipette off ethanol and salt. Add two volumes (2 x 10 = 20 µl) 70% EtOH and centrifuge again for 10 minutes. Pipette off ethanol and salt. Let dry in vacuum oven (no heat) for 25 minutes.

In-house sequencing: re-suspend in 12 µl TSR (template suppression reagent).

Sending it out: do not re-suspend, send out dry. (GeneGateway re-suspends with ABI Hi-Dye Formamide).

Sequencing

Denature samples at 98ºC for 5 minutes (Thermocycler program “HOTCOLD”). Keep samples on ice until ready to put them in the sequencer.

Appendix C – Sequences

ITS

G. arboreum
ATTGTCGAACCCTGCACAGCAGAGCGACCCGCGAACTCGTTAACAAACCGCGGGGAGCGGGT
GGTGCCTGCGCCCCCCGCAACCCGATGTCGGGTGATTGGGGGAAGCCCACTCTGCCCGACAA
AAAACGTACCCCCGGCGCGGTCCGCGCCAAGGAATCNAAACGAAGCAACGTGTGCAGTCCGC
CCGTTCGCGGGAAGCGACGGCAACACGGTCTTCCAATGTATACTAAACGACTCTCGGCAACG
GATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG
AATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCAC
GCCTGCCTGGGCGTCACGCGCTCCGTCGCCCCGCAACCCCTAACCCCGGAAACGGGCGAGGG
TGCTTGCGGTGCGGACAGTGGTCTCCCGTGTGCCCTGCTCGCGGCTGGCCTAAATTTGAGTCC
CGGACGCTCTGCTCTGCAGCCGACGGTGGTTGAGAAGCCCTCGAAAACGTGCTGCTGCATTGC
TGCCCGATGTGGACCCTGTGACCCTTGCGCGACCTCTCCCCACCGGGCGAGGGAGCTCCATGT
GNNACCCCAGNNCAGGCGGGGCACCCGCNAAT

G. multiflorum
ATTGTCGAACCCTGCACAGCAGAGCGACCCGCGAACTCGTTAACAAACCGCGGGGAGCGGGT
GGTGCCTGCGCCCCCCGCAACCCGATGTCGGGGGATTGGGGGAAGCCCACTCTGCCCGACAA
AAAACGTACCCCCGGCGCGGTCCGCGCCAAGGAATCGAAACGAAGCAACGTGTGCAGTCCGC
CCGTTCGCGGGAAGCGACGGCAACACGGTCTTCCAATGTATACTAAACGACTCTCGGCAACG
GATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG
AATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCAC
GCCTGCCTGGGCGTCACGCGCTCCGTCGCCCCGCAACCCCTAACCCCGGAAACGGGCGAGGG
TGGTTGCGGTGCGGACAGTGGTCTCCCGTGTGCCCTGCTCGCGGCTGGCCTAAATTTGAGTCC
CGGACGCTCTGCTCTGCAGCCGACGGTGGTTGAGAAGCCCTCGAAAACGTGCTGCTGCATTGC
TGCCCGATGTGGACCCTGTGACCCTTGCGCGACCTCTCCCCACCGGGCGAGGGAGCTCCATCT
GCGACCCCAGGTCAGGCGGGGCTACCCGCTGAAT

G. hanaense
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G. hillebrandii
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G. kauaiense
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G. c. tridens
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G. c. hololeucum
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G. c. hypoleucum
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G. richardsonii
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G. subulatostipulatum
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G. vulcanicola
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G. grandiflorum
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G. arboreum
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G. multiflorum
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G. hanaense
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G. hillebrandii

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G. kauaiense

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G. c. tridens

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G. c. hololeucum

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G. c. hypoleucum

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G. richardsonii

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G. subulatostipulatum
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G. vulcanicola
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G. grandiflorum
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