

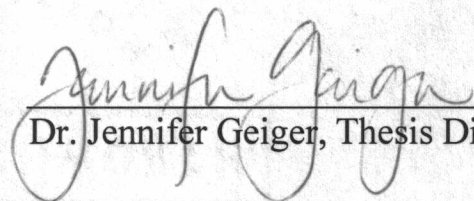
Biogeographical Origins of Hawaiian *Diplopterygium pinnatum*  
by Long Distance Wind Dispersal

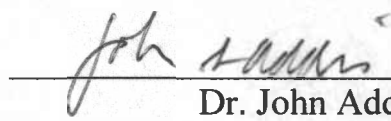
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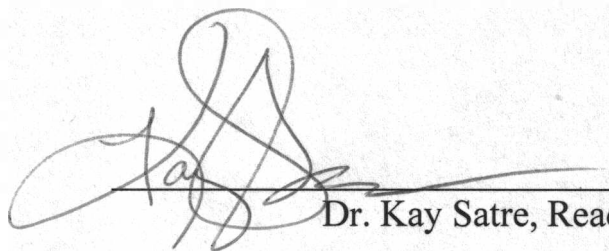
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**Abstract**

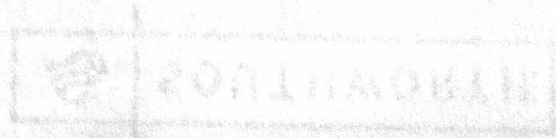
This study was designed to investigate a possible biogeographical origin and mechanism of dispersal of the Hawaiian fern *Diplazium pinnatum*. Molecular phylogenetic analyses were conducted on *trnL-F* and *rbcL*, cpDNA regions, for seven species of the genus *Diplazium* and three outgroups. Maximum parsimony and maximum likelihood analyses were used for phylogenetic reconstruction. Three hypotheses were tested: that the ancestor of *D. pinnatum* originated from 1) an Indo-Pacific source and traveled to Hawaii via the jet stream, 2) an American source and traveled to Hawaii via the trade winds, or 3) an Austral source and traveled to Hawaii by a combination of an intertropical convergence zone (ITCZ) shift, Hadley cell air movement, and the trade winds. The two methods of reconstruction, maximum parsimony and maximum likelihood, produced similar trees with similar support. The second hypothesis was rejected and the first and third hypotheses cannot be distinguished.

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## Introduction

Hawaii gives scientists unique opportunities to understand evolution on islands; both the archipelago's young age and its seclusion provide an excellent model for island evolution (Carlquist 1974). The great distance of Hawaii from any landmass, approximately 3500 km from North America (Clague and Dalrymple 1987), presents a formidable barrier to dispersal methods common to plants and animals that occur on the continental land masses (Lindqvist and Albert 2002). The Hawaiian Islands were formed, and continue to form, by volcanic activity from a point under the Pacific tectonic plate due to the exceptional heat at this point (Carson and Clague 1995). The Pacific tectonic plate moves in a northwesterly direction and, as it moves, the islands are displaced in the same direction and the volcanoes become extinct (volcanic activity ceases). After removal from the hotspot, erosion affects the islands over a long time period, and the islands eventually subside into the ocean. Thus, although the entire chain is about 80 million years old, the current oldest high island is only around 5 million years old (Carson and Clague 1995). For these reasons, dispersal methods must be suggested and tested so that an understanding develops for how organisms came to live on the Hawaiian Islands (Carlquist 1974). Dispersal models that are developed from studies of Hawaiian flora and fauna then may serve as models for other islands, and may even impact models already developed for intercontinental dispersal.

In many respects, the first steps of developing models of dispersal have already been undertaken Carlquist (1996). Lindqvist and Albert (2002) used the *rbcL* gene, the *trnL* intron, and the 5S ribosomal spacer to construct phylogenetic cladograms to find the closest living relative of the Hawaiian mints. Finding where a plant originated along with

understanding other characteristics of that plant, such as reproductive strategies, may help investigators to propose a specific dispersal model for that species. For example, the Hawaiian mint's closest living relative is in North America (Lindqvist and Albert 2002). This information alone may not be telling, but when other characteristics of the plant are considered, certain models may gain favor over others. For instance if a plant uses animals for seed dispersal, then dispersal models that involved these animals would gain favor. In Lindqvist and Albert (2002) Hawaiian mints were believed to have originated from North America and have been brought to the Hawaiian Islands by migratory birds.

For pteridophytes, the wind dispersal model has been favored over others. Spores from ferns range in size from 20 to 50 microns, which allows these spores to be carried by wind over large distances (Carlquist 1980). There are possible concerns, however, with this model of dispersal for spores, including those of desiccation (Carlquist 1974) and of UV exposure (Gradstein and van Zanten 2001). Yet, it has been shown that some spores are able to germinate after being in high altitudes, where most high-speed winds occur (Gradstein and van Zanten 2001).

The flora of the Hawaiian Islands provides some insight into the relative importance of spore dispersal in understanding biogeographical patterns. About one-sixth of the Hawaiian flora is comprised of pteridophytes (Palmer 2003). With such a significant number of spore-producing organisms, a model of dispersal is necessary to explain their presence, and our understanding of island biology is incomplete without information about spore dispersal (Geiger and Ranker 2005).

To better understand the continental origins of Hawaiian pteridophytes and spore dispersal via winds over long distances, this project's major goal was to study an endemic

Hawaiian pteridophyte and attempt to find its closest living relative using molecular phylogenetic methods. *Diplopterygium pinnatum*, a pteridophyte, was chosen for four main reasons: 1) it is endemic to the Hawaiian Islands (Kramer and Green 1990); 2) other species in the genus *Diplopterygium* have a wide geographic range (Kramer and Green 1990); 3) little research has been done on this species, and 4) there are relatively few species in this genus, so obtaining a complete phylogeny of the genus was realistic.

A member of the family Gleicheniaceae, the genus *Diplopterygium* contains 28 species (Hassler and Swale 2002). This genus is described by a creeping protostelic stem with a dormant rachis-apex often at the second node (Kramer and Green 1990). The spores from this genus are trilete tetrahedrals composed of wide angles (Kramer and Green 1990). The genus *Diplopterygium* is characterized by growing in tight clusters (Holtum 1957) and in Hawaii *D. pinnatum* usually occurs between 350 and 1,500 m, where there is abundant moisture and often growing next to two other ferns, *Dicranopteris linearis* and *Sticherus owbyhensis* (Palmer 2003). *Diplopterygium pinnatum* occurs only on the high islands of Kauai, Oahu, Molokai, Lanai, Maui, and Hawaii (Hassler and Swale 2002).

Seven of the 28 species of *Diplopterygium* were studied, representing three distinct geographical ranges: Asia, Australia, and the Americas. Eventually all species of this genus will be sampled, but this thesis focuses on the seven available.

Three distinct wind patterns could have contributed to a long distance dispersal of *D. pinnatum*'s ancestor to Hawaii: 1) the jet stream coming from south-eastern Asia, 2) the northeasterly trade winds originating from northern America, 3) and finally the

intertropical convergence zone (ITCZ) shift combined with Hadley cells from the Austral region (Wright et al. 2001).

The jet stream, moving at roughly 150 m.p.h., comes from an Indo-Pacific source, but only has strength at a width of a few miles in the middle latitudes (Blumenstock 1959). In tropical and sub-tropical areas, it is found in the tropopause about 15-16 km above the ocean, where the temperatures can reach from  $-70^{\circ}$  to  $-85^{\circ}\text{C}$  (Flohn 1969). These cold temperatures, along with extremely dry conditions, present a possible barrier for spores (Carlquist 1996).

Trade winds, in the Northern Hemisphere, are weaker winds that originate from the American region and blow toward the equator, in the southwesterly direction (Flohn 1969). At the equator, the trade winds meet with their counterpart from the Southern Hemisphere and create a band of low pressure (ITCZ) around the earth (Flohn 1969). Spores in the trade winds would not face the harsh conditions that other spores in the jet stream face, because as Flohn (1969) states, the trade winds are lower in altitude.

The ITCZ is a band of low pressure that surrounds the globe at the equator and occurs roughly at a latitude of  $30^{\circ}\text{N}$  in the summer months, then moves to  $21^{\circ}\text{S}$  in the winter months (Flohn 1969). This band of low pressure likely acts as a barrier for wind dispersal between the North and South hemispheres. The southern shift in the winter months provides an opportunity for spores from the Southern Hemisphere to be carried away to the Northern Hemisphere, but only from land that falls within the ITCZ shift (Wright et al. 2001). A wind cycle that blows northward, called a Hadley cell, can pick up material (spores) and deposit it northeast of Hawaii, where the cycle reaches its end

(Wright et al. 2001). The spores then could be picked up by the trade winds and carried to the Hawaiian Islands (Wright et al. 2001).

I hypothesized that *D. pinnatum*'s closest living relative is from the Indo-Pacific; therefore, the spores of the ancestor of *D. pinnatum* may have traveled in the jet stream to Hawaii. The other two hypotheses that I tested are that the closest living relative of *D. pinnatum*, and thus the ancestor of *D. pinnatum*, is either from the Austral region (dispersed by the ITCZ/Hadley cell) or from North/Central America (dispersed by the trade winds).

## Materials and Methods

### *Collection and DNA extraction*

Seven species of *Diplopterygium* were analyzed (although some species were represented multiple times), and species from the genera *Dicranopteris*, *Sticherus*, and *Gleichenia* were used as outgroups. The three genera were chosen as outgroups because of their close relationship to *Diplopterygium* (Kramer and Green 1990). The seven species of *Diplopterygium* analyzed were chosen based on availability of plant tissue; however, the ultimate goal is to sample all species of this genus.

Species were collected from Hawaii, Taiwan, Borneo, and Tahiti by Dr. Tom Ranker and Dr. Jennifer Geiger, and the leaf tissue was stored in silica gel until DNA extraction. Specimens from Costa Rica, Borneo and Ecuador were obtained from herbarium collections or provided by colleagues. Table 1 lists the area, date collected, and collector for each specimen.

One cm<sup>2</sup> of sample tissue was used for total cellular DNA extraction using the FastDNA Kit from Q-Biogene. Gel electrophoresis verified that DNA was extracted. The extracted DNA band in the gel was compared to a DNA ladder also run in the gel to determine an approximate concentration of the sample DNA.

#### *PCR amplification and sequencing*

The primers for the *trnL-F* intergenic spacer (IGS) and *rbcL* came from Taberlet et al. (1991) and Pryer et al. (2001) respectively. The primers Tab e and f were used for the *trnL-F* IGS amplification and sequencing. The primers af and 1379R were used for the *rbcL* amplification, and the primers af, J520F, and M955F were used for the sequencing reactions (Pryer et al. 2001). The PCR conditions used were outlined in Geiger and Ranker (2005), but the annealing temperature and MgCl<sub>2</sub> concentrations were modified as follows: annealing temperatures were 52°C (29 repetitions) for *rbcL* reactions; 45°C (4 repetitions)/60°C (37 repetitions) for most *trnL-F* reactions, and 48°C (4 repetitions)/60°C (37 repetitions) for the rest of the *trnL-F* IGS reactions; amount of MgCl<sub>2</sub> (2.5 mM) varied from 0.5-2 µL per 50 µL reaction to achieve successful amplification. Gel electrophoresis verified successful amplification. After PCR-amplification, 2 µL of ExoSAP-IT (USB Corporation, Cleveland, Ohio) was added to each reaction and then run through the thermocycler at 37°C for 15 minutes and then 80°C for another 15 minutes. Following this step, the reactions were vacuum dehydrated and rehydrated with dH<sub>2</sub>O to a DNA concentration of 50ng/µl.

Amplified samples were sent to Macrogen (Seoul, South Korea) where sequencing reactions were performed on an ABI3730XL sequencer and sequences were

obtained. Sequences were edited using ChromasPro version 1.15 (Technelysium Pty Ltd., Helensvale, Australia). The *trnL-F* IGS sequences were aligned using Clustal X (Thompson et al. 1997) and then manually adjusted to achieve more accurate alignments. The *rbcL* sequences were manually aligned.

### *Phylogenetic analysis*

Maximum parsimony and maximum likelihood analyses were performed on the *trnL-F* IGS and the *rbcL* sequences separately and then a combined maximum parsimony analysis was performed as implemented in PAUP\* 4.0 (Swofford 1998). In the maximum parsimony analyses, all characters were equally weighted and unordered. All uninformative characters were excluded and the heuristic search algorithm with 1000 random addition sequence replicates with MulTrees activated, and TBR branch swapping was employed. I also performed a bootstrap analysis with 1000 repetitions and 10 random addition sequence replicates each.

For each data set I used ModelTest 3.7 (Posada and Crandall 1998) to determine the model of molecular evolution that best explained the data for use in maximum likelihood analysis. Under the Akaike Information Criteria, the model of molecular evolution that best explained the *rbcL* data set was GTR+G (Tavaré 1986 modified by Posada and Crandall 1998) and for *trnL-F* IGS the K81uf+G model (Kimura 1981 modified by Posada and Crandall 1998) best explained the data. Using these models maximum likelihood bootstrap analysis was performed as implemented in PAUP\* 4.0 (Swofford 1998) with 100 repetitions and 10 random addition sequence replicates each.

Table 1: Sample Information: Species list, area collected, date collected, and collection number

| <b>FAMILY GLEICHENIACEAE</b>               |             |             |                          |
|--|-------------|-------------|--------------------------|
| <b>Species</b>                             | <b>Area</b> | <b>Date</b> | <b>Collection number</b> |
| Dicranopteris linearis (Burm)              | Moorea      | unavailable | Ranker 1930              |
| Dicranopteris linearis (Burm)              | USA         | unavailable | Ranker 1869              |
| Dicranopteris linearis var. Montana (Burm) | Taiwan      | 6/4/04      | Ranker 2075              |
| Diplopterygium pinnatum (Kunze)            | USA         | unavailable | Ranker 1983              |
| Diplopterygium longissimum (Blume)         | Tahiti      | unavailable | Ranker 1896              |
| Diplopterygium pinnatum (Kunze)            | USA         | unavailable | Ranker 1877              |
| Diplopterygium blotianum (C. Chr.)         | Taiwan      | 6/4/04      | Ranker 2076              |
| Diplopterygium chinesis (Rosenstock)       | Taiwan      | 6/1/04      | Ranker 2013              |
| Diplopterygium glaucum (Thunb.)            | Taiwan      | 6/1/04      | Ranker 2022              |
| Diplopterygium bancroftii (Hook.)          | Costa Rica  | 5/1/04      | Lemieux 2254             |
| Diplopterygium bancroftii (Hook.)          | Ecuador     | 2/12/04     | Moran 6837               |
| Diplopterygium bullatum (T. Moore)         | Borneo      | 6/1/05      | Ranker 2133              |
| Diplopterygium longissimum (Blume)         | Borneo      | 6/1/05      | Ranker 2198              |
| Diplopterygium longissimum (Blume)         | Borneo      | 6/1/05      | Ranker 2120              |
| Diplopterygium longissimum (Blume)         | Fiji        | 6/1/05      | Motley 2927              |
| Diplopterygium longissimum (Blume)         | Borneo      | 6/1/05      | Ranker 2114              |
| Gleichenia microphylla (Brown)             | Borneo      | 6/1/05      | Ranker 2122              |
| Sticherus owhyensis (Hook.)                | Kawaii      | unavailable | Ranker 1982              |
| Sticherus owhyensis (Hook.)                | Maui        | unavailable | Ranker 1880              |

## Results

### *Variability of Sequences*

Sixteen sequences of *trnL-F* IGS were obtained for *Diplopterygium* and outgroups *Gleichenia microphylla* and *Sticherus owbyhensis*. These sequences contained 320-394 base pairs, 417 total aligned characters, 272 of which were constant characters, 34 variable characters but not parsimony-informative, and 111 parsimony-informative characters. Sixteen sequences of *rbcL* were obtained for *Diplopterygium* and outgroups *Gleichenia microphylla*, *Dicranopteris linearis*, and *Sticherus owbyhensis*. These sequences contained 1305 total base pairs, 1147 of which were constant characters, 94 variable characters but not parsimony-informative, and 64 parsimony-informative characters. The combined analysis contained 1722 total characters: 1446 constant characters, 147 variable characters, and 129 parsimony-informative characters.

### *trnL-F IGS analysis*

A single most parsimonious tree based on maximum parsimony heuristic analysis for the *trnL-F* IGS data set was obtained. This tree had a length of 177 steps, a consistency index of 0.961, and a retention index of 0.967. A maximum likelihood analysis for the same data set resulted in a topology with an  $-\ln$  likelihood value of 2225.38. The topology from the maximum likelihood bootstrap analysis is shown in figure 1. Both analyses resolved similar relationships with no conflict among them. Analysis of the *trnL-F* IGS data set (Fig. 1) supports a closer relationship of *D. pinnatum* with *Diplopterygium longissimum* than with any other species. The bootstrap support between the Hawaiian species and *D. longissimum* is 64% for the maximum likelihood

analysis and 63% for the maximum parsimony analysis (Fig. 1). *Diplopterygium longissimum* samples from Tahiti, Fiji, and Borneo are clustered in the same clade with a confidence level of 85% (maximum likelihood, ml) and 90% (maximum parsimony, mp). *Diplopterygium pinnatum* and *D. longissimum* are strongly supported as part of an Indo-Pacific clade with 89% (ml) and 100% (mp) support. *Diplopterygium bancroftii* from Ecuador and Costa Rica are the furthest of the *Diplopterygium* genus from the Hawaiian species (100% ml and 100% mp). *Diplopterygium* was supported as monophyletic with 100% bootstrap support in both analyses. Other relationships within *Diplopterygium* cannot be determined due to the low level of resolution among species.

#### *rbcL* analysis

Parsimony analysis of *rbcL* sequences produced one most parsimonious tree. This tree had a length of 187 steps, a consistency index of 0.898, and a retention index of 0.826. The tree resulting from the MP bootstrap analysis is shown in Figure 2. An identical topology resulted from the maximum likelihood bootstrap analysis (Fig. 2) and the most likely tree had a  $-\ln$  likelihood of 2865.45. The analyses did not fully resolve terminal relationships. The analysis of the *rbcL* data set supports a closer relationship of *D. pinnatum* to Asian and Austral samples than to American samples. The clade with species from Borneo, Taiwan, Fiji, Tahiti, and Hawaii had a bootstrap support level of 97% (mp) and 86% (ml; Fig. 2). Other relationships cannot be determined due to the low level of resolution.

### *Combined analysis*

The maximum parsimony analysis of the two datasets produced one most parsimonious tree. This tree had a length of 332 steps, a consistency index of 0.937, and a retention index of 0.895. This bootstrap consensus tree is shown in Figure 3. The combined analysis had similar results to that found in the *rbcL* analysis, with *D. pinnatum* in the same clade as the species from Borneo, Taiwan, Tahiti, and Fiji (100% mp).

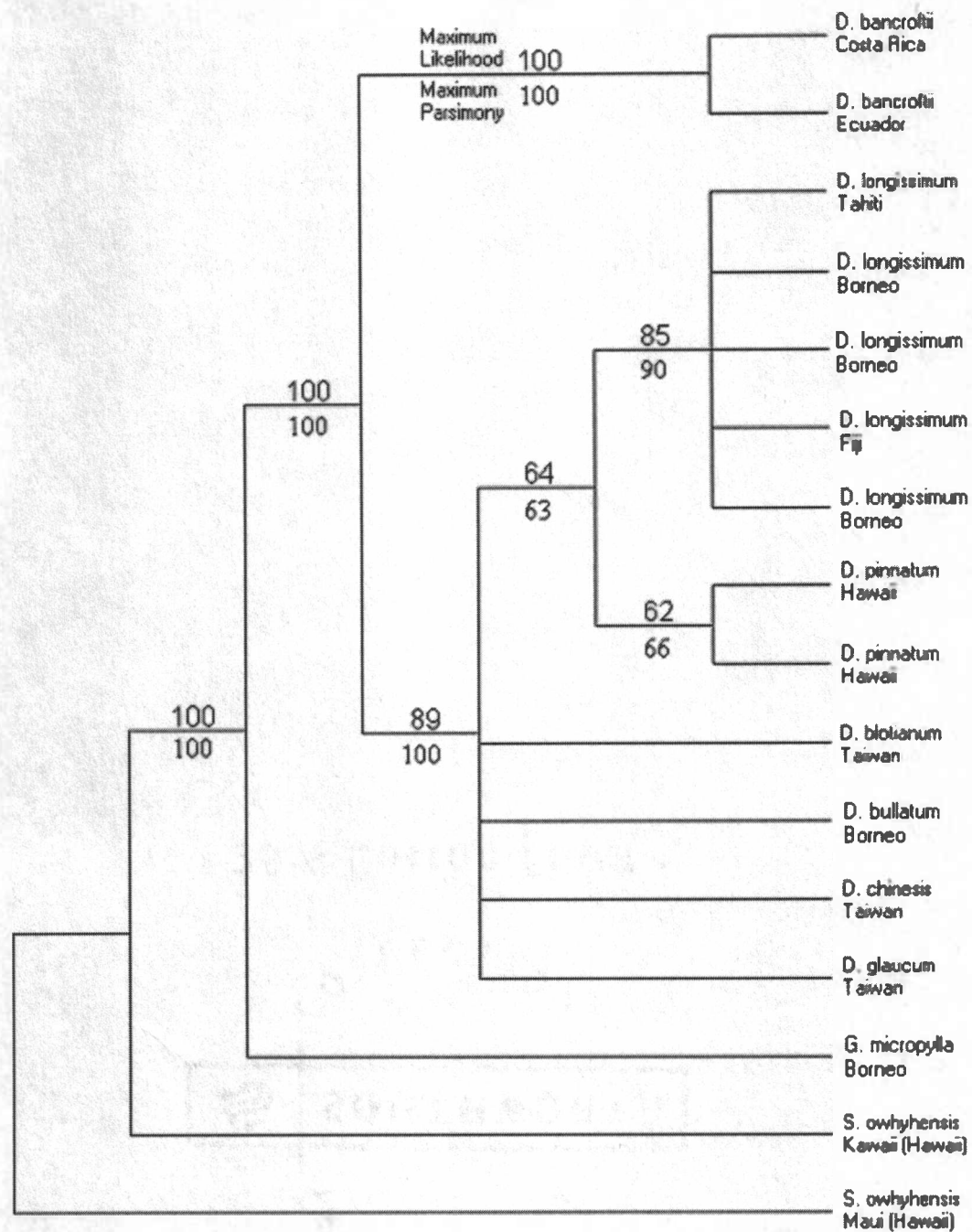


Figure 1. Maximum likelihood bootstrap consensus tree for *trnL-F* IGS data with maximum parsimony bootstrap values added. The values on top of each branch are from maximum likelihood analysis and the values below each branch are from maximum parsimony analysis. Collection location is listed below each species name.

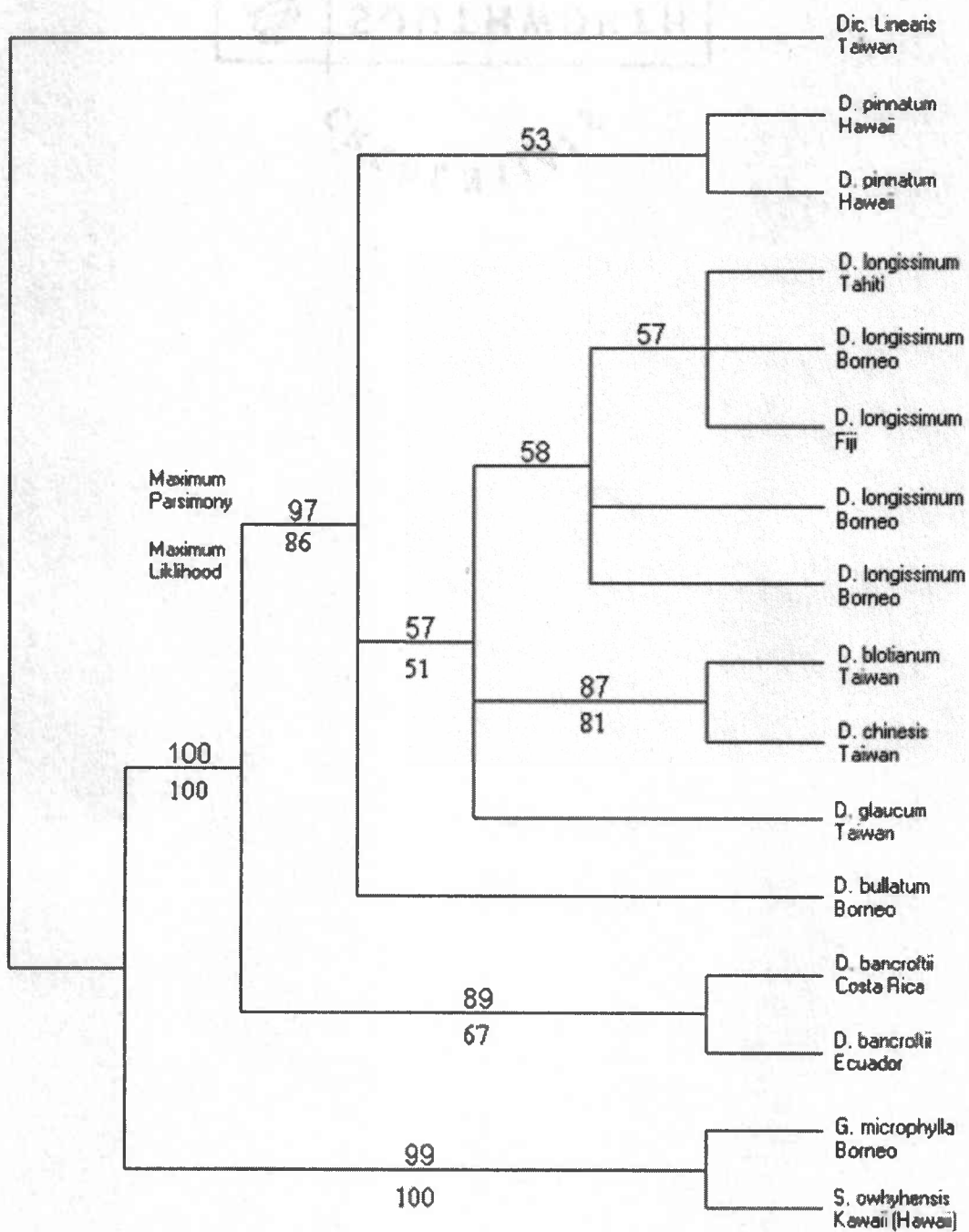


Figure 2. Maximum parsimony bootstrap consensus tree for *rbcL* data with maximum likelihood bootstrap values added. The values on top of each branch are from maximum parsimony analysis and the values below each branch are from maximum likelihood analysis. Collection location is listed below each species name.

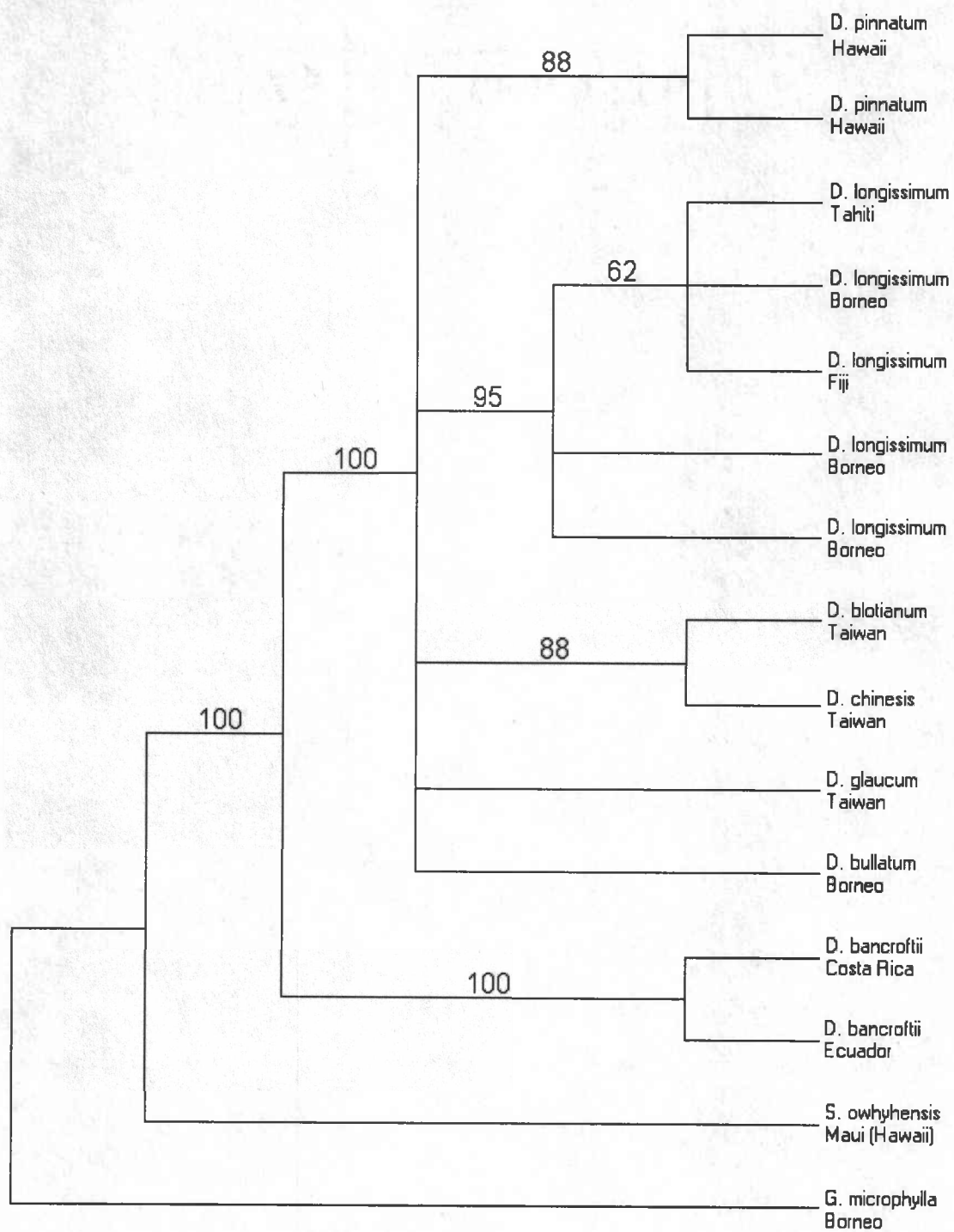


Figure 3. Maximum parsimony bootstrap consensus tree for the combined data set. Collection location is listed below each species name.

## Discussion

The goal of this project was to infer the dispersal mechanism by which the ancestor of *D. pinnatum* arrived on the Hawaiian Islands. To this end, molecular phylogenetics was used to reconstruct relationships with available members of the genus *Diplopterygium*. From these reconstructed phylogenies, I inferred *D. pinnatum*'s closest living relative and which of the three dispersal hypotheses was supported based on the region the closest living relative inhabits.

### *Relationships*

While the closest living relative of *D. pinnatum* was not determined for both cpDNA regions, several relationships have been elucidated by the three data sets, *trnL-F* IGS, *rbcL*, and the combined analysis (Fig. 1, Fig. 2, and Fig. 3). *Diplopterygium pinnatum* is resolved with *D. longissimum* (Tahiti, Borneo, and Fiji), *D. blotianum* (Taiwan), *D. chinesis* (Taiwan), *D. glaucum* (Taiwan), and *D. bullatum* (Borneo) with high support from both cpDNA regions. For the *trnL-F* IGS, the closest living relative of *D. pinnatum* supported by maximum likelihood and maximum parsimony analysis was *D. longissimum* (64% ml 63% mp; Fig. 1). This gives support that the Hawaiian *Diplopterygium* is more closely related to the South Asian and Austral species than to the species from the Americas. In the *trnL-F* IGS maximum likelihood tree a larger clade contained *D. longissimum* (Tahiti, Borneo, and Fiji), *D. blotianum* (Taiwan), *D. chinesis* (Taiwan), *D. glaucum* (Taiwan), and *D. bullatum* (Borneo) with *D. pinnatum* with support of 89%/100% (ml/mp; Fig. 1). The two samples of *D. bancroftii* from Costa Rica and Ecuador clustered together with high support in the *trnL-F* IGS cladogram (100% ml

and mp; Fig. 1). The Costa Rican and Ecuadorian species were the farthest from the Hawaiian species except for outgroups (100% ml and mp; Fig. 1). Similar results and similar support can be seen in the *rbcL* mp tree and the combined analysis tree for all of the relationships mentioned, with the main exception of the closest living relative (Fig. 2 and Fig. 3). The relationship between *D. pinnatum* and *D. longissimum* was not resolved with the *rbcL* or the combined analysis, and the other relationships seen were poorly resolved in all of the analyses. Additionally, not all *Diplopterygium* species are represented. However, *D. pinnatum* always fell within an Indo-Pacific/Austral clade to the exclusion of the South American species *D. bancroftii* (the only species of *Diplopterygium* found in the Americas).

### *Biogeography*

Based on the analysis of the *trnL-F* IGS sequences for these seven species, I can reject the hypothesis that *D. pinnatum*'s ancestor is of American origin but cannot reject either an Indo-Pacific or Austral as possible origins. The analyses of the *rbcL* and combined datasets do not allow any of the hypotheses to be rejected, but there is more confidence in the *trnL-F* IGS sequences because those sequences are more variable. *Diplopterygium pinnatum*'s ancestor appeared to have originated from an Indo-Pacific or Austral source (this information is consistent with a jet stream mechanism or an ITCZ shift for dispersal). These two regions have been implicated in other papers focusing on wind dispersal to the Hawaiian Islands. In Geiger and Ranker (2005) researchers found support for a jet stream model in a study of the Hawaiian endemic ferns *Dryopteris fusco-atra*, *Nothoperanema rubiginosum*, and a clade including *D. glabra* and close relatives.

In a study on *Metrosideros polymorpha*, Wright et al. (2001) found that *M. polymorpha*'s ancestor likely traveled via the ITCZ/Hadley cell mechanism. *Metrosideros polymorpha* has small seeds that may be carried by the wind and these seeds may have used this pathway to move from the Marquesas Islands (Southern Hemisphere) to the Hawaiian Islands (Wright et al. 2001).

#### *Further research*

The most important goal for the future would be to sample the rest of the genus. With only seven species analyzed, any assertions made will not be strongly supported. To gain higher resolution, different DNA regions should be studied for these species. These regions should be more variable than the *trnL-F* IGS regions. The two regions *trnL-F* IGS and *rbcL* were sufficient in a similar study by Geiger and Ranker (2005). That study sampled 18 Hawaiian taxa, 45 non-Hawaiian taxa, and two outgroup species (Geiger and Ranker 2005). In Lindqvist and Albert (2002) Hawaiian endemic mints were found to have originated from North America using the same cpDNA regions. Other studies, however, found that these regions did not resolve all species level relationships (Shaw et al. 2005). Shaw et al. (2005) argues that other cpDNA regions could be used with more success than the two widely used—*trnL-F* IGS and *rbcL*. Some of the regions suggested include: *trnD-trnT*, *rpoB-trnC*, and *trnS-trnG* regions since they are highly variable (Shaw et al. 2005). These studies were performed on angiosperms, but they may still be applicable.

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