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Phylogenetic Relationships of *Gunnera* based on Nuclear Ribosomal DNA ITS Region, *rbcL* and *rps16* Intron Sequences

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ABSTRACT. A previous analysis of two chloroplast gene regions, *rbcL* and the *rps16* intron, showed a clear phylogenetic pattern in *Gunnera*. However, these regions were not informative enough to completely resolve the phylogeny. In this study the nuclear ITS region was sequenced for 24 specimens representing 22 species of *Gunnera*. 223 characters out of 819 were informative and supported the same monophyletic groups as the chloroplast gene regions. Because of its greater information content, the ITS region identified additional well-supported clades. In an analysis based on the three gene regions together, 272 characters out of 3154 were informative. The results show that the South American annual, *G. herteri* is sister to all other species. The African *G. perpensa* is well-supported as sister to the remaining species, which form two well-defined clades, one with the Malayan *G. macrophylla* as sister to subgenus *Milligania* from New Zealand and Tasmania. In the other clade, the South American subgenus *Misandra* is sister group to subgenus *Panke* from South America and Hawaii. Within *Panke* the two Hawaiian species form a sister group to the American species. The morphological classification of Schindler, together with some biogeographical and morphological features, is discussed.

The systematically isolated genus *Gunnera*, with 30–40 species in Central and South America, Africa, Madagascar, Tasmania, New Zealand, Hawaii, and the Malayan Archipelago (Bader 1961), has long intrigued botanists. It has usually been included in the Haloragaceae in the Myrtales, but the systematic position has remained doubtful, and the genus is sometimes placed in a family of its own. Even though the disjunct southern distribution and its unique relationship with endosymbiotic cyanobacteria continued to attract attention, it was not until recently that the isolated position of *Gunnera* in the tricolpate clade was recognized (Chase et al. 1993).

The only monograph of the genus was published by Schindler in 1905. He divided *Gunnera* into five subgenera, *Perpensum*, *Pseudogunnera*, *Panke*, *Misandra*, and *Milligania*, mainly based on size, means of vegetative propagation, and geographic distribution. Schindler's subgenus *Perpensum* only includes the African *G. perpensa*, which is the type species for the genus (Linnaeus 1767). For nomenclatural reasons (Greuter et al. 2000), the subgenus name *Perpensum* should therefore be replaced with *Gunnera*. The species from New Zealand and the single Australian species *G. cordifolia* were included in subgenus *Milligania*. Subgenus *Misandra* includes only two species, *G. lobata* and *G. magellanica*, restricted to the southern part of South America. To subgenus *Panke* belong all the huge South and Central American species of *Gunnera* as well as the two Hawaiian species. Subgenus *Pseudogunnera* includes only the Malayan *G. macrophylla*. Schindler's classification was completed in 1933 by the addition of a new subgenus, *Ostenigunnera* (Mattfeld 1933), in connection with the discovery of the new, morphologically distinct, species *G. herteri*, among the sand dunes of the Atlantic coast in Uruguay. That *G. herteri* is an annual species, the only in the genus, was noted only recently (Wanntorp et al. 2001).

In 1995, some preliminary results on the phylogeny of *Gunnera* based on morphological and anatomical characters were summarized in an abstract by Fuller. In 2001, Wanntorp et al. published a phylogenetic study of *Gunnera* based on sequences from the chloroplast genome (Fig. 1). It was found that the South American *Gunnera herteri* is sister to all other species. *Gunnera perpensa* from Africa was weakly supported as sister group to the remaining species, which in turn formed two, well-supported clades. One included the Malayan *G. macrophylla*, the single Australian species *G. cordifolia* and the species from New Zealand. The second clade comprised the South American and Hawaiian species.

The *rbcL* and *rps16* intron regions used in that study gave only 49 informative characters, which was not enough to completely resolve the phylogeny. The position of the African *G. perpensa* remained doubtful. The relationship was still unresolved between *G. magellanica* and the *Panke* group in the South American species as was much of the relationship within the Malayan-Australasian clade (Fig. 1).

A better resolved phylogeny of *Gunnera* will aid in studying its disjunct distribution as well as in understanding the evolution of characters such as means of propagation and those traits connected with the symbiosis with the cyanobacteria (Wanntorp in prep.).

The aim of the present study is to further investigate the phylogeny of *Gunnera* by including several more species and by studying another gene region, the

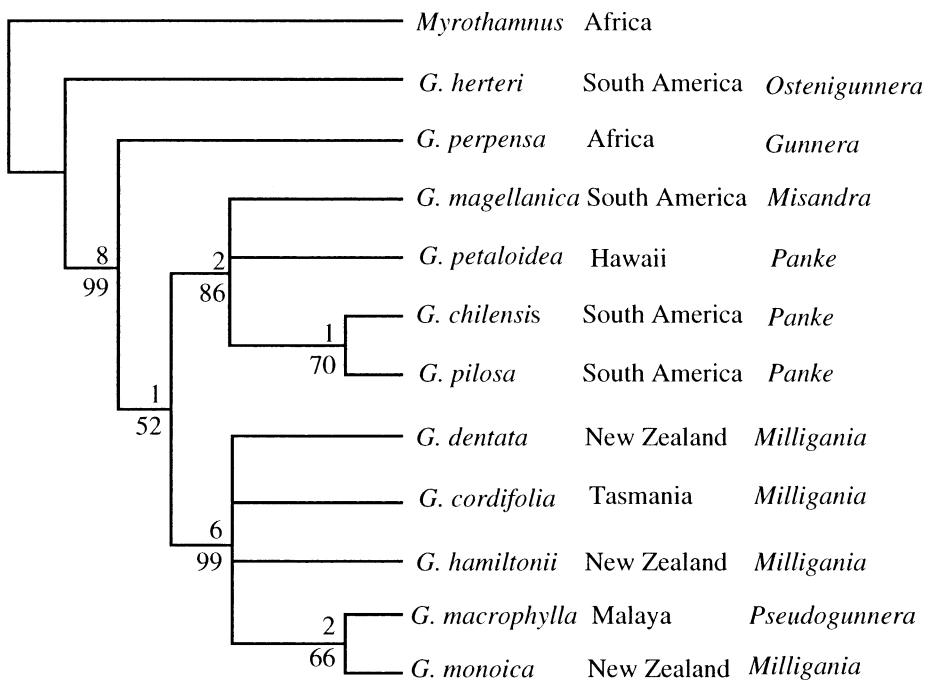

FIG. 1. Combined analysis of *rbcL* and *rps16* intron: Strict consensus tree of four most parsimonious trees (tree length = 268; CI = 0.71; RI = 0.77). Support values for internal nodes are reported as decay indices (above) and bootstrap values (below). (Redrawn from Wanntorp et al. 2001).

internal transcribed spacer (ITS) of the nuclear ribosomal DNA.

MATERIAL AND METHODS

Choice of Outgroup. A small African genus of shrubby plants, *Myrothamnus*, is well supported as sister group and the only close relative of *Gunnera* in recent molecular studies based on *rbcL*, *atp*B and 18S (Hoot et al. 1999; Soltis et al. 2000). *Myrothamnus* includes two species, *M. flabellifolius* from parts of Africa and *M. moschatus* endemic to Madagascar (Endress 1989). Of these two species, only *M. flabellifolius* has been sequenced so far. This species was also used as outgroup in the previous *rbcL*/*rps16* intron study of *Gunnera* (Wanntorp et al. 2001). *Myrothamnus* could unfortunately not be used as outgroup in the present study because the primers used for the amplification of the ITS region in *Gunnera* did not work for *M. flabellifolius*. The lack of adequate primers led us to try universal primers that produced DNA of low yield. Multiple copies of ITS were also detected. The *rbcL*/*rps16* intron analysis identified *G. herteri* as sister group to all the other included species of *Gunnera* (Wanntorp et al. 2001). The support for this placement was very high, so *G. herteri* was used as outgroup in the present ITS analysis. In the analysis combining *rbcL*, *rps16* intron, and ITS sequences, *M. flabellifolius* was used as outgroup and its missing ITS sequence was replaced by questions marks.

Ingroup Sampling. The intention of this study was to include as many species as possible of *Gunnera*. Sequences of 24 specimens representing 22 different species of *Gunnera* (Table 1) were included in the combined analysis. Twelve species belonging to subgenus *Panke* were sampled to cover as much of geographic distribution as possible. Unfortunately, *Panke* is still under-represented from Colombia, where many endemic species occur (Mora-Osejo 1984). *Gunnera chilensis* was collected in mainland Chile; *G. peltata*, *G. bracteata*, and *G. masafuerae* were collected in the Juan Fernández Islands, and the ITS sequences from the latter two species were kindly provided by Dr. E. Ruiz (Universidad de Concepción, Chile). Material of *G. brasiliensis* collected in the Serra do Mar in southeastern Brazil as well as a cultivated specimen of *G. manicata* were included in the present study. In the previous molecular study of Wanntorp et al. (2001), the *rbcL* sequence of *G. manicata* was retrieved from GenBank but the *rps16* intron for this species was not sequenced due to the lack of material from the same source. In this study, a cultivated specimen of *G. manicata* not studied previously was sequenced. Although we are working with different specimens for the *rbcL* and ITS sequences, we treat these cultivated specimens as representing the same taxon in the combined analysis. Subgenus *Panke* is also represented by *G. boliviana*, *G. pilosa*, *G. talamancana*, and *G. insignis* collected in Bolivia, Equador, Costa Rica, and Panama, respectively. From Hawaii, *G. petaloidea* was collected on Maui and *G. kauaiensis* on Kauai. *Gunnera perpensa* has sometimes been considered to consist of a complex of different species or varieties (Schindler 1905; Skottsberg 1936). For this reason two specimens, one collected in Tanzania, the other in South Africa, were included. *Gunnera macrophylla* from subgenus *Pseudogunnera*, sometimes regarded as a complex species (van der Post 1855) was represented by two different specimens from Sumatra and Java, respectively. The previous discovery of structurally different sequences of *rbcL* in these specimens (Wanntorp et al. 2001) was another reason for including both in the present analysis. Subgenus *Misandra* from South America was represented by *G. magellanica* and *G. lobata*, both collected in Chile. From subgenus *Milligania*, four species from New Zealand were included (*G. dentata*, *G. hamiltonii*, *G. monoica*, and *G. prorepens*), together with *G. cordifolia* from Tasmania. Lastly, *Gunnera herteri* collected in Uruguay was added to the ingroup in the *rbcL*/*rps16* intron/ITS analysis.

DNA Extraction, Amplification and Sequencing. Extracts were made from living plants or from silica-gel dried leaf fragments collected in the field. DNA extractions, PCR amplification, and sequencing were conducted following Wanntorp et al. 2001. The primers (18SF and 26SR) used for amplification and sequencing of *Gunnera* were situated at the 3′-end of 18S and the 5′-end of the 26S, respectively (the primer sequences were obtained from C. Ry-

TABLE 1. Names of the specimens, voucher and GenBank accession numbers for the sequences of *rbcL*, *rps16* intron and ITS. The *rbcL* gene sequences of *Gunnera manicata* (L11186), *G. monoica* (AF307918), *G. lobata* (AF060707) and *Myrothamnus flabellifolius* (AF060707) were retrieved from GenBank. Herbarium abbreviations follow Holmgren et al. (1990).

Subgenus/Species	DNA Source/Voucher	Accession No (ITS)	Accession No. (*rbcL*)	Accession No. (*rps16* intron)
GUNNERA				
Gunnera perpensa L.	L. & H-E. Wanntorp 558 (S); Tanzania	AF447748	AY008154	AY008165
Gunnera perpensa L.	B. Wallace 556/87 (S); South Africa	AF447749		
MILLIGANIA				
Gunnera cordifolia Hook. F.	L. & H.-E.Wanntorp 515 (S); Tasmania	AF447731	AY008146	AY008158
Gunnera dentata Kirk	L. & H-E.Wanntorp 534 (S); New Zealand	AF447733	AY008147	AY008159
Gunnera hamiltonii Kirk	L. & H.-E.Wanntorp 559 (S); New Zealand	AF447732	AY008148	AY008160
Gunnera monoica Raoul	L. & H-E.Wanntorp 551 (S); New Zealand	AF447734		AY008164
Gunnera monoica Raoul	C. Ezcurra 2184; New Zealand		AF307918	
Gunnera prorepens Hook.F.	A. Watkins 1 960748 (AK); New Zealand	AF447735		
MISANDRA				
Gunnera lobata Hook. F.	C. Ezcurra 2184 (CRP); Chile	AF447747	AF307919	
Gunnera magellanica Lam.	L. & H-E.Wanntorp 551 (S); Chile	AF447746	AY008152	AY008163
OSTENIGUNNERA				
Gunnera herteri Osten	L. & H.-E.Wanntorp 555 (S); Uruguay	AF447728	AY008149	AY008161
PANKE				
Gunnera boliviana Morong	C. Persson & C. Gustafsson 369 a,b (S); Bolivia	AF447743		
Gunnera bracteata Steud.	E. Ruiz 106 (Conc); Juan Fernandez Is.			
Gunnera brasiliensis Schindler	A. Reis; Brazil	AF447741		
Gunnera chilensis Lam.	L. & H-E.Wanntorp 551 (S); Chile	AF447738	AY008145	AY008157
Gunnera insignis (Oerst.) A. DC.	A. Madura 561 (S); Panama	AF447736		
Gunnera kauaiensis Rock	K. R. Wood 5914 (PTBG); Hawaii	AF447745		
Gunnera manicata Linden	L. & H-E. Wanntorp 560 (S), from cultivation	AF447740		
Gunnera manicata Linden	Kruckenberg (WTU); origin unknown		L11186	
Gunnera masafuerae Skottsb.	E. Ruiz 108 (Conc); Juan Fernandez Is.			
Gunnera peltata Phil.	M. Ricci, 501; Juan Fernandez Is.	AF447739		
Gunnera petaloidea Gaudich.	K. R. Wood 5968 (PTBG); Hawaii	AF447744	AY008155	AY008166
Gunnera pilosa HBK.	B. Ståhl et al. 3770 (AAU); Equador	AF447742	AY008156	AY008167
Gunnera talamancana Weber & Mora	B. Hammel & M. M Chavarría 22027 (INBIO); Costa Rica	AF447737		
PSEUDOGUNNERA				
Gunnera macrophylla Blume	L. & H.-E.Wanntorp 540 (S); Sumatra	AF447729	AY008150	
Gunnera macrophylla Blume	L. & H.-E.Wanntorp 542 (S); Java	AF447730	AY008151	AY008162
OUTGROUP				
Myrothamnus flabellifolius Welw.	P. Craven 4982 (WIND); Namibia			AY008168
Myrothamnus flabellifolius Welw.	Winter 72 (JHB); origin unknown		AF060707	

din, Stockholm University, Sweden, who designed them). In addition, the primers C26A (TTTCTTTTCCTCCGCT, designed by Dr. Youngbae Suh at Seoul National University, Korea) and 340F (GGCAACGGATATCTCGGCTCTCG, designed by L. Wanntorp) were used for sequencing. All sequences were assembled and edited using the Staden package (Staden 1996) and GDE (Smith et al. 1994). The sequences have been deposited in GenBank. The Genbank accession numbers are given in Table 1.

In the previous study of Wanntorp et al. (2001) the *rbcL* sequences of *G. monoica* and *G. macrophylla* were shorter. It was suspected that the phylogenetic relationship between these two species was due to a retrieved pseudogene. Recently the *rbcL* gene of *G. monoica* was sequenced once again (Wardle et al. 2001), and this time a sequence of usual length was found. In the combined analysis of the three genes in the present study we felt more confident in using this *rbcL* sequence instead of the sequence used in the previous molecular study. New in this analysis is also the *rbcL* sequence of *G. lobata* (Wardle et al. 2001).

Phylogenetic Analyses. A variety of parameters was explored in the alignment of the ITS sequences performed with the package ClustalX 1,8-PPC (Thomson et al. 1997). The final alignment was adjusted manually, so as to keep the number of the total mutational changes to a minimum (the aligned data matrix can be obtained from the first author upon request). Indels were included in the analyses and coded according to the principles suggested by Simmons and Ochoterena (2000). Substitutions and indels were given equal weight in all phylogenetic analyses.

An analysis of the ITS region alone was performed using *Gunnera herteri* as outgroup. A combined analysis based on *rbcL*, *rps16* intron, and ITS was then performed using *Myrothamnus flabellifolius* as outgroup. For some of the species, sequences of all the three gene regions were not available (Table 1). Question marks were therefore used for these sequences. We chose to do this, rather than to prune all these sequences from the analysis, to get as much information as possible from our material. As a result, 37% of the total data set was scored as missing data, representing 16% of the informative data matrix.

All phylogenies were inferred using the program PAUP* version 4.0b6 (Swofford 2001). Bootstrap analyses based on 1,000 replicates were performed to explore the robustness of the trees. The following options were used for all analyses: parsimony, branch and bound search, stepwise furthest addition sequence, collapse zero-length branches. Bremer support (1994) was calculated using the program Autodecay version 4.0.2′ PPC (Eriksson 1998).

RESULTS

The ITS Analysis. The sequenced 22 nucleotides of the 18S region did not contain any informative characters, six informative characters were detected in the sequenced part of the 26S region (79 nucleotides), ITS1 and ITS2 contributed 105 and 92 informative characters, respectively. The conserved 5.8 S subunit yielded four informative characters. A complication sometimes encountered working with the ITS region is the presence of polymorphic copies of this region. This problem was not encountered in *Gunnera*.

The ITS analysis included 24 sequences representing 22 species. Using *G. herteri* as outgroup, 223 characters out of 819 were informative (including 16 informative indel characters). The strict consensus tree of 30 most parsimonious trees is shown in Fig. 2 (tree length = 452; CI = 0.84; RI = 0.94).

The two specimens of *G. perpensa* group strongly together (bootstrap value 100%; decay index 47) and form the sister group of all other species of the ingroup (bootstrap value 100%; decay index 16). These remaining species are divided into two very well-supported clades, one including the American and Hawaiian species (bootstrap value 100%; decay index 31), and another including the species from Malaya, New Zealand, and Tasmania (bootstrap value 100%; decay index 26). The South American / Hawaiian clade forms a trichotomy. One subclade is formed by the two species *G. magellanica* and *G. lobata* (bootstrap value 100%; decay index 8). The second subclade consists of the Hawaiian species, *G. petaloidea* and *G. kauaiensis* (bootstrap value 100%; decay index 15). The third subclade consists of all American species of subgenus *Panke* (bootstrap value 99%; decay index 5). Within this subclade there is generally little resolution. *Gunnera manicata* and *G. brasiliensis* group strongly together (bootstrap value 98%; decay index 4), forming a poorly supported group with *G. boliviana* (bootstrap value 52%; decay index 1). This group is weakly supported as sister group to the remaining American species. Among them, the Chilean species form a weakly supported group (bootstrap value 51 %; decay index 1). This was in turn divided into two subclades: *Gunnera chilensis* groups strongly with *G. masafuerae* (bootstrap value 98%; decay index 4), *G. peltata* and *G. bracteata* are sister species (bootstrap value 69%; decay index 1).

In the second main clade, the two specimens of the Malayan *G. macrophylla* form the sister group (bootstrap value 87%; decay index 2) to a subclade including all the species from New Zealand and Tasmania (bootstrap value 86%; decay index 4). Within this subclade *G. monoica* is sister taxon to the other species (bootstrap value 84%; decay index 3). Next, *Gunnera hamiltonii* is the well-supported (bootstrap value 96%; decay index 4) sister to a subclade formed by *G. cordifolia*, which is in turn sister to *G. dentata* and *G. prorepens*, the latter two forming a weakly supported group (bootstrap value 62%; decay index 1).

The Combined Analysis. In the combined analysis of *rbcL*, *rps16* intron, and ITS, 272 of 3154 characters were informative. The strict consensus tree for the 15 most parsimonious trees recovered is shown in Fig. 3 (tree length = 722; CI = 0.82; RI = 0.92). As in the previous *rbcL* / *rps16* intron analysis, *G. herteri* emerged as sister to all other *Gunnera* species with a very high support (bootstrap value 100%, decay index 8). Apart from some different support values, the general topology obtained by the ITS analysis was again retrieved in the combined analysis. The only significant change from the ITS topology is that subgenus *Misandra* (*G. magellanica* and *G. lobata)* now emerges as a poorly supported sister group to a monophyletic *Panke* (bootstrap value 57%, decay index 1). The Hawaiian species (*G. kauaiensis* and *G. petaloidea*) are now the sister group of the American species of the subgenus (bootstrap value 99%, decay index 5).

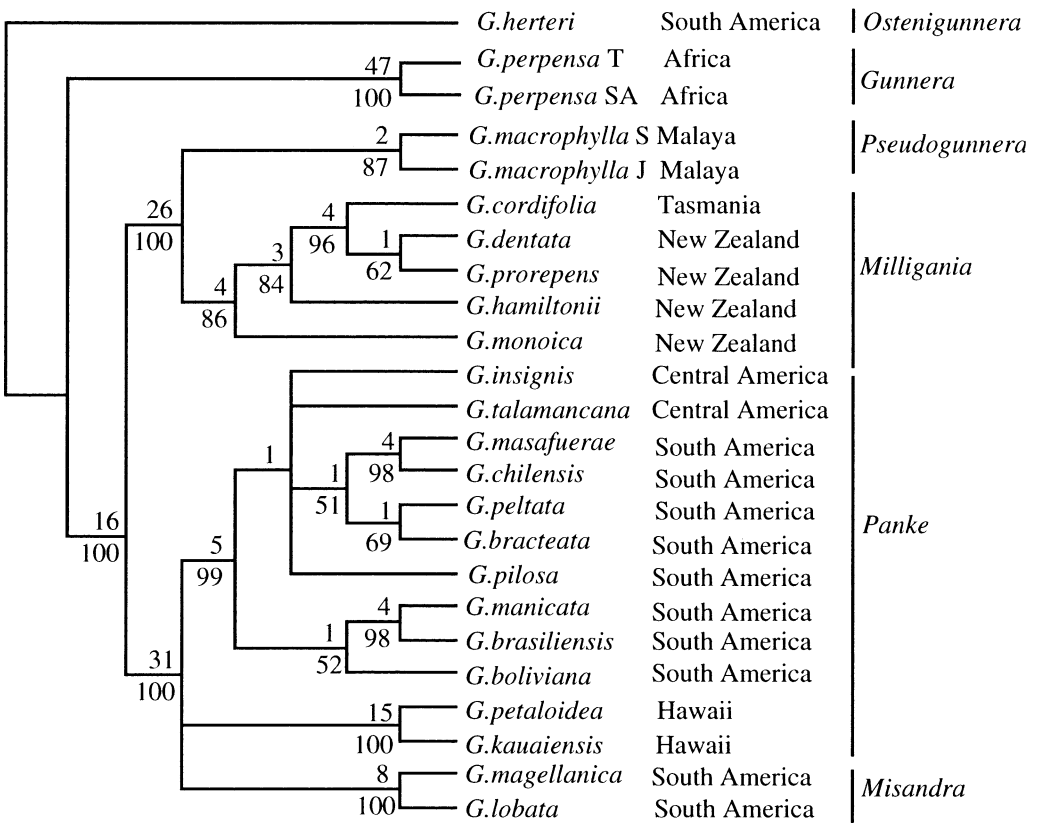

FIG. 2. ITS Strict consensus tree of 30 most parsimonious trees (length = 452, CI = 0.84, RI = 0.94). Support values for internal nodes are reported as decay indices (above) and bootstrap values (below). J = Java, S = Sumatra, SA = South Africa, T = Tanzania

DISCUSSION

The present study has generally corroborated the results obtained in the *rbcL*/*rps16* intron study (Wanntorp et al. 2001). The considerably higher number of informative characters in the ITS region and the enhanced number of taxa of this study led to the further resolution of the tree, and some of the previously suggested monophyletic groups received higher support. The well-supported position of *Gunnera herteri* as sister group to the remaining species, found by the chloroplast data (Wanntorp et al. 2001), was not further investigated in the present study (see Choice of Outgroup). Here, *Gunnera perpensa* was found as sister to two main clades, one containing all species from New Zealand, Tasmania, and Malaya, the other formed by South American and Hawaiian representatives. Within the former clade there was good resolution. Within the South American/Hawaiian clade, the position of the Hawaiian species as sister to the American *Panke* was retrieved with considerable support. The phylogenetic relationships of the 22 species of *Gunnera* examined in the present study is discussed below in relation to the classification of Schindler. Some morphological and biogeographical issues are also introduced.

Subgenus* Ostenigunnera, *the Annual Species. *Gunnera herteri* was originally thought to be a perennial like all other *Gunnera* species (Mattfeld 1933). Its annual habit was first discovered in the field by the two first authors and this was corroborated through cultivation (Wanntorp et al. 2001). This species is placed at the base of the cladogram based on the chloroplast sequences. *Gunnera herteri* is somewhat divergent from the rest of the species in its molecular sequences. Several of the traits in its habit are also unique, which would make *G. herteri* a poor standard for deducing ancestral morphological traits in *Gunnera*. *Gunnera herteri* was also supported as sister to the other species of the genus in the morphological study of Fuller (1995).

Subgenus* Gunnera, *the African Species. The ITS and the *rps16* intron data suggested that *G. perpensa* is sister group to a clade consisting of all remaining species of *Gunnera*. The *rbcL* data, instead, placed *G. perpensa* as sister to the South American subgenera *Misandra* and *Panke*. The placement of *G. perpensa* in a sep-

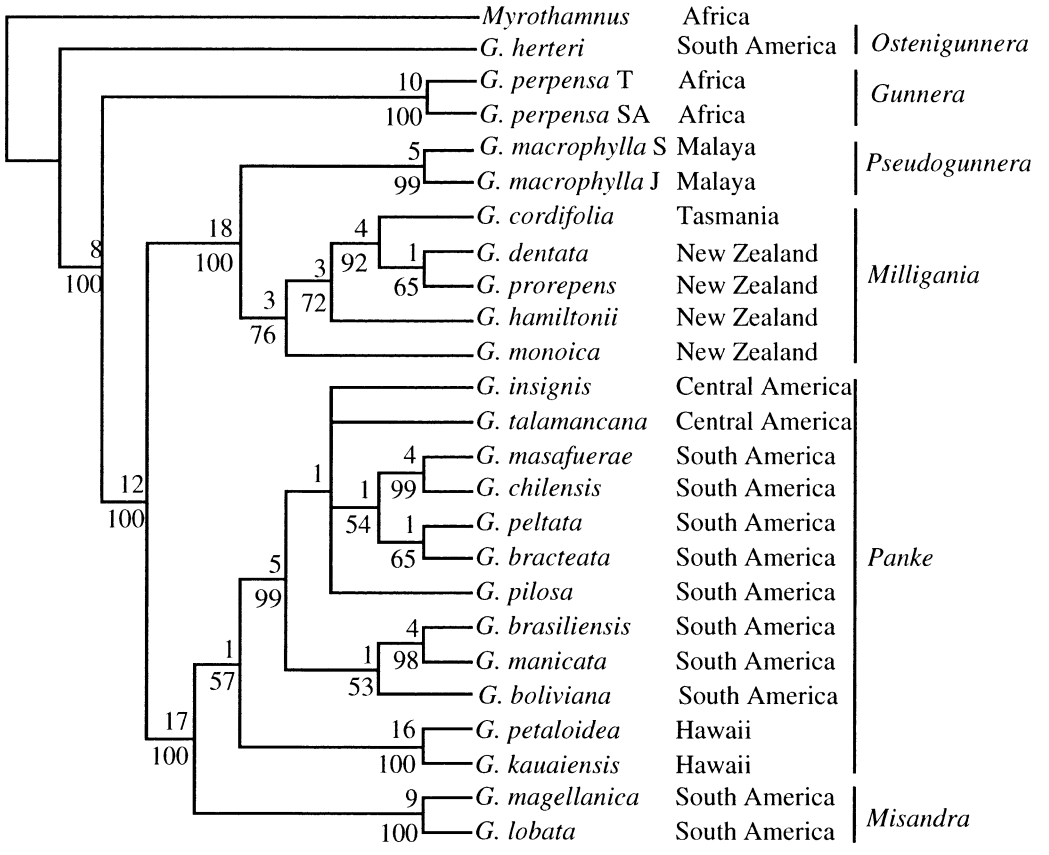

FIG. 3. Combined analysis of *rbcL, rps16* intron and ITS: Strict consensus tree of 15 most parsimonious trees (tree length = 722; CI = 0.82; RI = 0.92). Support values for internal nodes are reported as decay indices (above) and bootstrap values (below). J = Java, S = Sumatra, SA = South Africa, T = Tanzania.

arate subgenus as suggested by Schindler (1905) is confirmed by our study (Fig. 3). Like *Panke*, *Gunnera perpensa* is characterised by thick, polystelic rhizomes (Wilkinson 2000), but in contrast to the erect or ascending unbranched stems of *Panke*, the rhizomes of *G. perpensa* are creeping and branching. According to our results (Fig. 3), the thick, polystelic rhizomes are not homologous in these two subgenera. The morphological study of Fuller (1995) suggested that the African species *G. perpensa* and the Malayan *G. macrophylla* could ''best be merged into a single lineage.'' We did not find any support for this result. The position of *G. perpensa* as sister to the other species of *Gunnera*, excepting only *G. herteri*, is, in contrast, a well-supported result of the present study as well as the position of *G. macrophylla* as sister to *Milligania*.

Gunnera perpensa occurs in South Africa and on mountains along the Great Rift Valley from Zimbabwe and Mozambique, over Congo, Rwanda, Burundi, Kenya, Tanzania, and Uganda all the way to Sudan and Ethiopia (Boutique and Verdcourt 1973). It is also found in the central parts of Madagascar. Schindler (1905) considered *G. perpensa* to consist of three varieties different in geographic distribution. The variety growing in Madagascar was named *angusta* due to its narrow inflorescence. The variety *kilimandscharica* from East Tropical Africa and Ethiopia is said to differ from the South African type specimen by hairless petals and stalked drupes. Skottsberg (1936) considered the subgenus to include four species, one growing in South Africa, one in Tanzania, one in Ethiopia, and one on Madagascar, and he thought the distribution to be very ancient. We had access to two of Skottsberg's presumed taxa of *G. perpensa*, the one from Tanzania and the other from South Africa. Their ITS sequences were not completely identical, but still very similar, differing at a few base pairs only, and grouped together with high support. We could also notice how similar the two plants were in morphology, but the differences reported between at least some of the ''varieties'' seem to us no less than those considered to separate species in the subgenus *Panke*.

Subgenus* Pseudogunnera, *the Malayan Species. Subgenus *Pseudogunnera* consists of the single species *G. macrophylla*, which is widespread in the Malayan area, occurring on mountains in New Guinea, the Sol-

omon Islands, Sulawesi, Java, Sumatra, Borneo, and the Philippines. The analyses based on the single ITS region, as well as on all three gene regions, clearly place *G. macrophylla* as a well-supported sister to subgenus *Milligania*, which includes all species from New Zealand and Tasmania (Figs. 2, 3).

In the cladogram from Wanntorp et al. (2001) based on *rbcL* and *rps16* intron only, *G. macrophylla* was placed inside *Milligania* as sister group of *G. monoica* from New Zealand. Because the *rps16* intron alone instead placed *G. macrophylla* as sister group to *Milligania*, the previous result was suspected to have been caused by the isolation of the same paralogous pseudogene copy of the *rbcL* in these two species (Wanntorp et al. 2001). A recent study (Wardle et al. 2001) identified a *rbcL* sequence from *G. monoica* of usual length. When this sequence was used in the *rbcL* analysis there was no longer any indication of a close relationship between *G. monoica* and *G. macrophylla*. This supports the presence of two copies of the *rbcL* gene in these species, and that the apparent close relationship between *G. monoica* and *G. macrophylla* was due to the isolation of a pseudogene.

In its large size and reniform leaf shape *G. macrophylla* is somewhat similar to *G. perpensa* and the species of subgenus *Panke*, but the Malayan species shares the propagation by slender stolons with the species of subgenus *Milligania* and *Misandra*. As in subgenus *Milligania*, the stolons have two opposite apical bracts, subtending the short erect leafy stem. Also the bright-colored juicy drupes of *G. macrophylla*, are more similar to the fruits of the species in *Milligania* than to the greenish, drier drupes mostly present in *Panke* and *G. perpensa*.

Just as in *G. perpensa*, individuals of *G. macrophylla* from different parts of the Malayan area have sometimes been hypothesized to belong to different varieties or species. For example, plants from Sumatra were identified by van der Post in 1855 as different from specimens from Java. Our study includes one specimen from Sumatra and one from Java. These specimens showed *rbcL* sequences of different length (Wanntorp et al. 2001). The ITS sequences for these two specimens, however, were identical.

Subgenus* Milligania, *the Species from New Zealand and Tasmania. This subgenus includes all species from New Zealand and *G. cordifolia* from Tasmania which are characterized by small size and propagation by stolons. The present study found four well-supported clades. *G. monoica* is well-supported as sister to the remainder of *Milligania*. It has bisexual inflorescences carrying female flowers basally and male flowers apically, and is in this respect similar to *G. macrophylla*. In contrast, the other New Zealand species, as well as the Tasmanian *G. cordifolia*, have male and female flowers on separate inflorescences.

The position of the Australian *G. cordifolia*, securely nested among the New Zealand species, suggests a comparatively late dispersal from New Zealand rather than an ancient vicariance involving the Antarctic continent. If our hypothesis is correct, then the fossil *Gunnera* pollen recorded from upper Cretaceous Campanian strata in Australia, (Jarzen and Dettmann 1989) must derive not from this species but from more distant relatives, possibly closer to *G. macrophylla*.

Subgenus* Misandra, *the Stoloniferous South American Species. South America is the area with the highest diversity of *Gunnera* with representatives of three different subgenera. Of these, subgenus *Misandra* includes small-sized dioecious, perennial species that spread by stolons. The shoot apex is protected by a membranous hood or ochrea, a character that does not occur in any other species of *Gunnera*. In most other traits these species are quite similar to the species of *Milligania*, however. Indeed, it was recently suggested that these two subgenera are not clearly distinguishable on morphological grounds and could perhaps be amalgamated (Wilkinson 2000).

Gunnera magellanica is the most widespread of all South American *Gunnera* species and occurs from Cape Horn and the Falkland Islands along the Andean Cordillera to Colombia (Mora-Osejo 1984). *Gunnera lobata* occurs in Tierra del Fuego and Patagonia. In the former study (Wanntorp et al. 2001), *Misandra* was represented by *G. magellanica* only, which was placed in an unresolved position with members of the subgenus *Panke*. However, the analysis based on the single *rps16* intron showed clear support for *G. magellanica* as closely related to *Panke*. The ITS study revealed a close relationship between *G. lobata* and *G. magellanica*. When combining the three different gene regions, the group formed by *G. magellanica* and *G. lobata* was found to be the sister group to subgenus *Panke*. The phylogenetic signal in the *rbcL* gene had obviously been complemented by the ITS data.

Despite the general morphological similarity there is no evidence for a close relationship between the subgenera *Misandra* and *Milligania*. Their inclusion in two clearly separate clades is among the most well-supported results of the entire analysis. It seems reasonable to conclude that stolons, which are the main uniting character, may have been an ancestral feature in *Gunnera*, possibly excluding *G. perpensa* and *G. herteri*. Their absence in subgenus *Panke*, then, is due to a loss of this means of vegetative dispersal.

Subgenus* Panke, *the Giants of the Cordilleras. The Andean Cordilleras from Patagonia to Mexico in South and Central America contain the highest species diversity of subgenus *Panke*, with at least 20 endemic species, often allopatric and similar in morphology. Outside the Andes, the two species of *Gunnera* from Hawaii also belong in *Panke* as do the species from the

Juan Fernández Islands. *Gunnera brasiliensis* is also disjunct from the rest of the subgenus, being restricted to the Serra do Mar near the Atlantic coast in southern Brazil. The difficult demarcation of species and their frequent hybridization (Palkovic 1978; Pacheco et al. 1991) indicate that speciation within *Panke* is still ongoing. It seems reasonable to connect at least most of the diversification of *Panke* with the uprising of the Andes. This was initiated in the South, already during the Cretaceous, while the Colombian Cordilleras in the north are of Plio-Pleistocene age (Putzer 1968).

Neither the previous *rbcL*/*rps16* intron analysis nor the ITS study strongly supported the monophyly of subgenus *Panke*, which morphologically is very clearly defined. Subgenus *Panke* are usually large plants with ascending to erect polystelic rhizomes covered by large often frilled scales, palmate leaves and huge compound inflorescences. The well-supported association of *Misandra* and *Panke* in our analysis, on the other hand, has almost no support in morphological traits, unless, as proposed by Skottsberg (1936), the ''ochrea'' of *Misandra* is indeed homologous to the large scales, which are one of the distinguishing features of *Panke*.

As in the *rbcL*/*rps16* intron analysis, the Hawaiian species of subgenus *Panke* are the sister group of the South American species, but now this relationship is well-supported. Several *Gunnera* species have been described from the Hawaiian islands (St. John 1946). Though there seems to be considerable geographical variation, the number has recently been reduced to the two species included in this study, *G. petaloidea* and *G. kauaiensis* (Wagner et al. 1990). The Hawaiian species form a very well-supported clade.

The resolution within subgenus *Panke* was higher than in the previous study, but the information in the ITS region is still insufficient to fully resolve the phylogeny of the American species. However, a significant result was the well-supported position of *G. manicata* in the tree (Fig. 3) and its corroborated identity with *G. brasiliensis*. There exists considerable confusion about the identity and origin of the frequently cultivated *G. manicata*. This species is sometimes considered to have been introduced from Brazil (Linden 1867; Ayres Fevereiro and Perazzo Barbosa 1976) and to be possibly identical to the wild *G. brasiliensis* (Schindler 1905). However, it has also been claimed to be of Colombian origin (Mora-Osejo 1984). This study constitutes a first attempt to clarify the matter phylogenetically. The ITS region of a cultivated plant of *G. manicata* of garden origin was sequenced as well as the ITS of *G. brasiliensis*. These sequences were completely identical and the two specimens grouped together with strong support.

Among the remaining species, those from the South form a poorly supported clade. Among these, *Gunnera chilensis* grows in mainland Chile. It is sister to *G. masafuerae*, an endemic to Masafuera (the youngest, and outermost, of the Juan Fernández Islands). These two species are the sister group of *G. peltata* and *G. bracteata* which also appear as sister species. The latter two grow on Masatierra (the inner and more ancient of the Juan Fernández Islands), where they form hybrids (Skottsberg 1914, Pacheco et al. 1991). Generally these four ITS sequences differ in very few base pairs. The sequences from the morphologically quite distinct *G. peltata* and *G. bracteata* were completely identical. The Juan Fernández Islands are only two to three million years old (Stuessy et al. 1984), and the diversification of these species is likely to have taken place within this time span.

In 1959, Macbride suggested that the Peruvian *G. bolivari* could be closely related to *G. bracteata* based on smaller leaf size and also on the glabrousness of these plants. A phylogenetic study of the relationship of the Juan Fernández species was undertaken by Pacheco et al. (1993). This was based on flavonoids and morphology and indicated that *G. bolivari* is more closely related to other species such as *G. mexicana* and *G. boliviana* than to *G. bracteata*. On the other hand, flavonoid compounds unique to *G. chilensis* and to the species from Juan Fernández Islands were identified. Based on morphological as well as flavonoid data they considered *G. chilensis* to be the closest mainland relative of all the Juan Fernández species. In a phylogenetic analysis of the relationship of the Juan Fernández species they chose *G. chilensis* as an outgroup. *Gunnera masafuerae* was most closely related to *G. peltata*, but this clade was supported by one morphological character only, while the flavonoid data were uninformative. The present phylogenetic study for the first time allows the comparison of the species from the Juan Fernández Islands with species not only from Chile, but also from Tropical South America, Central America and Hawaii. The close relationship with *G. chilensis* suggested by Pacheco et al. (1993) is supported (even though *G. bolivari* is not included in our analysis). The relationship among the Juan Fernández species, however, is in conflict since in our analysis *G. masafuerae* is most closely related to the mainland *G. chilensis*, rather than to *G. peltata* from Masatierra. This indicates two separate colonizations of the Juan Fernandez Islands, one by the ancestor of *G. peltata*/*G. bracteata* and the other by *G. masafuerae*. More information is needed to resolve the positions of *G. pilosa* from Equador, *G. insignis* from Panama, and *G. talamancana* from Costa Rica.

The South American species of *Panke* show a high degree of similarity, morphologically as well as in ITS sequences, which could support a recent origin for this part of the subgenus. If so, the late Cretaceous findings of *Gunnera* pollen (Jarzen and Dettmann 1989), as well as putative macrofossils of leaves attributed to subgenus *Panke* (Wilkinson 2000) from North America comes

into new light. If *Panke*, including the Hawaiian species, is of southern origin, a late diversification seems to rule out the possibility that the North American fossils could belong to this clade. If these fossils, on the other hand, truly represent *Panke*, a quite different and more complicated history for *Gunnera* may be envisioned, involving colonization of South America also from the north. This scenario is in agreement with the basal position of the Hawaiian species. While few or no other close South American relatives of Hawaiian species are known, a relationship between North America and Hawaii is a well-known biogeographic feature (Skottsberg 1936; Vargas et al. 1998).

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LITERATURE CITED

AYRES FEVEREIRO, P. C. and V. PERAZZO BARBOSA. 1976. Gunneráceas. Pp. 3–18 in *Flora Ilustrada Catarinense*, ed. P. R. Reitz. Itajaí.

BADER, F. W. J. 1961. Das Areal der Gattung *Gunnera* L. Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 80: 281–293.

BOUTIQUE, R. and B. VERDCOURT. 1973. Haloragaceae. Pp. 1–4 in *Flora of tropical East Africa*, eds. E. Milne-Redhead and R. M. Polhill. London, Rotterdam: Balkema.

BREMER, K. 1994. Branch support and tree stability. Cladistics 10: 295–304.

CHASE, M. W., D. E. SOLTIS, R. G. OLMSTEAD, D. MORGAN, D. H. LES, B. D. MISHLER, M. R. DUVALL, R. A. PRICE, H. G. HILLS, Y.-L. QIU, K. A. KRON, J. H. RETTIG, E. CONTI, J. D. PALMER, J. R. MANHART, K. J. SYTSMA, H. J. MICHAELS, W. J. KRESS, K. G. KAROL, W. D. CLARK, M. HEDRÉN, B. S. GAUT, R. K. JANSEN, K.-J. KIM, C. F. WIMPEE, J. F. SMITH, G. R. FURNIER, S. H. STRAUSS, Q.-Y. XIANG, G. M. PLUNKETT, P. S. SOLTIS, S. M. SWENSEN, S. E. WILLIAMS, P. A. GADEK, C. J. QUINN, L. E. EGUIARTE, E. GOLENBERG, G. H. LEARN, S. W. GRAHAM, S. C. H. BARRETT, S. DAYANANDAN and V. A. ALBERT. 1993. Phylogenetics of Seed Plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. Annals of the Missouri Botanical Garden 80: 523–785.

ENDRESS P. K. 1989. The systematic position of the Myrothamnaceae. Pp. 193–200 in *evolution, systematics, and fossil history of the Hamamelidae 1*., eds. P. R. Crane and S. Blackmore. Oxford: Clarendon Press.

ERIKSSON, T. 1998. Autodecay, vers. 4.0.2′ Stockholm: Department of Botany.

FULLER, D. Q. 1995. Systematics and leaf architecture of the Gunneraceae. American Journal of Botany 82(6, Supplement): 130–131.

GREUTER, W., J. MCNEILL, F. R. BARRIE, H. M. BURDET, V. DEMOULIN, T. S. FILGUEIRAS, D. H. NICOLSON, P. C. SILVA, J. E. SKOG, P. TREHANE and N. J. TURLAND. 2000. *International code of botanical nomenclature (Saint Louis Code)*. Königstein: Koelts Scientific Books.

HOLMGREN, P. K., N. H. HOLMGREN and L. C. BARNETT. 1990. *Index herbariorum 1: the herbaria of the world*. New York: New York Botanical Garden.

HOOT, S. B., S. MAGALLÓN and P. R. CRANE. 1999. Phylogeny of basal eudicots based on three molecular data sets: *atp*B, *rbc*L and 18S nuclear ribosomal DNA sequences. Annals of the Missouri Botanical Garden 86: 1–32.

JARZEN, D. M. and M. E. DETTMANN. 1989. Taxonomic revision of *Tricolpites reticulatus* Cookson ex Couper 1953 with notes on the biogeography of *Gunnera* L. Pollen & Spores 31: 97–112.

LINDEN, J. J. 1867. Relation d'un voyage scientifique executé par J. J. Linden au Venezuela et a la Nouvelle Grenade 1841–1844. La Belgique Horticole, Annales d'Horticulture Belge et Étrangère 17: 35–256.

LINNAEUS, C. 1767. *Mantissa Plantarum*. Stockholm: Salvius.

MACBRIDE, J. F. 1959. Haloragaceae. Pp. 3–7 in *Flora of Peru*, ed. B. E. Dahlgren. Vol. 13. Chicago: Field Museum of Natural History.

MATTFELD, J. 1933. Weiteres zur Kenntnis der *Gunnera Herteri* Osten. Ostenia 102–118.

MORA-OSEJO, L. E. 1984. Halogaceae. Pp. 1–173 in *Flora de Colombia*, ed. Colciencias. Bogotá: Instituto de Ciencias Naturales-Museo de Historia Natural de la Universidad Nacional de Colombia.

PACHECO, P., D. J. CRAWFORD, T. F. STUESSY, and M. O. SILVA. 1993. Flavonoid chemistry and evolution of *Gunnera* (Gunneraceae) in the Juan Fernandez Islands, Chile. Gayana Botanica 50: 17–28.

———, T. F. STUESSY, and D. J. CRAWFORD. 1991. Natural interspecific Hybridization in *Gunnera* (Gunneraceae) of the Juan Fernandez Islands, Chile. Pacific Science 45: 389–399.

PALKOVIC, L. A. 1978. A hybrid of *Gunnera* from Costa Rica. Systematic Botany 3: 226–235.

PUTZER, H. 1968. Ueberblick über die geologische Entwicklung Südamerikas. Pp. 1–41 in *Biogeography and ecology in South America*, eds. E. J. Fittkau, H. Klinge, G. H. Schwabe and H. Sioli. The Hague: W. N. V. Junk Publishers.

SCHINDLER, A. K. 1905. Halorrhagaceae. Pp. 1–133 in *Das Pflanzenreich*, ed. A. Engler. Leipzig: Engelmann.

SIMMONS, M. P. and H. OCHOTERENA. 2000. Gaps as Characters in Sequence-Based Phylogenetic Analyses. Systematic Biology 49: 369–381.

SKOTTSBERG, C. 1914. Studien über die Vegetation der Juan-Fernandez-Inseln. Kungliga Svenska Vetenskapsakademiens Handlingar 51: 9: 1–73.

———. 1936. Antarctic plants in Polynesia. Pp. 291–311 in *Essays in geobotany in honour of William Albert Setchell*, ed. T. H. Goodspeed. Berkeley: University of California Press.

SMITH, S. W., R. OVERBEEK, C. R. WOESE, W. GILBERT, and P. M. GILLEVET. 1994. The genetic data environment, an expandable guide for multiple sequence analyses. Cabios 10: 671–675.

SOLTIS, D. E., P. S. SOLTIS, M. W. CHASE, M. E. MORT, D. C. ALBACH, M. ZANIS, V. SAVOLAINEN, W. H. HAHN, S. B. HOOT, M. F. FAY, M. AXTELL, S. M. SWENSEN, L. M. PRINCE, W. J. KRESS, K. C. NIXON, and J. S. FARRIS. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbc*L and *atp*B sequences. Botanical Journal of the Linnean Society 133: 381–461.

ST. JOHN, H. 1946. Endemism in the Hawaiian flora, and a revision of the Hawaiian species of *Gunnera* (Haloragidaceae) Hawaiian plant studies 11. Proceedings of the California Academy of Sciences XXV, No 16: 377–420.

STADEN, R. 1996. The staden sequence analysis package. Molecular Biotechnology 5: 233–241.

STUESSY, T. F., K. A. FOLAND, J. F. SUTTER, R. W. SANDERS, and M. SILVA. 1984. Botanical and geological significance of potassium-argon dates from the Juan Fernández Islands. Science 225: 338–346.

SWOFFORD, D. 2001. PAUP*: Phylogenetic Analysis Using Parsimony, vers.4.0b6. Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers.

THOMSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, and D. G. HIGGINS. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleid Acids Research 24: 4876–4882.

VAN DER POST, C. G. 1855. *Florae Indiae Batavae.* Amsterdam: Frid. Fleischer.

VARGAS, P., B. G. BALDWIN, and L. CONSTANCE. 1998. Nuclear ribosomal DNA Evidence for a western North American origin of Hawaiian and South American species of Sanicula (Apiaceae). Proceedings of the National Academy of Sciences of the United States of America 95: 235–240.

WAGNER, W. L., D. R. HERBOT, and S. H. SOHNER. 1990. *Manual of the flowering plants of Hawaii.* Bishop: University of Hawaii Press, Bishop Museum Press.

WANNTORP, L., H.-E. WANNTORP, M. KÄLLERSJÖ, and B. OXELMAN. 2001. Phylogeny of *Gunnera*. Plant Systematics and Evolution 226: 85–107.

WARDLE, P., C. EZCURRA, C. RAMIRIZ and S. WAGSTAFF. 2001. Floristic Comparison of Southern Andean and New Zealand Plant Communities. New Zealand Journal of Botany 39: 69–108.

WILKINSON, H. P. 2000. A revision of the anatomy of Gunneraceae. Botanical Journal of the Linnean Society 134: 233–266.