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Multiple origins of Southern Hemisphere *Anemone* (Ranunculaceae) based on plastid and nuclear sequence data

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Abstract. Using two molecular data sets, the plastid *atp*B-*rbc*L intergenic spacer region and the nuclear ribosomal internal transcribed spacer regions (ITS), the taxonomic affinities of two newly available *Anemone* species from the Southern Hemisphere were tested. From previous work based on morphology and geographic distribution, it was assumed that *A. tenuicaulis* from New Zealand was most closely related to the Tasmanian *A. crassifolia*, whereas the affinity of *A. antucensis* from Chile and Argentina was regarded as uncertain. Analyses of molecular sequence data from these and 18 other species of *Anemone* s.lat. (with *Clematis* as outgroup) result in trees largely congruent with past analyses based on morphology and plastid restriction site data. They strongly support *A. richardsonii* and *A. canadensis* (with boreal distributions in the Northern Hemisphere) as paraphyletic to a well supported Southern Hemisphere clade consisting of *A. antucensis* and *A. tenuicaulis*. This group of four species is part of an otherwise predominantly Northern Hemisphere assemblage (subgenus *Anemonidium* s.lat., chromosome base number $x = 7$), including *A. narcissiflora*, *A. obtusiloba*, *A. keiskeana* and *A.* (= *Hepatica*) *americana*. All other austral species included in the present sampling, *A. crassifolia* (Tasmania), *A. knowltonia* (= *Knowltonia capensis*), and *A. caffra* (both South African), form a separate clade, sister to *A.* (= *Pulsatilla*) *occidentalis* and other Northern Hemisphere anemones (subgenus *Anemone* s.lat., $x = 8$). Possible phytogeographical links of the Southern Hemisphere species are discussed.

Key words: *Anemone*, Ranunculaceae, *atp*B-*rbc*L intergenic spacer, ITS, phylogeny, biogeography.

The genus *Anemone* s.str. consists of approximately 150 species (Tamura 1995) with the vast majority of species found in the Northern Hemisphere. However, a few species also occur in the cooler regions of the Southern Hemisphere. Three of these species, *A. knowltonia* (= *Knowltonia capensis*), *A. caffra* (both South Africa), and *A. crassifolia* (Tasmania) were included in a recent phylogenetic study of the genus based on both morphological and molecular data (Hoot et al. 1994). It was found that these species formed a well-supported, monophyletic group within subgenus *Anemone* ($x = 8$). From this analysis, it was hypothesized that all species found in the Southern Hemisphere were somewhat closely related, possibly reflecting a former Gondwanan distribution (Hoot et al. 1994, Hoot 1995).

Leaf material from two additional anemones from the Southern Hemisphere, *A. tenui-*

caulis (New Zealand) and *A. antucensis* (South America), recently became available, allowing two independent research teams to test this hypothesis. Based on *atp*B-*rbc*L spacer and ITS sequence data and a sampling of 17 species of *Anemone*, Schuettpelz and Hoot (2000) reported the inclusion of *A. tenuicaulis* within a N. Hemisphere clade, most closely related to *A. canadensis*. Ehrendorfer and Samuel (2000, 2001), using *atp*B-*rbc*L spacer sequence data and sampling seven species of *Anemone* (including the South American *A. antucensis*) obtained similar results and were able to demonstrate a sister group relationship between *A. antucensis* and *A. tenuicaulis*. Both of these preliminary works highlighted the need to pool data and publish a joint paper with a broader sampling than either could obtain alone.

A. tenuicaulis is found on both the South and North Islands of New Zealand where it prefers subalpine to lower alpine habitats, usually confined to damp sites in snow tussock grassland and herbfields (Allan 1961). It is a low-growing, herbaceous perennial with a vertical to rhizomatous rootstock (Parkin and Sledge 1935). Leaves are tripartite with long petioles (>3 cm). Inflorescences are usually one- or two-flowered, with an involucre consisting of three linear, entire or bi- to tri-lobed leaves that are dissimilar to the basal leaves. Flowers are dull red in color with 5–6(-7) linear sepals with acute apices and rounded bases. Stamens number 6–14, filaments are threadlike with the connectives extending slightly beyond the pollen sacs. Carpel number varies from 14 to 34; achenes are glabrous and have a long hooked style. Pollen is spiraperturate (Huyhn 1970b). $2n=28$, making the base chromosome number most likely $x=7$ (Hair 1963; Ehrendorfer 1995). Mainly based on morphology, Parkin and Sledge (1935) placed *A. tenuicaulis* into sect. *Rivularidium* (comparing it with *A. antucensis*). Hoot et al. (1994) tentatively placed *A. tenuicaulis* together with *A. crassifolia* into the informal *Knowltonia* group of sect. *Pullsatilloides*, subgenus *Anemone* ($x=8$). Tamura (1995) also combined *A. tenuicaulis* and *A. crassifolia* into sect. *Crassifolia*, subgen. *Rivularidium*, though far away from his subgen. *Pulsatilloides*. Ehrendorfer (1995) expressed doubts about this affinity of *A. tenuicaulis* because of its deviating chromosome base number.

A. antucensis occurs in mountain forests of central Chile and Neuquén, Argentina. It is a low-growing, somewhat rhizomatous, herbaceous perennial with basal, tripartite leaves with long (5–14 cm) petioles. Inflorescences are solitary to two-flowered, with involucral leaves three-lobed and similar to basal leaves (Lourteig 1951). Flowers are whitish and have five elliptical to suborbicular sepals, 20–33 stamens with threadlike filaments and slightly extended connectives, 25–35 glabrous carpels with a relatively long hooked style. Pollen is either tricolpate or 6- to 9-pantocolpate (Huynh 1970a). No chromosome count is available. Based on morphology, Hoot et al. (1994) speculated that *A. antucensis* along with three other South American species, may have affinities with both the South African, New Zealand, and Tasmanian species and provisionally placed them all into the *Knowltonia* group of sect. *Pulsatilloides*. In contrast, Tamura (1995) listed *A. antucensis* in subgen. and sect. *Rivularidium*.

To solve these deviating opinions on Southern Hemisphere *Anemone* species, DNA sequences from the plastid *atp*B-*rbc*L intergenic spacer region and the nuclear ITS regions were chosen as data sources after preliminary tests indicated the level of variation was appropriate within the genus. The *atp*B-*rbc*L intergenic spacer of the plastid genome is approximately 750 bp in length, and has been used successfully in a variety of phylogenetic studies (Golenberg et al. 1993, Hoot and Douglas 1998). The ITS I and II regions are located in the nuclear genome between the 18S and 26S ribosomal genes. The ITS regions together with the 5.8S gene are approximately 600 bp long. Although there are multiple copies of this ribosomal array in the genome, they often appear to evolve in concert, and are therefore frequently identical (Baldwin et al. 1995). The ITS regions have been used exten-

sively to study angiosperms at the species and generic level.

Materials and methods

Sampling. Sampling for the proposed study was done using a placeholder approach, selecting species to represent the major subdivisions of the genus based on the molecular and morphological results of Hoot et al. (1994). Once the affinities of *Anemone tenuicaulis* and *A. antucensis* were determined, additional species were added to further resolve their placement. Included in the sampling (Table 1), are one species each of the traditional genera *Hepatica* (*A. americana*), *Pulsatilla* (*A. occidentalis*), *Knowltonia* (*A. knowltonia*), and 18 species of *Anemone* s.str. Because of its close affinities, *Clematis* was included as an outgroup to root the tree (Hoot et al. 1994, Johannson 1995, Hoot 1995). The grouping of taxa within *Anemone* s.lat. (=Anemoninae) follows the provisional and informal arrangement presented by Hoot et al. (1994: Fig. 4 and Appendix 2) of subgenera, sections, and species groups.

DNA sequencing. Total DNA was extracted from either fresh, silica-dried, or herbarium leaf material for each sample. When sufficient amounts of material were present, DNA was extracted using the procedure of Doyle and Doyle (1987). When only small amounts of leaf material were available, DNA easy columns (Qiagen) were utilized according to the manufacturer's protocol. If necessary, DNA was further purified using DNA easy columns (Qiagen).

The ITS regions, including the 5.8S gene, were amplified by the polymerase chain reaction (PCR) using primers 1830F, located in the 18S gene, and 25R, located in the 26S gene (for primer sequences, see Nickrent et al. 1994). Double-stranded amplifications in 100 μL reactions were conducted with the following reagents: 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 M DMSO, 50 μM each dNTP, 0.25 μM of each amplification primer, and 2.5 U Taq polymerase. After overlaying each reaction with mineral oil, genomic DNA (210–500 ng) was added. Reaction conditions consisted of 40 cycles of 94 °C for 30 sec., 45 °C for 30 sec., and 72 °C for 2 min. for the denaturation, annealing, and extension steps, respectively. The first cycle was preceeded by a 4 min denaturation step and the last cycle by a 5 min extension step. The *atp*B-*rbc*L spacer regions were amplified using the PCR as described in Hoot and Douglas (1998).

The PCR products were purified using one of two methods: 1) the PCR products were separated from impurities on a low-melt agarose gel, excised from the gel as a plug, and separated from the agarose and concentrated using Wizard Columns (Promega) according to the manufacturer's protocol; or 2) the PCR products were separated from impurities and concentrated using QIAquick Spin Columns (Qiagen) according to the manufacturer's protocol. Sequencing was carried out in both directions for each purified double-stranded PCR product using the same amplification primers as above and Dye Terminator Cycle Sequencing (ABI) according to the manufacturer's protocol.

Data analysis. The two contigs for each sample were aligned (providing complete or near complete overlap) and any ambiguous bases corrected using the computer program Sequencher (Gene Codes Corporation). The resulting consensus sequences for each species were aligned with each other using Sequencher, then further adjusted manually. Alignment procedures were as described in Hoot and Douglas (1998), paying careful attention to repeated motifs (Type Ib indels) and runs of the same nucleotide (Type Ia indels). Using MacClade (Maddison and Maddison 1992), insertions and deletions (indels) were scored as single events, then deleted if otherwise uninformative. Regions of ambiguous alignment were removed from the data set without scoring.

Parsimony analyses of the *atp*B-*rbc*L spacer and ITS data were conducted for each gene independently (results not shown) and in combination using PAUP* version 4.0b2 (Swofford 1999) and the branch and bound search option. In the case of multiple shortest trees, strict consensus trees were constructed. To estimate the confidence to be placed in the topology, bootstrap values were calculated using the branch and bound search option and 1000 replications (Felsenstein 1985).

Before combining the data sets, several methods of assessing congruence among the two data sets were implemented: visual comparison of the various clades found in the minimal trees, their bootstrap support, and implementation of the incongruence length difference (ILD) test (Farris et al. 1995), which tests whether the predefined partitions in the data differ significantly from random partitions of the combined data set. The

Table 1. Species included in the study, sectional affinities (informal, from Hoot et al. 1994), geographic distribution, vouchers, and GenBank numbers (*atp*B-*rbc*L spacer first line, ITS second line). RBGE = Royal Botanical Garden, Edinburgh

Species	Sectional affinities	Vouchers	GenBank numbers
Anemone americana DC. [= *Hepatica americana* (DC.) H. Hara]	Hepatica	S. Hoot 883, MICH	AY055407 AY055386
Anemone canadensis L.	Anemonidium	S. Hoot 867, MICH	AY055408 AY055387
A. richardsonii Hook. f.	Anemonidium	C.L. Parker 9801, ALA	AY055409 AY055388
A. antucensis Poeppig	Anemonidium	L. & F. Ehrendorfer: Chile, Concepcion, Nahuelbuta, 24.01.98, WU	AF311735 AY056049
A. tenuicaulis (Cheeseman) Parkin & Sledge	Anemonidium	Garnock-Jones 2147, CHR	AY055410 AY055389
A. flaccida F. Schmidt	Keiskea	S. Hoot 8952, MICH	AY055412 AY055391
A. keiskeana Ito	Keiskea	S. Hoot 8951, MICH	AY055411 AY055390
A. narcissiflora L.	Homalocarpus	R. Meyers 88–8	AY055414 AY055393
A. demissa Hook. f. & Thomson	Homalocarpus	RBGE 841910	AY055413 AY055392
A. obtusiloba D. Don	Homalocarpus	RBGE 851867	AY055415 AY055394
A. trullifolia Hook. f. & Thomson	Homalocarpus	RBGE 812614	AY055416 AY055395
A. rivularis Buch.-Ham.ex DC.	Anemonospermos	S. Hoot 8853, MICH	AY055417 AY055396
A. hupehensis Lemoine	Anemonospermos	S. Hoot 911, MICH	AY055418 AY055397
A. crassifolia Hook.f.	Pulsatilloides	S. Hoot 8855, MICH	AY055419 AY055398
A. caffra (Eckl. & Zeyh.) Harvey	Pulsatilloides	RBGE 770617	AY055420 AY055399
A. knowltonia Burtt-Davy [= *Knowltonia capensis* (L.) Huth	Pulsatilloides	Univ. Of Copenhagen, Bot. Garden, Denmark	AY055421 AY055401
Anemone occidentalis S. Waston [= *Pulsatilla occidentalis* (S. Watson) Freyn]	Pulsatilloides	S. Hoot 8817, MICH	AY055426 AY055400
A. blanda Schott & Kotschy	Anemone	Matthaei Bot. Garden, Ann Arbor, MICH	AY055422 AY055402
A. caroliniana Walter	Anemone	L. Raymond s.n.	AY055423 AY055403
A. drummondii S. Watson	Anemone	R. Meyers 88–7	AY055424 AY055404
A. multifida Poir	Anemone	B. Polastri s.n.	AY055425 AY055405
Clematis hexapetala Pall.		S. Hoot 9150, MICH	AY055406 AY055385

ILD analysis was conducted using PAUP* with the following settings: 1000 replications, heuristic search with simple addition, TBR (tree bisection-reconnection) branch swapping, and saving up to 2000 trees for each replicate.

Results

Nuclear internal transcribed spacers (ITS). The average length of the sequences obtained for this region was 570 bases, with the longest at 599 bases (*Anemone caffra*) and the shortest at 494 bases (*A. hupehensis*). Thirteen insertions/deletions (indels) were scored. After the removal of unalignable regions and uninformative characters, the data set consisted of 193 variable characters, 134 of these were parsimony informative. The parsimony analysis of the ITS data resulted in a single most parsimonious tree (tree not presented) with a consistency index excluding parsimony-uninformative characters (CI) of 0.65, and a retention index (RI) of 0.74. The clades found with the ITS data are largely identical to those found in the tree resulting from the combined data sets (Fig. 1). They differ only in the placement of *A.* (= *Hepatica*) *americana*; it is sister to all other anemones with the ITS data.

Chloroplast *atp*B-*rbc*L intergenic spacer. The average length of the sequences was 793 bases, with the longest at 884 bases (*A. flaccida*) and the shortest at 632 bases (*A. hupehensis*). 23 indels were scored. After removal of unalignable regions, the data set consisted of 125 variable characters, 62 of which were parsimony informative. The parsimony analysis of the *atp*B-*rbc*L intergenic spacer data resulted in 42 equally parsimonious trees with CI = 0.91 and RI = 0.97.

The strict consensus tree of the 42 most parsimonious trees is identical in topology to the tree derived from the combined data (Fig. 1) but exhibits less resolution. The clade consisting of *A.* (= *Pulsatilla*) *occidentalis* through to *A. caroliniana* is a polytomy with the following clades recognized: ((*A. rivularis*, *A. hupehensis*), (*A. drummondii* (*A. blanda*, *A. multifida*, *A. caroliniana*))).

Combined analysis. The ILD test revealed no significant difference ($P = 0.78$) between the partition defined by the two genes and random partitions, indicating a high degree of data congruence. Because of these results and the largely congruent topologies of the ITS and *atp*B-*rbc*L spacer trees, the data sets were combined. Analysis of the combined data (196 parsimony informative characters) resulted in a single, most parsimonious tree (Fig. 1) with CI = 0.72 and RI = 0.81 (Fig. 1). Two large clades are evident, corresponding to the two subgenera *Anemone* and *Anemonidium* previously found by Hoot et al. (1994): 1) Subgenus *Anemone* (base chromosome number $x = 8$) consisting of *A.* (= *Pulsatilla*) *occidentalis*, *A. knowltonia* (= *Knowltonia vesicatoria*), and assorted other *Anemone* species from South Africa, Tasmania, and the Northern Hemisphere and 2) Subgenus *Anemonidium* ($x = 7$), including three well-supported clades (bootstrap = 100%): sect. *Keiskea* (*A. flaccida* and *A. keiskeana*), sect. *Anemonidium* consisting of the Northern Hemisphere species *A. canadensis*/*A. richardsonii* and the Southern Hemisphere species *A. antucensis*/*A. tenuicaulis*, and section *Homalocarpus* consisting of various species from the Northern Hemisphere (Fig. 1).

Discussion

The combined phylogeny resulting from the present study is nearly identical to that derived by Hoot et al. (1994) using morphological and plastid restriction site data. Only two clades have somewhat different placements. In the previous study, subg. *Anemone* Sect. *Anemonospermos* (including *A. rivularis* and *A. hupehensis*) was resolved as sister to sect. *Pulsatilloides* including *A. pulsatilla* (= *Pulsatilla vulgaris*), *A. crassifolia*, *A. knowltonia* (= *Knowltonia capensis*), and *A. caffra*, but with weak bootstrap support (64%). In the present study, sect. *Anemonospermos* is moderately supported (73%) as sister to sect. *Anemone* (Fig. 1).

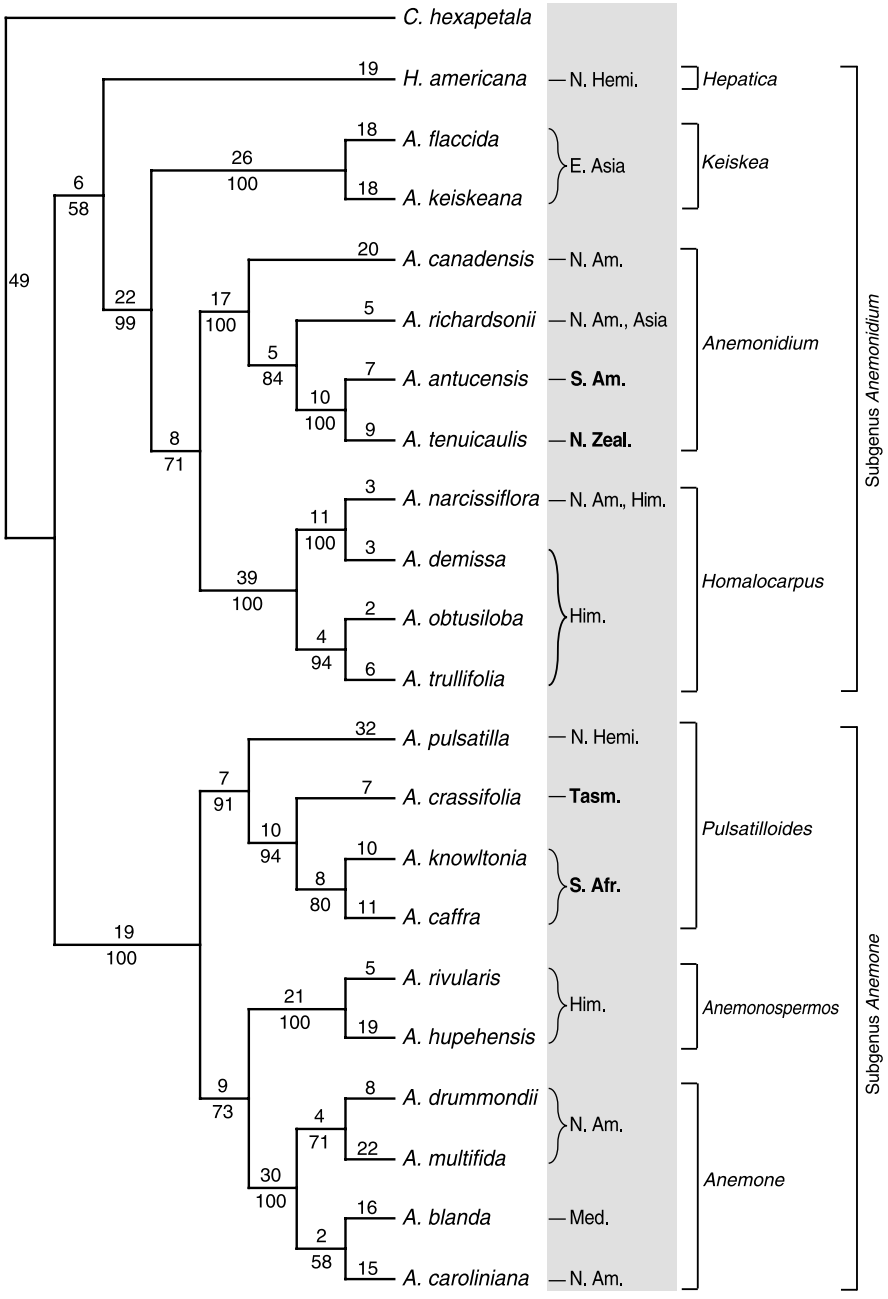

Fig. 1. Single most parsimonious tree for *Anemone* s.lat. resulting from analysis of the combined *atp*B-*rbc*L spacer and ITS data, using *Clematis hexapetala* as the outgroup. Abbreviated geographical distributions are indicated in shaded column; Southern Hemisphere species in bold. Informal sectional names (Hoot et al. 1994) are listed after geographical distributions

The second difference relates to the position within subgen. *Anemonidium* of sect. *Keiskea* (including *A. keiskeana* and *A. flaccida*) which in Hoot et al. (1994) is placed as sister to sect. *Homalocarpus* (including *A. narcissiflora*, *A. demissa*, *A. obtusiloba*, and *A. trullifolia*). In the present study, sect. *Keiskea* is sister to both sect. *Homalocarpus*

and sect. *Anemonidium* (including *A. canadensis*, *A. richardsonia*, *A. antucensis*, and *A. tenuicaulis*). In both studies, these relationships received moderate (93%) to high (99%) bootstrap support. Both of these contradictions may be due to sampling differences.

Available data on *Anemone* support a Northern Hemisphere origin of the genus and two major clades: subgenus *Anemone* ($x = 8$) and subgenus *Anemonidium* ($x = 7$). The combined sequence data (Fig. 1) clearly support the close affinities of the South African *A. caffra* and *A. knowltonia* with the Tasmanian *A. crassifolia* (bootstrap = 94%) as already suggested by morphological similarities and plastid restriction data (Hoot et al. 1994, bootstrap = 92%). This verifies the taxonomic placement of these taxa into the informal sect. *Pulsatilloides* (sensu Hoot et al. 1994).

The combined sequence data (Fig. 1) also demonstrate that the New Zealand *A. tenuicaulis* is sister to the South American *A. antucensis* (bootstrap = 100%) and that these two species are included within sect. *Anemonidium* along with the Northern Hemisphere *A. richardsonii* and *A. canadensis* (and most certainly, although not sampled, its Eurasian sister species *A. dichotoma*). Species in sect. *Anemonidium* have the following morphological and karyological characters in common (but not unique to just this section): leaves trilobed, no stomata on adaxial leaf surface; inflorescences 1-, 2-, (3-) flowered; sepals in low number (4–7); stamens with narrow filaments (< 0.4 mm); carpels in limited number (< 40); stigmas slender; and $x = 7$ (not yet documented for *A. antucensis*).

The moderately supported clade of *A. richardsonii*, *A. tenuicaulis*, and *A. antucensis* is characterized by low-growing, somewhat rhizomatous habit and achenes tapering to an extended hooked style. Differences between the species are found in the nature of the involucral leaves (similar to basal leaves or not), sepal shape and color, and pollen morphology (*A. richardsonii* and *A. antucensis* have tricolpate to eupantocolpate, *A. tenuicaulis* spiraperturate pollen). According to Parkin and Sledge (1935), *A. tenuicaulis* differs from *A. antucensis* by single flowered scapes and thread-like rather than flattened filaments. However, examinations of herbarium specimens (Hoot, preliminary data) do not support this: both *A. antucensis* and *A. tenuicaulis* have fairly similar filament widths and single to two-flowered scapes.

Considering the numerous examples of links between Tasmania (and Southeast Australia) and New Zealand (Wardle 1978), the lack of closer phylogenetic relationships between *A. crassifolia* and *A. tenuicaulis* from these two areas is remarkable. For an explanation of the present day distribution of the relevant *Anemone* clades, one has to account for links between South Africa and Tasmania (for sect. *Pulsatilloides*) and between North America, South America, and New Zealand (for sect. *Anemonidium*). If one includes the three other Ranunculaceae genera with taxa in both hemispheres (*Caltha*, *Clematis*, and *Ranunculus*; Hoot 1995; Schuettpelz, preliminary data), at least three additional Southern Hemisphere links must be considered.

Achene morphology and the relatively restricted ranges of many of the anemones in question make long-distance dispersal events unlikely. Therefore, a more parsimonious explanation invokes a vicariance model: *Anemone* and other ranunculacean genera must have been present in the N. Hemisphere and Gondwanaland, probably in the mid to late Cretaceous. From what we know about the fossil record: the oldest definite angiosperm pollen dating at ~130 mya (Brenner 1996), the oldest tricolpate pollen at ~120 mya (Doyle 1992, Hughes 1994), and the rapid diversification of angiosperms by the mid Cretaceous (Drinnan and Crane 1990), *Anemone* could have already undergone considerable radiation by ~100 mya.

Geological reconstructions of the continents (Smith et al. 1981, Storey 1995) indicate that as late as the Santonian of the late Cretaceous (80 mya); North America, South America, Antarctica, New Zealand, and Australia were contiguous to each other or close enough to be accessible by short distance dispersal. In addi-

tion, North America, Eurasia, and Africa were more or less contiguous at this time. The only Gondwanan land masses that may not have been accessible by short-distance dispersal are Madagascar and India. Thus, the differentiation of *Anemone* s.lat. into two major clades, the subgenera *Anemone* ($x = 8$) and *Anemonidium* ($x = 7$) and their subgroups, could have occurred before short distance dispersal was prevented between the austral land masses. In this way ancestors of sect. *Pulsatilloides* could have linked South Africa to Tasmania, and ancestors of sect. *Anemonidium* could have extended from North to South America via Antarctica to New Zealand.

Further support for a vicariance model to explain geographical distribution patterns in *Anemone* comes from the Proteaceae, a family that, like the Ranunculaceae, is among the earliest branching eudicots (Hoot et al. 1999). Proteaceae include several well-supported lineages containing "Gondwanan" elements, indicating that the family had substantially differentiated before gene flow was compromised by the separation of the austral landmasses (Hoot and Douglas 1998). This is documented by phylogenetic links from Australia (probably via New Zealand and Antarctica, both with diverse Proteaceae fossils) to South America (e.g. *Cardwellia – Gevuina – Euplassa*, *Floydia – Roupala*, *Telopea – Embothrium*), between Australia and South Africa (e.g. *Petrophile – Aulax*, *Adenanthos – Leucadendron – Protea*) or between all three areas (*Macadamia – Panopsis – Brabejum*). The sister group to the Proteaceae, Platanaceae, has a reliable fossil record dating back to the Albian (Crane et al. 1993, Magallón-Pueblo et al. 1997), lending support to the presence of early eudicots in the period between Lower and Upper Cretaceous. Work is continuing on *Anemone* and other Ranunculaceae genera to further test this biogeographical hypothesis.

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