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PHYLOGENETIC POSITION OF THE SOUTH AMERICAN DIOECIOUS GENUS *HAMADRYAS* AND RELATED RANUNCULEAE (RANUNCULACEAE)

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The endemic southern South American genus *Hamadryas* (Ranunculaceae), consisting of five species, has been regarded as closely related to or part of *Ranunculus* s.l. (tribe Ranunculeae) based on morphological observations. However, it differs from most other *Ranunculus* in its dioecious breeding system. Its phylogenetic placement within Ranunculaceae has never been rigorously examined. Here we report the results of two DNA sequence analyses: one using the chloroplast genes *atpB* and *rbcL* to broadly place *Hamadryas* within the family; the second using nuclear ribosomal internal transcribed spacer (ITS) regions to further pinpoint *Hamadryas*'s position within Ranunculeae. The chloroplast data confirm a monophyletic Ranunculoideae consisting of Ranunculeae and Anemoneae, with a well-supported rooting for Ranunculeae between a clade consisting of *Hamadrayas*, *Halerpestes*, and *Trautvetteria* (also including *Ranunculus ficaria*, but with weak support) and a core *Raununculus* clade. With the ITS data for Ranunculeae, *Hamadryas* is within a moderately supported clade consisting of *Oxygraphis* (Asia, Alaska), *Peltocalathos* (South Africa), and *Callianthemoides* (Chile, Argentina), with *Callianthemoides* weakly supported as sister to *Hamadryas*. Based on this and other data, we recommend that *Hamadryas* (along with many other segregate genera within Ranunculeae) be subsumed within the genus *Ranunculus*. We characterize the morphology and other attributes that occur in *Hamadryas* and sister genera and discuss possible causes for the development of dioecy in *Hamadryas*.

Keywords: Hamadryas, Ranunculaceae, Ranunculeae, *Ranunculus*, dioecy, *atpB*, *rbcL*, internal transcribed spacer (ITS).

Introduction

Hamadryas (Ranunculaceae), consisting of long-lived perennial herbs, is restricted in distribution to the extreme south of South America (southern Chile and southern Argentina) and the adjacent Falkland Islands, with one species, *Hamadryas delfinii*, extending northward to San Juan Province in central Andean Argentina. *Hamadryas argentea* is endemic to the Falkland Islands and is listed on the International Union for Conservation of Nature and Natural Resources (IUCN) threatened species list (Broughton and McAdam 2005). Species of *Hamadryas* grow predominantly in alpine and subalpine habitats, where they can reach the upper limits of the vegetation, as well as in the Patagonian steppe in grasslands and on rocky outcrops. They are characterized by a low-growing habit; basal, variously divided, usually tomentose leaves; solitary or few-flowered cymes or panicles; unisexual flowers with 5–8 sepals and (7–)12–16(–20) petals with basal nectaries; tricolpate pollen; and numerous achenes (Santisuk 1979; Moore 1983; Tamura 1993).

The phylogenetic affinities of *Hamadryas* within Ranunculaceae have never been rigorously tested using either morphological or molecular data. The genus is considered to be closely related to *Ranunculus*, within tribe Ranunculeae, subfam. Ranunculoideae (Lourteig 1951; Tamura 1993). However, *Hamadryas* differs most markedly from most members of Ranunculeae in its dioecious breeding system (although *Paroxygraphis*, whose phylogenetic placement has never been tested, may comprise a second dioecious member of this tribe). Lourteig (1951) recognized five species within the genus: *Hamadryas kingii*, *H. argentea*, *H. delfinii*, *H. sempervivoides*, and *H. magellanica*. She considered both *H. tomentosa* DC. and *H. paniculata* Hook. f. as synonyms of *H. magellanica* f. *paniculata* (Hook. f.) Lourt. We follow Lourteig's taxonomy for *Hamadryas* in this article.

While no phylogenetic work to date includes *Hamadryas*, excellent molecular work on a broad sampling of *Ranunculus* and closely related species has been performed (Johansson 1998; Hörandl et al. 2005; Paun et al. 2005). These results indicate a basal grade or clade of *Ranunculus* and other genera that, in turn, were sister to a well-supported "core *Ranunculus* clade" consisting of most *Ranunculus* species. With *Trautvetteria* and *Ficaria* as outgroups (Hörandl et al. 2005), *Arcteranthis*, *Callianthemoides*, *Peltocalathos*, *Oxygraphis*, *Halerpestes*, and five species of *Ranunculus* were found in the basal grade. *Ficaria* was actually embedded within the core *Ranunculus* despite its outgroup designation.

The goals of this study were (1) to clarify the position of the root within Ranunculeae, (2) to test the phylogenetic

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affinities of *Hamadryas* within Ranunculaceae using the chloroplast genes *rbcL* and *atpB*, (3) to further pinpoint the phylogenetic position of *Hamadryas* within tribe Ranunculeae sensu Tamura (1993) using the nuclear internal transcribed spacer region (ITS) sequence data, and (4) to characterize the five species of *Hamadryas* using morphological characters.

Material and Methods

Taxon Sampling

Using the chloroplast data to test the position of *Hamadryas* within Ranunculaceae, 28 genera were chosen to represent all the major clades in the family, based on previous molecular work (Hoot 1995; Johansson 1995; table A1). Using ITS data to test the affinities of *Hamadryas* within tribe Ranunculeae, a broad sampling of *Ranunculus* and related genera were selected as placeholder species (table A2) by using the extensive work of Hörandl et al. (2005) as a guide. Attempts to amplify the other putative dioecious member of Ranunculeae, *Paroxygraphis* (Tamura 1993), from DNA extracted from herbarium material have been unsuccessful to date. For comparative purposes, nomenclature and group labels (e.g., *Ranunculus* grade, core *Ranunculus*) used in this article follow those used by Hörandl et al. (2005).

For the morphological work, information was distilled from the literature (see table 2 for citations). In addition, a limited number of herbarium specimens were examined (table 2) using stereo microscopy.

DNA Sequencing and Alignment

DNA extractions were done according to Doyle and Doyle (1987) or as in Qiagen or Sigma DNA extraction kit protocols. Amplification of the chloroplast genes (*atpB*, *rbcL*) was as described in Hoot et al. (1995) for all specimens except those noted in table A1. The protocol for ITS and the remaining chloroplast amplifications (25-μL reaction volume) was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM amplification primer, 1.5 U Taq polymerase, 4 μg BSA, 0.1% TritonX-100 and Tween 20, and 0.3–2.0 μL template DNA. Cycling parameters were 94°C for 5 min; 40 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min; 72°C for 5 min. PCR products were purified using either Wizard Columns (Promega) or QIAquick (Qiagen) PCR purification columns (sometimes after agarose gel purification). Sequencing reactions were as described by Hoot et al. (1995) or prepared and run on an ABI 373-Stretch or a Beckmann Coulter CEQ 2000 automated sequencer following the manufacturers' protocols.

Alignment of ITS sequences was accomplished to a rough approximation using Sequencher 3.0 (Gene Codes) with subsequent manual corrections. The criteria for indel alignment were as described by Hoot and Douglas (1998). Indels were not scored, and regions of ambiguous alignment were removed from the data set.

Data Analyses

We used three analytical approaches: equally weighted maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). Trees for the *atpB* and *rbcL* analyses were rooted with *Glaucidium* (Hoot 1995). The ITS data were rooted with three placeholder species from the sister tribe Anemoneae (*Anemone americana*, *Anemone canadensis*, and *Anemone keiskeana*), selected based on previous molecular work on that tribe (Hoot et al. 1994; Hoot 1995; Schuettpelz et al. 2002).

Before combining the *atpB* and *rbcL* data sets, tree congruence was assessed by visual comparisons of the MP bootstrap trees for each data set and by conducting the partition homogeneity test (Farris et al. 1995) using PAUP* with 100 heuristic search replications.

Parsimony analyses of the combined *atpB*-*rbcL* and ITS data sets were performed with PAUP* version 4.0b2a (Swofford 1998), using the heuristic search option with 500 random addition sequence replicates and TBR branch swapping with MULTrees in effect. To assess branch support for the combined data, MP bootstrap (MPBS; Felsenstein 1985) analyses were performed using PAUP*, full heuristic search, with 500 replications of 10 random additions each. MPBS values of $\geq$70% were considered moderate to strong support (Hillis and Bull 1993; Alfaro et al. 2003).

For the ML analyses, the best-fitting model of nucleotide substitution was identified for the combined data set using the hierarchical likelihood ratio test (hLRT) approach as implemented in MrModeltest 2.2 (Nylander 2004). Heuristic ML searches were conducted in PAUP* using the appropriate model of evolution with the associated parameter estimates, 10 random addition sequence replicates, and TBR branch swapping with MULTrees in effect. ML bootstrap (MLBS) values were calculated using the above parameters with 100 replicates, 10 random additions each.

Bayesian inference was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Using the models for each individual data set as identified by MrModeltest 2.2 and flat priors, four chains (three heated) were run for one million generations, sampling trees every 100 generations. The first 100,000 generations (1000 trees) were excluded as the burn-in phase, allowing the analysis to reach stationarity. After importing the trees into PAUP*, a 50% majority rule consensus tree with posterior probabilities (PP) was computed.

Results

Chloroplast Data

The chloroplast data consisting of the *atpB* and *rbcL* genes were used in their entirety except for pruning of data from the beginning and end of the sequences (table 1). Several taxa were missing more than 100 bases of sequence due to amplification or sequencing difficulties: *atpB*: *Hamadryas*—229 bases (sites 792–1021); *rbcL: Ficaria*—150 (1257–1407), *Krapfia*—404 (632–948, 1319–1407); *Ranunculus trichophyllus*—175 (1232–1407).

The phylogenies resulting from the chloroplast genes were topologically highly congruent, and the partition homogeneity test resulted in a *P* value of 0.56, indicating considerable congruence between the two data sets. For these reasons, only the combined results for these genes are considered in this article.

Table 1

Parsimony Analyses Tree Statistics

	atpB, *rbcL*	ITS
Alignment length	2,816	637
Characters pruned[a]	178	33
Missing data %	2.18	2.00
Variable characters	621	266
Informative characters	334	186
Number of trees	4	9
Consistency index	.53	.48
Retention index	.71	.66

[a] Characters were pruned at the beginning or end of chloroplast genes due to large amounts of missing data. Internal transcribed spacer uninformative insertions with large amounts of missing data were also pruned.

As determined with MrModeltest, the best-fit model selected by hLRT for the likelihood analyses of chloroplast data was GTR + I + C with the following parameters: base frequencies: A = 0.2789; C = 0.1989; G = 0.2488; T = 0.2734; Nst = 6; rate matrix: A–C = 1.7353, A–G = 2.9930, A–T = 0.2506, C–G = 0.7102, C–T = 3.9033; rates = γ; shape = 0.8915; and proportion of invariable sites (Pinvar) = 0.5572.

The trees resulting from the MP, ML, and BI analyses of the combined chloroplast data were highly congruent, especially where support values were significant. The MP consensus tree (fig. 1), derived from the four shortest trees, indicates that the backbone of the tree is not well supported by either bootstrap or posterior probability values. However, many of the more terminal clades are moderately to well supported (MPBS and MLBS $\geq$ 70; PP $\geq$ 0.95). As predicted by earlier classifications of *Hamadryas*, the genus is found within a well-supported Ranunculeae (MPBS and MLBS = 100, PP = 1.00), consisting of *Ranunculus* and other closely related genera. It is strongly supported as sister to *Trautvetteria* and *Halerpestes* (MPBS = 95, MLBS = 97, PP = 1.00). It is not in the same clade as two other representatives of the *Ranunculus* alliance found in Andean South America, *Krapfia* and *Laccopetalum*, which are part of the core *Ranunculus* clade as defined by Hörandl et al. It is also not closely related to other genera (*Clematis*, *Thalictum*) in the family that have species that exhibit dioecy (fig. 1).

ITS Data

The ITS nuclear ribosomal data were, for the most part, easy to align. In two regions within ITS 1, due to alignment uncertainty involving the outgroup taxa only, bases were replaced by "?" at 14 sites within the outgroup (*Anemone* spp.). In addition, uninformative insertions consisting largely of missing data were removed (table 1).

As determined with MrModeltest, the best-fit model selected by hLRT for the likelihood analyses of chloroplast data was GTR + G with the following parameters: base frequencies: A = 0.2688, C = 0.2507, G = 0.2551, T = 0.2254; Nst = 6; rate matrix: A–C = 1.2445, A–G = 3.1953, A–T = 2.1338, C–G = 0.4873, C–T = 6.7417; rates = γ; shape = 0.3679; and proportion of invariable sites (Pinvar) = 0.0.

As with the chloroplast genes, the phylogenies resulting from the MP, ML, and BI analyses were for the most part congruent, especially where support values were high. The ML bootstrap tree was least resolved (tree not presented), collapsing several clades in the core *Ranunculus* that are resolved in the MP and BI trees. The BI tree weakly resolves *Halerpestes* and *Ranunculus andersonii* as sister to the core *Ranunculus* (PP = 0.61; tree not shown).

All analyses (figs. 2, 3) place the clade consisting of *Arcteranthis* and *Trautvetteria* as sister to a weakly supported clade consisting of all remaining *Ranunculus* and closely related segregate genera (MPBS < 50, MLBS = 51, PP = 0.63). *Ranunculus pallasii*, *Ranunculus lapponicus*, *Ranunculus kochii*, and *Ranunculus ficaria* are moderately to strongly supported (MPBS = 73, MLBS = 78, PP = 0.99) as sister to the core *Ranunuculus* (figs. 2, 3).

The ITS data place the three species of *Hamadryas* included in this study in an unresolved trichotomy (MPBS = 100, MLBS = 99, PP = 1.00) within the *Ranunculus* grade (figs. 2, 3). The *Hamadryas* clade is part of a moderately supported clade (MPBS = 74, MLBS = 78, PP = 0.99) including *Oxygraphis*, *Peltocalathos*, and *Callianthemoides*. Given the differences in sampling, this placement of *Hamadryas* is not incongruent with that found with the chloroplast data (fig. 1).

The lack of resolution along the backbone of the ITS tree (figs. 2, 3) does not allow exact placement of the *Hamadryas* clade (*Hamadryas*, *Oxygraphis*, *Peltocalathos*, and *Callianthemoides*) within the *Ranunculus* grade. However, it is clear that *Hamadryas* is not in the core *Ranunculus* (fig. 2), which includes the two other South American genera in our sampling, *Krapfia* and *Laccopetalum*.

Discussion

Phylogenetic Relationships

Unlike previous work, which rooted Ranunculeae using genera often considered within the tribe, namely *Trautvetteria* and *Myosurus* (Hörandl et al. 2005) or *Trautvetteria* alone (Paun et al. 2005), both our data sets were rooted with external outgroups. The combined chloroplast data (fig. 1) strongly support a root within Ranunculeae between a clade consisting of *Hamadryas*, *Halerpestes*, and *Trautvetteria* (with *Ranunculus ficaria* weakly associated) and the core *Ranunculus* as defined by Hörandl et al. (2005). This rooting is in agreement with previous chloroplast restriction site work (Johansson 1998) using *Caltha* and *Trollius* as outgroups. The ITS data (fig. 2), rooted with *Anemone*, places *Trautvetteria* and *Arcteranthis* as sister to all remaining *Ranunculus*, but the low support values found along the backbone of this tree make determination of the root difficult. Future work is needed to expand the DNA data and sampling, including taxa such as *Cyrtorhyncha*, *Kumlienia*, and *Paroxygraphis*, that may represent early diverging lineages and whose inclusion may alter the topology and/or the root.

Both the *atpB*/*rbcL* and ITS data strongly support (BS $\geq$ 100, PP $\geq$ 0.98) a monophyletic Ranunculeae (figs.1, 2). Both of our phylogenies agree with previous molecular studies

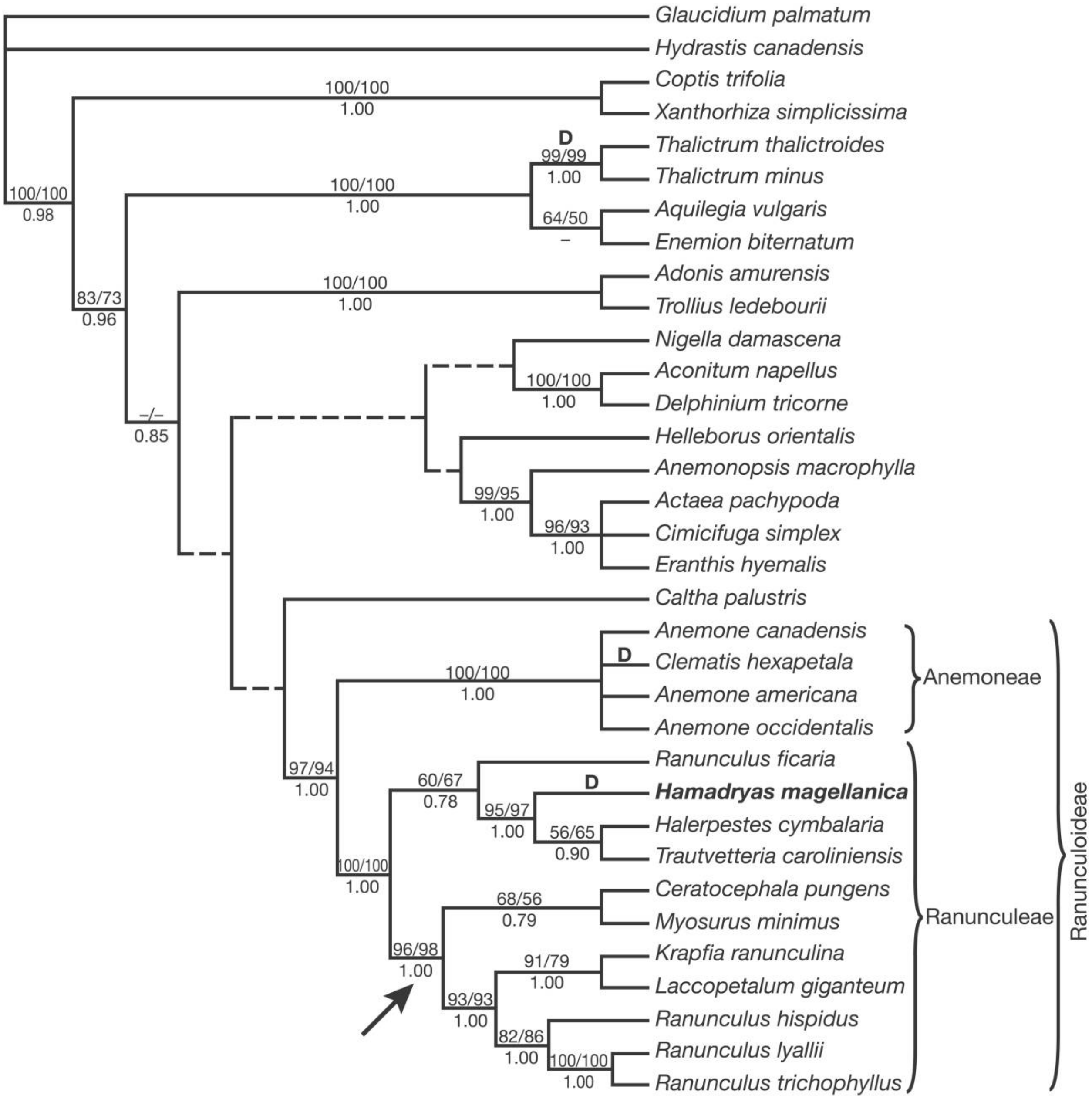

Fig. 1 Strict consensus maximum parsimony tree resulting from analysis of combined *atpB* and *rbcL* data for Ranunculaceae. Numbers above nodes are maximum parsimony and maximum likelihood bootstrap values; numbers below are posterior probabilities. Dashed branches indicate those not supported by any support measure; a hyphen indicates BS < 50 or PP < 95; the arrow indicates the base of the *Ranunculus* core clade; D = dioecious.

(Johansson 1998; Hörandl et al. 2005; Paun et al. 2005), recognizing a grade of taxa leading up to a well-supported and species-rich core *Ranunculus* (fig. 2). Our results essentially agree with previous work on the affinities of *Krapfia* and *Laccopetalum*; the work of Lehnebach et al. (2007) places the two taxa as sister to the core *Ranunculus*, although the strong support (BS = 100) for this clade could also be interpreted as placing them within the core *Ranunculus*. Our data strongly support (MPBS = 99, MLBS = 95, PP = 1.00) *Krapfia* and *Laccopetalum* as nested within the core *Ranunculus* but with little backbone support within the core *Ranunculus* (fig. 2). Both chloroplast and nuclear data strongly support past classifications that place *Hamadryas* in the tribe Ranunculeae, subfam. Ranunculoideae (fig. 1; Tamura 1993).

Tribe Ranunculeae is clearly delineated on the ITS ML phylogram by a long branch reflecting a high number of substitutions, and the tribe itself has a relatively shallow backbone (fig. 3). For these reasons, and to avoid paraphyly, we suggest that segregate genera such as *Trautvetteria*, *Arcteranthis*, *Halerpestes*, *Oxygraphis*, *Peltocalathos*, *Callianthemoides*, *Hamadryas*, *Laccopetalum*, *Krapfia*, *Myosurus*, and possibly

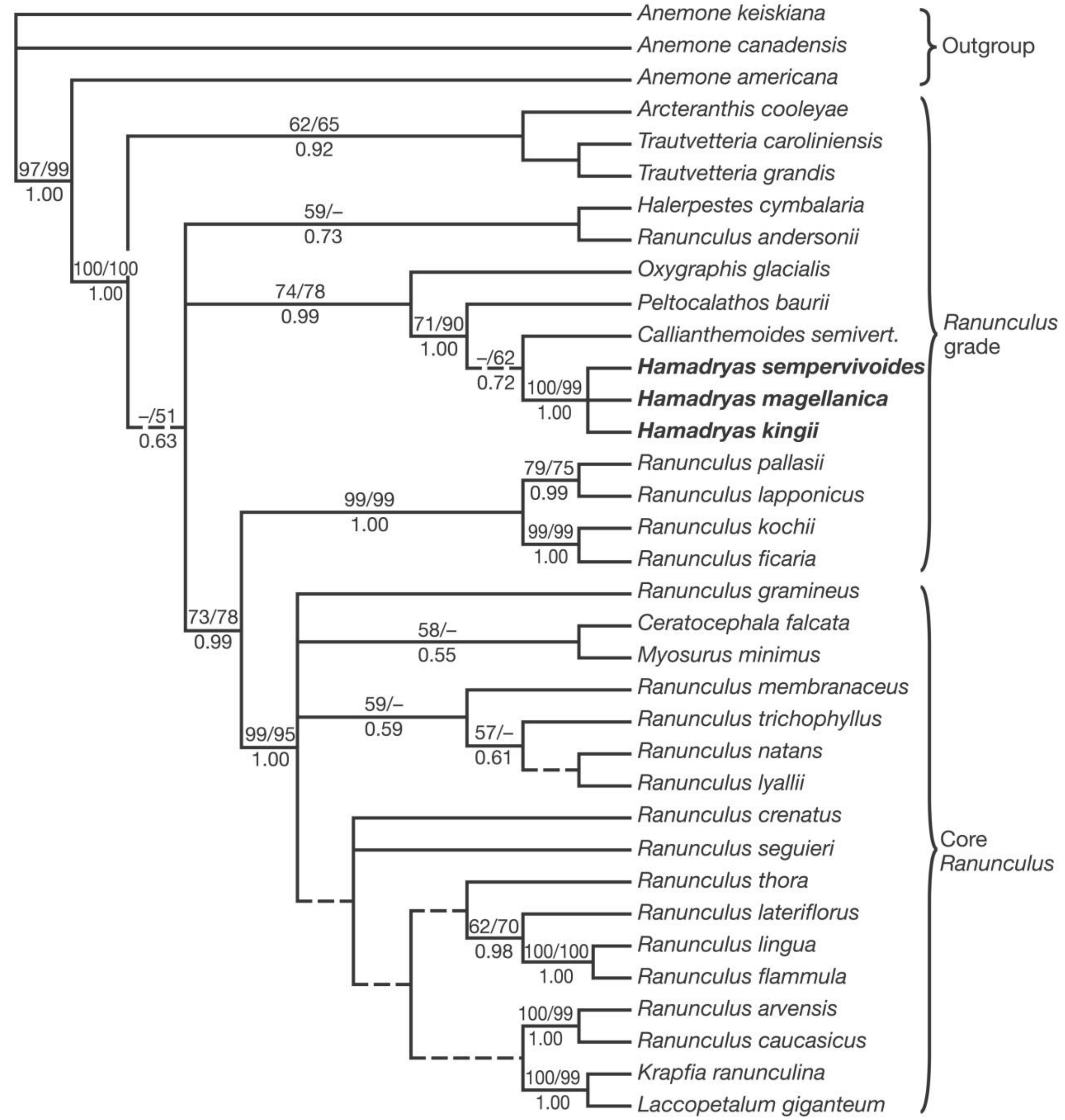

Fig. 2 Strict consensus maximum parsimony tree resulting from analysis of internal transcribed spacer data for tribe Ranunculeae and outgroups. Numbers above nodes are maximum parsimony and maximum likelihood bootstrap values; numbers below are posterior probabilities. Dashed branches indicate those not supported by any support measure; a hyphen indicates BS < 50 or PP < 95; *Ranunculus* labels on the right are as in Hörandl et al. (2005); *semivert.* = *semiverticillatus*.

others not included in this study should be subsumed within *Ranunculus*. Many of these taxa (e.g., *Callianthemoides*, *Peltocalathos, Laccopetalum*, *Krapfia*) are already commonly referred to as *Ranunculus* rather than their segregate names (table A2).

Hamadryas is weakly supported (fig. 2; MPBS < 50, MLBS = 62, PP = 0.72) as sister to another South American genus, *Callianthemoides semiverticillatus* (=*Ranunculus semiverticillatus*), also found in alpine regions of southern Argentina and Chile. *Hamadryas* and *Callianthemoides* are part of a moderately supported to well-supported clade (MPBS = 74, MLBS = 78, PP = 0.99) including *Oxygraphis glacialis* (arctic and alpine regions of East Asia and North America) and *Peltocalathos baurii*. *Peltocalathos baurii* (=*R. baurii*) of alpine to subalpine South Africa is sister to *Hamadryas* and *Callianthemoides* (MPBS = 71, MLBS = 90, PP = 1.00), suggesting a possible previous Gondwanan distribution. Paun et al. (2005), in dating divergence times for *Ranunculus*

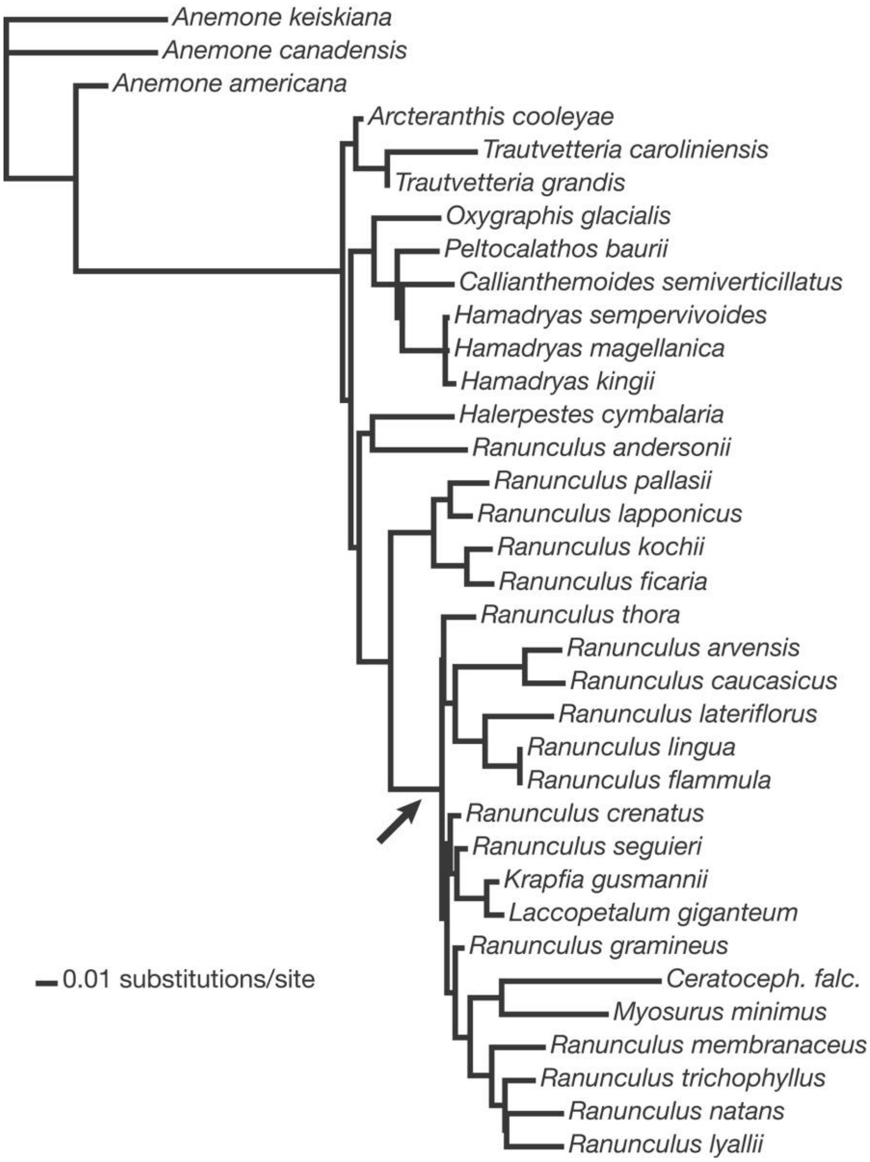

Fig. 3 Maximum likelihood tree resulting from analysis of internal transcribed spacer data for tribe Ranunculeae and outgroups. The arrow indicates the base of the *Ranunculus* core clade; *Ceratoceph. falc.* = *Ceratocephala falcata*.

s.l. using *matK*, estimated the divergence of the *Ranunculus* grade and core *Ranunculus* at approximately 42.0 Ma (Eocene). If correct, this date makes vicariance an unlikely explanation for the geographic distribution found in the *Hamadryas* clade. During the Eocene, South America and Africa were well separated (McLoughlin 2001; Scotese 2001), although long-distance dispersal would have been easier than at present. In any case, numerous examples of long-distance dispersal are appearing in predominantly alpine lineages (cf. Winkworth et al. 2005). Further testing with more precise dating techniques and the inclusion of better fossil calibration points for Ranunculaceae are needed.

Morphology

All the taxa included in the *Hamadryas* clade (*Oxygraphis*, *Peltocalathos*, *Callianthemoides*, and *Hamadryas*) prefer an alpine to subantarctic habitat and share a low-growing to cushionlike habit, four to five colorful sepals (except *Oxygraphis*), variable number of petals with open pockets or slits for nectaries, and large numbers of stamens and carpels. *Callianthemoides* is most like *Hamadryas* on the basis of its distribution in southern South America, preference for higher altitudes, leaves petiolate and highly divided (most similar to *Hamadryas delfinii* and *Hamadryas sempervivoides*; table 2; Lourteig 1951), and flowers often solitary (as in *H. delfinii*, *Hamadryas kingii*, and *H. sempervivoides*). *Callianthemoides* differs most markedly from *Hamadryas* in having bisexual flowers instead of unisexual, sepal and petal color white and larger in size (11–19 × 5–10 mm and 13–25 × 4–8 mm, respectively), and achenes with four prominent nerves.

Oxygraphis, consisting of four to five species, is found in the colder regions of eastern Asia and western North America

Table 2

Characteristics of *Hamadryas* Species

Character or trait	*Hamadryas argentea*	*Hamadryas delfinii*	*Hamadryas kingii*	*Hamadryas magellanica*	*Hamadryas sempervivoides*
Geographic distribution	Falkland Islands	Argentina: Tierra del Fuego to Neuquén Province, San Juan Province; Chile: Magallanes, including Tierra del Fuego	Argentina: Chubut to Río Negro Province; Chile: Magallanes, including Tierra del Fuego	Argentina: Tierra del Fuego, Isla de los Estados; Chile: Magallanes, including Tierra del Fuego	Argentina: Santa Cruz Province; Chile: Magallanes
Zone	Cold steppe 6–615 m	Cold steppe, subalpine, alpine 400–1200 m	Alpine 600–1600 m	Cold steppe and subalpine 150–850 m	Alpine 650–1700 m
Habitat	Grasslands, heath, cliffs, and slopes	Grasslands, herbfield, rocky outcrops	Feldfield and rocky outcrops	Grasslands, heaths, herbfield, and occasionally at forest edge	Wet herbfield and streamsides in rocky places
Petiole	Petioles not wide, up to 18 cm long, not laminate	~1.2 mm wide, 2–10 cm long, not laminate	3–5 mm wide, up to 9 cm long, laminate	~1.0 mm wide, up to 20 cm long, laminate at base	2–6 mm wide, laminate
Leaf lamina	3 lobed, margins crenate with glands; tomentose both surfaces	3 lobed, each lobe 2- to 3-pinnate; tomentose both surfaces	3–5 lobed, margins lobed or crenate; tomentose both surfaces	3 lobed to trifoliate, margins lobed; tomentose, especially abaxially	3-lobed to highly dissected; essentially glabrous on both surfaces
Inflorescence	Cyme of 2–3 flowers	Solitary (2)	Solitary	Cyme or panicle of 2–5 flowers	Solitary
Sepal number:					
Male	5–6	5–7	5–6	5–7	5–6
Female	5–6	5	5–6	5	5–6
Petal color	Yellowish brown to reddish	Copper brownish to brownish red with yellow center	Dark bluish brown, with yellow center	Pale yellow to greenish yellow	Yellow
Petal number:					
Male	15	(8)–11–18	10–15	(7)–12–16	7–8
Female	15	10–14	10–15	7	7–8
Petal morphology	Lanceolate or sublinear, acute; 11–13 mm × 1–1.5 mm; tomentose abaxially	Linear, acute to acuminate; 6–12 mm × 1–2 mm; glabrous	Spatulate to sublinear, obtuse; 9–15 mm × 1–5 mm; tomentose abaxially	Linear to lanceolate, acute to acuminate; 8–10 mm × .5–1.65 mm; glabrous	Linear; ~14 mm × .6–1.0 mm; glabrous
Nectaries	Pocket with thick inferior edge	Small, thick, “scale”	Shallow pocket of thickened tissue	Shallow pocket of thickened tissue	Narrow, shallow pocket of thickened tissue
Stamen number	30–40	30–60	30–60	35–50	40–45
Carpel number	50–90	30–65	100–230	35–50	30–50
Achenes	Glabrous, ovoid-to-ovoid fusiform	Glabrous, asymmetrically compressed, angular, rugose dorsally	Pubescent, longitudinal striations present	Glabrous, triangular in cross-section, compressed, longitudinal striations present	Glabrous, triangular in cross-section, compressed, with prominent veins dorsally, smooth

Sources. Hooker 1847; Lourteig 1951; Moore 1968, 1983; Arroyo et al. 1989; Zuloaga and Morrone 1999; S. B. Hoot, J. Kramer, and M. T. K. Arroyo, field and herbarium observations.

and differs most from *Hamadryas* in its largely undivided, glabrous leaves, usually solitary bisexual flower, green sepals (usually 5), and achenes longitudinally one-or-more veined on each side (Whittemore 1997; Wencai et al. 2001). The monotypic genus *Peltocalathos* (=*Ranunculus baurii*), occurring in moist places along streams and waterfalls at altitudes ≥1500 m, is found from the eastern Cape into Lesotho, Natal, Swaziland, and eastern Transvaal. It differs most from *Hamadryas* in having peltate, glabrous leaves with veins markedly lighter in color, bisexual flowers, and glabrous ridged achenes (Tölken 1967).

The only other dioecious species putatively within Ranunculeae (not included in any molecular work to date), *Paroxygraphis sikkimensis* W.W. Sm., is endemic to alpine eastern Himalayan regions. It is a diminutive perennial with undivided, glabrous leaves, solitary unisexual flowers, open pocket nectaries, tricolpate pollen, and achenes with one longitudinal vein on each lateral face (Santisuk 1979; Tamura 1993). Most of these characteristics occur in the earliest branching Ranunculeae and suggest that this genus may be within the *Ranunculus* grade (but not necessarily in the *Hamadryas* clade).

There is some confusion in the literature concerning the terminology used to described nectaries in *Hamadryas* and other early-diverging *Ranunculus*. Lourteig (1951) describes some species of *Hamadryas (H. delphinii*, *H. magellanica*) as having nectary scales (table 2). After looking at herbarium specimens of all *Hamadryas* species except *H. delfinii* (floral material not available), we would characterize the nectaries as either open slits or pockets. This characterization is in agreement with Tamura's (1993) terminology. However, our observations were from dried material only; there is a need for scanning electron microscopy of nectaries from freshly collected material for the *Hamadryas* clade.

The *Hamadryas* clade plus *Halerpestes* and *Arcteranthis* have been characterized in the literature as having prominent, longitudinal, parallel veins on the achenes (Hörandl et al. 2005). Such achene venation patterns do not appear in all species of *Hamadryas* (e.g., *H. delfinii, H. sempervivoides*; table 2) or in *Callianthemoides*, which has smooth, angular, four-faceted achenes with raised veins along each face edge.

Evolution of Dioecy

Dioecy has evolved at least three times in Ranunculaceae—within the genera *Clematis*, *Thalictrum*, and *Hamadryas* (fig. 1), and possibly four times, depending on the position of *Paroxygraphis* (see above). Dioecy is known to have evolved repeatedly at the familial (e.g., Renner and Won 2001) and generic (e.g., Gleiser and Verdú 2005) levels and from a number of breeding systems. The direction of evolution in the *Hamadryas* clade (fig. 2) is clearly from bisexual flowers to dioecy.

It is interesting to speculate why dioecy evolved in *Hamadryas* and not other *Ranunculus*. Of the characters suggested to be correlated with dioecy (Thomson and Brunet 1990; Renner and Ricklefs 1995; Vamosi et al. 2003)—perennial habit, plastic phyllotaxy, abiotic pollination, inconspicuous greenish yellow or white flowers, inconspicuous inflorescences, monoecy, climbing habit, fleshy fruits, and island distributions—the first three appear to be most applicable to *Hamadryas*. The perennial habit and plastic phyllotaxy (especially variation in petal number) is found in all *Hamadryas* species (table 2) and all other members of the *Hamadryas* clade (*Callianthemoides*, *Peltocalathos*, and *Oxygraphis*).

In relation to the third correlate, abiotic pollination, quantified phenological observations show that alpine species of *Hamadryas* are among the earliest flowering species in their habitat (Squeo 1991), with peak flowering occurring in early December at around 50°S shortly after snowmelt. Observations made on *Hamadryas kingii* and *H. sempervivoides* in a community-wide pollination survey in the Patagonian alpine, where pollination rates reach their lowest for temperate latitudes in the Andes (Arroyo and Squeo 1990), revealed casual visits by Dipterans (Muscidae) on *H. kingii*, with no visitors recorded on *H. sempervivoides*. The prominent anthers and presence of these particular species in the upper alpine belt, where they are exposed to continuous strong winds, make it highly probable that species of *Hamadryas* are at least facultatively wind pollinated. Judging by the fairly conspicuous flowers that bear nectaries, reported to secrete nectar in both sexes in lowland *Hamadryas argentea* (Moore 1968), it seems likely that insect pollination is the primitive condition in *Hamadryas*. Thus, any trend to move toward wind pollination in the high-elevation species would have occurred after the initial appearance of sexual dimorphism.

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Appendix

Table A1

Species Sampled, Voucher Information, and GenBank Accession Numbers for *atpB* and *rbcL* Sequences

		GenBank accession no.	
Species	Voucher information	*atpB*	*rbcL*
Aconitum napellus L.	S. Hoot 926 (UWM)	EU053868	EU053898
Actaea pachypoda Ell.	M. Chase 550 (K)	EU053869	EU053899
Adonis amurensis Regel and Radde	S. Hoot 933 (UWM)	EU053870	EU053900
Anemone americana (DC.) Hara = *Hepatica americana* (DC.) Ker-Gawl.	S. Hoot 883 (MICH)	EU053871	EU053901
A. canadensis L.	S. Hoot 867 (MICH)	EU053872	EU053902
A. occidentalis S. Wats. = *Pulsatilla occidentalis* Freyn	S. Hoot 8817 (MICH)	EU053873	EU053903
Anemonopsis macrophylla Siebold and Zucc.	A. Reznicek 9977 (MICH)	EU053874	EU053904
Aquilegia vulgaris L.	A. Reznicek 1108 (MICH)	EU053875	EU053905
Caltha palustris L.	S. Hoot 299 (MICH)	EU053876	EU053906
Ceratocephala pungens Garn.-Jones	B. Patrick 9-93 (OTA)	EU053877	EU053907
Cimicifuga simplex Wormsk.	A. Reznicek 9238 (MICH)	EU053878	EU053908
Clematis hexapetala Pall.	S. Hoot 9150 (MICH)	EU053879	EU053909
Coptis trifolia (L.) Salisb.	E. Voss s.n. (MICH)	AF093393	AF093730
Delphinium tricorne Michaux	A. Reznicek 9997 (MICH)	EU053880	EU053910
Enemion biternatum Raf. = *Isopyrum biternatum*	S. Hoot 9214 (UWM)	EU053881	EU053911
Eranthis hyemalis (L.) Salisb.	S. Hoot 920 (UWM)	EU053882	EU053912
Glaucidium palmatum Siebold and Zucc.	S. Hoot 924 (UWM)	AF093375	AF093723
Halerpestes cymbalaria (Pursh) Greene = *Ranunculus cymbalaria*	S. Hoot 004 (UWM)	EU053883	EU053913
Hamadryas magellanica Lam.[a]	E. Pisano et al. 8251 (CONC)	EU053884	EU053914
Helleborus orientalis Lam.	S. Hoot 9224 (UWM)	EU053885	EU053915
Hydrastis canadensis L.	R. Naczi 2883 (MICH)	AF093382	AF093725
Krapfia ranunculina DC.[a]	A. Hofreiter C25 (MSB)	EU053886	EU053916
Laccopetalum giganteum Ulbr.[a]	J. Pera s.n. (HUT)	EU053887	EU053917
Myosurus minimus L.	K. Ford 10-19-93 (CHR) M. Chase 532 (K)	EU053888	DQ09944
Nigella damascena L.	S. Hoot 9211 (UWM)	EU053889	EU053918
Ranunculus ficaria L.	S. Hoot 002 (UWM)	EU053890	EU053919
R. hispidus Michx.	S. Hoot 901 (MICH)	EU053891	EU053920
R. lyallii Hook. f.	RBGE 19331732[b]	EU053892	EU053921
R. trichophyllus Chaix[a]	S. Hoot 0011 (UWM)	EU053893	EU053922
Thalictrum minus Bess. 'adiantifolium'	A. Reznicek 10107 (MICH)	EU053894	EU053923
T. thalictroides (L.) Eames and Boivin = *Anemonella thallictroides*	S. Hoot 9223 (UWM)	EU053895	EU053924
Trautvetteria caroliniensis (Walt.) Vail	S. Hoot 9218 (UWM)	EU053896	EU053925
Trollius ledebourii Reichb.	A. Reznicek 100035 (MICH)	EU053897	EU053926
Xanthorhiza simplicissima Marshall	Y. Qui 91030 (NCU)	AF093394	L12669

[a] Specimens amplified using alternative protocol described in "Material and Methods."

[b] Royal Botanic Garden, Edinburgh, accession number.

Table A2

Species Sampled, Geographical Distribution, Source of Plant Material or Reference Cited in GenBank, and GenBank Accession Numbers for ITS Sequences

Species	Geographic distribution	Source of plant material or GenBank reference	ITS
Arcteranthis cooleyae (Vasey and Rose) Greene = *R. cooleyae* Vasey and Rose	Canada	Hörandl et al. 2005	AY680201
Callianthemoides semiverticillatus (Phil.) Tamura = *R. semiverticillatus* Phil.	Andean cordilleras of Chile and Argentina	Hörandl et al. 2005	AY680199
Ceratocephala falcata (L.) Pers. = *R. falcatus* L.	Austria	Hörandl et al. 2005	AY680191
Halerpestes cymbalaria (Pursh) Greene = *R. cymbalaria* Pursh	North America	Hörandl et al. 2005	AY680196
Hamadryas kingii Hook. f.	Chile and Argentina	M. T. K. Arroyo et al. 92-60 (CONC)	EU053929
H. magellanica Lam.	Chile and Argentina	E. Pisano et al. 8251 (CONC)	EU053928
H. sempervivoides Sprague	Chile and Argentina	M. T. K. Arroyo and F. Squeo 87162 (CONC)	EU053927
Krapfia ranunculina DC. = *R. krapfia* DC. ex Deless.	Ecuador and Peru	A. Hofreiter C25 (MSB)	EU053930
Laccopetalum giganteum Ulbr.	Andes, Peru	J. Pera s.n. (HUT)	EU053931
Myosurus minimus L. = *R. minimus* EHL Krause	Temperate regions worldwide	Hörandl et al. 2005	AJ347913
Oxygraphis glacialis (Fischer ex DC.) Bunge = *R. kamchaticus* DC.	Asia, Aleutian Alaska	Hörandl et al. 2005	AY680198
Peltocalathos baurii (McOwan) Tamura = *R. baurii* McOwan	South Africa	Hörandl et al. 2005	AY680200
Ranunculus andersonii A. Gray	Western North America	Hörandl et al. 2005	AY680197
R. arvensis L.	Western Asia/Europe; invasive North America	Hörandl et al. 2005	AY680177
R. caucasicus M. Bieb.	Caucasia	Hörandl et al. 2005	AY680178
R. crenatus Waldst. and Kit.	Montane Europe	Hörandl et al. 2005	AY680086
R. ficaria L.	Europe; invasive in North and South America	Hörandl et al. 2005	AY680192
R. flammula L.	Eurasia, North America	Hörandl et al. 2005	AY680185
R. gramineus L.	Europe	Hörandl et al. 2005	AY680076
R. kochii Ledeb.	Western Asia	Hörandl et al. 2005	AY680193
R. lateriflorus DC.	Mediterranean	Hörandl et al. 2005	AY680179
R. lapponicus L.	Circumboreal	Hörandl et al.2005	AY680194
R. lingua L.	Eurasia	Hörandl et al. 2005	AY680184
R. lyallii Hook f.	New Zealand	Hörandl et al. 2005	AF323283
R. membranaceus Royle	China, Tibet	Hörandl et al. 2005	AY680056
R. natans C. A. Mey.	Asia	Hörandl et al. 2005	AY680113
R. pallasii Schlecht.	Circumboreal	Hörandl et al. 2005	AY680195
R. seguieri Vill.	Alpine Europe	Hörandl et al. 2005	AY680079
R. thora L.	Alpine Europe	Hörandl et al. 2005	AY680188
R. trichophyllus Chaix = *R. aquatilis* L. var. *diffusa* With.	Eurasia, North and South America	Hörandl et al. 2005	AY680067
Trautvetteria grandis Nutt.[a]	Western North America	Hörandl et al. 2005	AY680202
T. caroliniensis (Walter) Vail	North America	Hörandl et al. 2005	AH007301
Outgroups:			
Anemone americana (DC.) Hara = *Hepatica americana* (DC.) Ker Gawl.	Eastern North America	Schuettpelz et al. 2002	AY055386
A. canadensis L.	North America	Schuettpelz et al. 2002	AY055387
A. keiskeana Ito	East Asia	Schuettpelz et al. 2002	AY055390

Note. Nomenclature in this article follows that used in Hörandl et al. 2005.

[a] Name as listed in Hörandl et al. 2005. Correct name is *T. caroliniensis* (Parfitt 1997).

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