

PHYLOGENETIC SYSTEMATICS AND CHARACTER EVOLUTION IN THE ANGIOSPERM FAMILY HALORAGACEAE¹

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The poorly known Haloragaceae R. Br. (Saxifragales) are highly diverse in habit (small trees to submerged aquatics) and labile in floral merosity (2–4), both uncommon among the core eudicots. This family has a cosmopolitan distribution, but taxonomic diversity is concentrated in Australia. An explicit phylogenetic approach has not previously been utilized to examine relationships or character evolution in this family. We used molecular evidence from nrDNA ITS and cpDNA *trnK* and *matK* regions under both Bayesian and parsimony analyses to address phylogenetic relationships. Combined molecular analyses defined a monophyletic Haloragaceae with the woody genera (*Haloragodendron*, *Glischrocaryon*) sister to the rest. Relationships among many genera were well resolved, with genera as currently delimited generally well supported, although there were notable exceptions; a new genus (*Trihaloragis*) is recognized, and the aquatic genus *Meionectes* is again distinct from *Haloragis*. Three new species combinations are also recognized. There are multiple (two or three) origins of the submerged aquatic habit in the family and potentially an intermediate reversal to the terrestrial habit, neither previously demonstrated in a core eudicot family using an explicit phylogenetic hypothesis. Ancestral character analyses suggest two origins of trimerous flowers and multiple reductions to dimerous flowers throughout Haloragaceae.

Key words: aquatic; Bayes; floral merosity; Haloragaceae; ITS; *matK*; phylogenetics.

Haloragaceae are a cosmopolitan family currently with eight genera and about 120 species (Table 1). They are extremely diverse in habit, ranging from small trees to submerged aquatics. Four genera (*Glischrocaryon*, *Gonocarpus*, *Haloragis*, *Haloragodendron*) are primarily terrestrial, whereas four (*Laurembergia*, *Meziella*, *Myriophyllum*, *Proserpinaca*) are aquatic/semiaquatic. The habitats of these taxa range from arid deserts to freshwater lakes exceeding 10 m in depth. Haloragaceae also are highly labile in floral merosity (2–4), uncommon among core eudicots. The center of diversity for the family is Australia, where four endemic genera and ≈70% of the species occur.

Early circumscriptions of the family included disparate genera such as the dimerous *Gunnera* and the aquatics *Hippuris* and *Callitriche* (Brown, 1814; Candolle, 1828). Schindler (1905) reinterpreted the family circumscription by removing *Hippuris* and *Callitriche* and merging *Gonocarpus* (terrestrial) and *Meionectes* (monotypic aquatic) into *Haloragis*. Until recently, this interpretation of Haloragaceae had been widely followed (Appendix S1, see Supplemental Data accompanying online version of this article). Shaw (1966)

further reduced the family by excluding the genus *Gunnera*. A thorough examination of the family by Orchard (1975) included a much wider sampling of herbarium material (especially from Australia) than had been available to previous authors, and he delimited a family that comprised eight genera (Table 1; Appendix S1). Orchard's (1975) treatment of the family restored *Gonocarpus*, which had been placed in synonymy with *Haloragis* by Brown (1814) followed by Schindler (1905). The recognition of *Gonocarpus* was based on a diversity of characters relating primarily to reproductive structures. The woody *Haloragodendron* was split from *Haloragis* by Orchard (1975), although the aquatic *Meionectes* remained in synonymy with *Haloragis*.

Prior to the advent of molecular-based phylogenetic studies, many authors suggested that Haloragaceae had a close relationship to Onagraceae (Schindler, 1905; Hutchinson, 1959; Melchior, 1964), Cornaceae (Thorne, 1968; Orchard, 1975), or the aquatic Podostemaceae (Cronquist, 1968; Takhtajan, 1969). These suggestions were based on features from embryology, pollen morphology, and floral vasculature. However, inclusive studies of angiosperms using molecular phylogenetic approaches have supported the placement of Haloragaceae within Saxifragales (Morgan and Soltis, 1993; Soltis et al., 1997). Fishbein et al. (2001) sampled two Haloragaceae taxa (*Myriophyllum* sp. and *Haloragis* sp.) in a phylogenetic treatment of the Saxifragales using sequence data from five genes and found Haloragaceae to form a clade with three genera (*Aphanopetalum*, *Penthorum*, *Tetracarpaea*) not considered previously as being closely allied to Haloragaceae. In turn, Crassulaceae resolved as a well-supported sister group to this clade (Fishbein et al., 2001). Consequently, inclusion of both *Tetracarpaea* and *Penthorum* in Haloragaceae has been proposed as an option (APG II 2003).

Intergeneric relationships—Disparate geographic distributions and highly divergent morphology (Orchard, 1975; Schindler, 1905) among the four aquatic/semiaquatic genera (Table 1) have made it difficult to hypothesize their

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TABLE 1. Distribution, habit, and species diversity of Haloragaceae genera.

Genus	Distribution	Habit	No. species
<i>Glischrocaryon</i>	Australia	Terrestrial	4
<i>Gonocarpus</i>	Australia, New Zealand, S. E. Asia	Terrestrial	≈36
<i>Haloragis</i>	Australia, New Zealand, S. Pacific	Terrestrial ^a	≈26
<i>Haloragodendron</i>	Australia	Terrestrial	5
<i>Laurembergia</i>	Pantropical	Semiaquatic	4
<i>Meziella</i>	S. W. Australia	Aquatic	1
<i>Myriophyllum</i>	Cosmopolitan	Aquatic	≈60
<i>Proserpinaca</i>	New World	Aquatic	3

^aThree species are aquatic.

evolutionary relationships within Haloragaceae. The aquatic genera have different distributions; neither *Proserpinaca* (New World) nor *Laurembergia* (Africa, Asia, and South America) is found in Australia. *Meziella* is an Australian endemic, while *Myriophyllum* has a global distribution with its center of diversity (≈60% of species) in Australia. In contrast, the four terrestrial genera (Table 1) occur almost exclusively in Australia and New Zealand. Three *Gonocarpus* species extend into Southeast Asia, and a group of four *Haloragis* species extends east from Australia into islands of the New Caledonia complex (*H. prostrata* J.R.Forst. & G.Forst.), Rapa Island (*H. stokesii* R.Br.), and the Juan Fernandez Islands off the west coast of Chile (*H. masatierrana* Skotts., *H. masafuerrana* Skotts.).

Though many morphological features have been examined in Haloragaceae, intergeneric relationships remain elusive. The circumscription of the aquatic genera (excluding *Meionectes*) has not been questioned and is supported by morphology (Orchard, 1975). However, relationships among these genera are unclear. Unisexual flowers (except in *Proserpinaca*) and pinnately dissected leaves (except in *Laurembergia*) are common among the aquatic/semiaquatic genera, but these characteristics also are convergent among many aquatic angiosperms (Sculthorpe, 1967; Cook, 1996). Other morphological characters less likely to be adaptive to the aquatic habit indicate that some aquatic taxa may not be as closely related to each other as they are to *Haloragis* or *Gonocarpus*. For example, pollen characters (Pragowski, 1970) associate *Haloragis*, *Gonocarpus*, *Laurembergia*, and *Myriophyllum*, whereas embryological features (Bawa 1969a, b) link only *Haloragis*, *Myriophyllum*, and *Laurembergia*. Furthermore, several reproductive and vegetative features (Orchard, 1975) are shared more closely among *Haloragis*, *Gonocarpus*, and *Laurembergia* but not *Myriophyllum*.

Aquatic taxa—*Myriophyllum* (60+ spp.) is among the most speciose genera of submerged aquatic “dicots” (Cook, 1996) and is unique within Haloragaceae in having fruits that separate into individual nutlets at maturity and carpellate flowers that lack petals and often sepals. *Meziella* is a genus thought to be extinct in Australia until its recent rediscovery by Orchard and Keighery (1993), who suggested a close affiliation of *Meziella* with *Myriophyllum*. *Laurembergia* may best be characterized as a helophyte (i.e., generally terrestrial but tolerant of prolonged inundation; Cook, 1996). Orchard (1975) considered the placement of *Proserpinaca* within Haloragaceae particularly problematic. He suggested with reservation a “transitional placement” of *Proserpinaca* between *Haloragis* and *Myriophyllum*. The deeply dissected submerged leaves of *Proserpi-*

naca are similar to those of *Myriophyllum* but also occur in the aquatic/semiaquatic members of *Haloragis* (*H. brownii* (Hook.f.) Schindl., *H. heterophylla* Brongn., and *H. tenuifolia* Benth.). The trimerous, perfect flowers of *Proserpinaca* rarely occur elsewhere in the family and are found otherwise only in *Gonocarpus hexandrus* (F.Muell.) Orchard, *Haloragis gossei* F.Muell., *H. tenuifolia*, *H. trigonocarpa* F.Muell., and occasionally in *H. digyna* Labill. The trimerous flower has been emphasized as an important feature in the generic circumscription of *Proserpinaca*.

Terrestrial taxa—Prior to Orchard’s (1975) revision of Haloragaceae, all of the terrestrial taxa except *Glischrocaryon* (= *Loudonia*) were usually included in *Haloragis* (Brown, 1814; Schindler, 1905). Orchard (1975) split *Gonocarpus* (the most speciose terrestrial genus; 38 spp.) from *Haloragis* based primarily on discordant fruit characters. He suggested (p. 274), “the relationship between [*Haloragis* and *Gonocarpus*] is probably not close” and instead proposed a closer relationship between *Gonocarpus* and the semiaquatic *Laurembergia*. He also split *Haloragodendron* from *Haloragis* based on its woody habit, large flowers, and winged fruits, all characters that were shared with *Glischrocaryon* spp. Orchard (1975) based the distinction between *Glischrocaryon* and *Haloragodendron* primarily on vegetative characters. Conspicuously, *Glischrocaryon* has adaptations common to arid environments, such as green stems and leaves reduced in size and number along the stem, with most located basally, characteristics not found in *Haloragodendron*.

Character evolution—**Aquatic habit**—Multiple origins of the aquatic habit are now generally regarded as having occurred across the angiosperms (Cook, 1996). However, multiple origins of submerged aquatic genera in a single family as defined by APG II (2003) are rare among the core eudicots. Character states often associated with aquatic taxa include those related to anemophily (i.e., reduced flowers, dioecy, monoecy, etc.) as well as highly divided or ribbonlike leaves (Sculthorpe, 1967). Submerged leaves of aquatic angiosperms undergo extreme structural modification. In contrast to their terrestrial ancestors, the leaves of submerged aquatics characteristically have a reduced/absent cuticle, are astomatal, and often are highly divided and/or ribbonlike to compensate for low light levels, limited CO₂ uptake, and water resistance (Cronk and Fennessy, 2001; Sculthorpe, 1967). All of these characteristics can be found among the aquatic Haloragaceae.

The aquatic habit manifests itself in various forms within Haloragaceae. *Myriophyllum*, *Meziella*, *Proserpinaca*, *Haloragis brownii*, and *H. tenuifolium* all spend part of their life

cycle underwater and are considered primarily submerged taxa. However, as in most aquatic plants, their reproductive structures are produced primarily on emergent stems that have variable leaf forms resembling those of their terrestrial relatives. The predominant vegetative form of aquatic Haloragaceae is a submerged stem with lacunal passages and pinnately lacinate leaves, although several *Myriophyllum* species have only linear, minute leaves. All the submerged aquatic genera have species that display some plasticity in form and/or habit, with some taxa adapted to survival on mudflats through thickened leaves that are reduced in size and segmentation, traits also found in the aquatic *Haloragis* (*H. brownii*, *H. tenuifolia*). In contrast, the helophytic *Laurembergia* does not include any species with a distinctive submerged form. Given this diversity, Haloragaceae are an ideal group for investigating the evolutionary transition from terrestrial to submerged aquatic habitats.

Floral merosity—Floral merosity often deviates from the basic tetramerous plan that occurs throughout Haloragaceae. Perfect, trimerous flowers are uncommon among the core eudicots (Soltis et al., 2003), yet occur in four different Haloragaceae genera (*Glischrocaryon*, *Gonocarpus*, *Haloragis*, *Proserpinaca*). Moreover, dimerous flowers, another unusual condition among core eudicots, are present in *Glischrocaryon behrii* (Schindl.) Orchard, *Haloragis brownii*, *Haloragis digyna* (usually), and some *Myriophyllum* spp. Reductions in locule or carpel number have also occurred in some species. Orchard (1975) considered these differences to represent derived reductions within genera, an opinion also shared by Schindler (1905). Assuming multiple reductions, neither author suggested close relationships between the pinnate-leaved, aquatic, trimerous- and dimerous-flowered species. This lability of floral merosity makes Haloragaceae an ideal group for investigating these evolutionary trends.

Data selection and analyses—Given the extreme diversity and contrasting systematic hypotheses that have been suggested for this family, a phylogenetic approach using molecular markers should help clarify relationships within Haloragaceae as it has for other families (Judd et al., 2002). It is important to use multiple data sets to address phylogenetic questions at different taxonomic levels (Swofford et al., 1996) and to account for hybridization and/or lineage sorting through incongruent phylogenetic hypotheses based on plastid vs. nuclear genes (Rieseberg and Wendel, 1993; Avise, 1994; Wendel and Doyle, 1998; Sang and Zhong, 2000). Hybridization can be restricted taxonomically, being more prominent in some groups than in others (Ellstrand et al., 1996; Rieseberg, 1997). Because hybridization is suspected or known in Haloragaceae (Orchard, 1975; Moody and Les, 2002), it is important to consider hybridization when interpreting phylogenetic hypotheses in this family. Although ancient hybridizations are difficult to determine using current phylogenetic methodologies (Linder and Rieseberg, 2004), more recent hybridization events can be assessed by comparing phylogenetic hypotheses derived from nuclear and plastid genomes and observing marked incongruence between the resulting tree topologies (Rieseberg, 1991; Wendel et al., 1991, 1995). We have chosen to use the nrDNA ITS and the cpDNA *trnK* introns + *matK* coding region for their combined effectiveness at elucidating phylogenetic relationships at the generic and subgeneric level (e.g., Moody et al., 2001; Les et al., 2002a, b).

Conflict among phylogenetic hypotheses can also arise because of unequal rates of evolution among data sets. This problem has been addressed by recent advances in Bayesian phylogenetic analyses (Ronquist and Huelsenbeck, 2003; Nylander et al., 2004), and we use multiple maximum likelihood (ML) models for separate, defined partitions in our combined analyses. Maximum parsimony was also used as an alternative source of reference to compare to Bayesian analysis results. Thus, both parsimony and Bayesian analyses were implemented for comparative purposes.

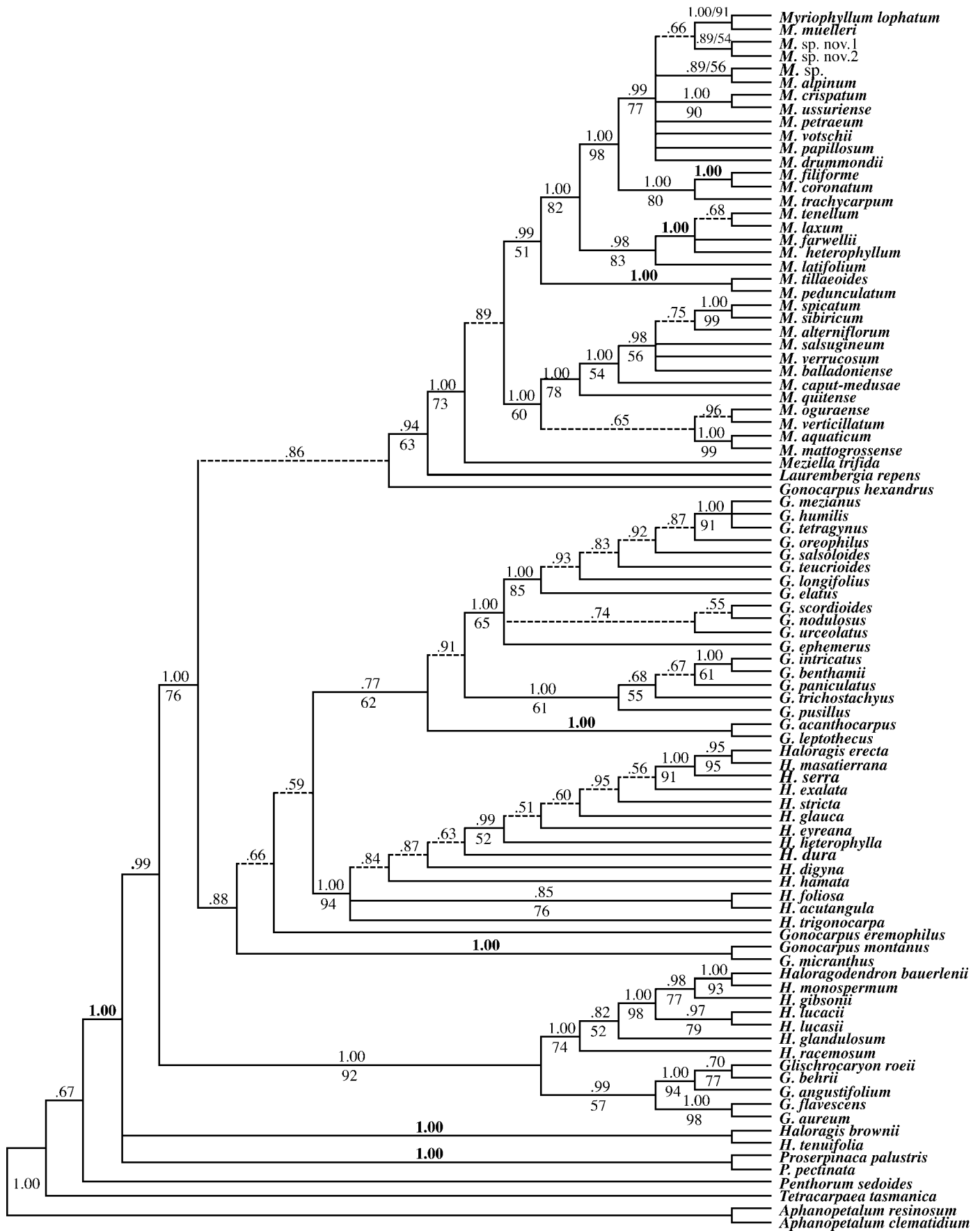
The major goals of this research were to (1) elucidate phylogenetic relationships among Haloragaceae genera and within some (2) evaluate generic limits as currently proposed and redefine them in the context of the phylogeny where necessary, and (3) examine the evolution of the aquatic habit and floral merosity among Haloragaceae genera using explicit phylogenetic hypotheses.

MATERIALS AND METHODS

Taxon sampling—Ninety-three taxa representing all genera of Haloragaceae and the hypothesized outgroups Aphanopetalaceae, Penthoraceae, and Tetracarpaceae (Fishbein et al., 2001) were sampled. All known species of *Glischrocaryon*, *Haloragadendron*, and *Meziella* were sampled. Other generic coverage included 23 of 36 (64%) *Gonocarpus* species and 16 of 26 (62%) *Haloragis* species, representing all major groupings recognized by Orchard (1975) and all the subsections of Schindler (1905). Also one of four (25%) *Laurembergia* species, two of three (67%) *Proserpinaca* species, and 31 of 65 (48%) of *Myriophyllum* species (including the major alliances of Orchard [1986] and most subsections of Schindler [1905]) were sampled. Most taxa were collected in the field and preserved with cetyl trimethylammonium bromide (CTAB) (Rogstad, 1992). Several taxa also were sampled from herbarium specimens, and multiple accessions of each taxon were sampled when possible (Appendix 1).

DNA extraction, PCR, and sequencing—Total genomic DNAs were extracted from fresh, CTAB-preserved, and herbarium specimen leaf material using a modified CTAB miniprep procedure (Doyle and Doyle, 1987). PCR was used to amplify the ITS-1, ITS-2, and 5.8S region of nuclear ribosomal DNA using the ITS4 and ITS5 primers or, in the case of several herbarium specimens, using ITS3 and ITS4 to amplify the ITS-2 and partial 5.8S region and ITS2 and ITS5 to amplify the ITS-1 and partial 5.8S region (White et al., 1990). The cpDNA *trnK* introns and *matK* coding-region were amplified using the primers *trnK*-3914F and *trnK*-2R (Johnson and Soltis, 1994). Several additional primers were used to amplify *trnK* and *matK* from DNA of herbarium leaf material including *matK*68F, *matK*1872R (Johnson and Soltis, 1994), *matK* 900F (Moody and Les, 2002), and newly developed *trnK*5R (5'-TCTTGGGTTATCAAATGATA) and *matK*70R (5'-GTTGTGTTGAC GAAAT). PCR protocols and conditions were the same as described in Moody et al. (2001). Cycle-sequencing of ITS used combinations of the ITS2, ITS3, ITS4, and ITS5 primers (White et al., 1990). The ITS region was cloned for some taxa using the TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA) to identify and exclude fungal contaminants. The *trnK* introns and *matK* region were sequenced using *trnK*-3914F, *matK*68F, *matK*1872R, *matK*900F, *trnK*360F, *trnK*2R, *matK*70R, *trnK*R, and a newly developed Haloragaceae specific primer *trnK*3F (5'-CGTCGATTGTGCGTA). Sequences were obtained using Big Dye terminator technology on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, California, USA).

Phylogenetic analyses—Sequences were edited using the program Sequencher 4.1.2 (Gene Code Corp., Ann Arbor, Michigan, USA) and manually aligned using the program MacClade 4.01 (Maddison and Maddison, 2001). Indel alignments were carefully assessed as in Graham et al. (2000). Parsimony analyses were performed with the program PAUP* version 4.0b8 (Swofford, 2000) using heuristic searches with random taxon-addition sequences, max trees undefined, and tree bisection-reconnection with unordered, equally weighted characters with 100 analysis replicates. Indels were treated as missing data, and indel regions lacking variable data were



removed. ITS data were easily aligned in conserved regions, but ITS regions with highly ambiguous alignment (84 bp from nine regions) were removed from the data set when necessary (Appendix S2; see Supplemental Data accompanying online version of this article). Standard measures of homoplasy, such as consistency index (CI), retention index (RI), and rescaled consistency index (RC) excluding uninformative characters, and the level of internal support (bootstrap values) were calculated using PAUP* 4.0b8. Bootstrap analyses were conducted using 200 replicate heuristic searches as indicated earlier with max trees set at 10000.

Bayesian analyses were performed using the program MrBayes version 3.0B4 (Huelsenbeck and Ronquist, 2001). The nrDNA ITS, *trnK*, and *matK* data sets were examined initially to determine the best-fit DNA substitution model using the program MrModeltest version 1.1b (Nylander, 2003). The *matK* data set was analyzed with each codon position representing a separate data set and each having an individual best-fit model. Initially, two separate analyses were performed: (1) nrDNA ITS and (2) cpDNA *trnK* and *matK*. Subsequently, the nrDNA and cpDNA data sets were combined. Bayesian analyses were performed twice for 3.0×10^6 generations using the best-fit model for each character partition. Trees were sampled every 1000 generations. Stability of the process was assessed by plotting model $-\ln$ likelihood scores against generations to determine equilibrium. Trees sampled before reaching equilibrium were discarded as burn in, with the remaining trees used to generate a 50% majority rule consensus tree where the percentage of the nodes recovered represented each node's posterior probability. Trees recovered from each individual run then were compared for topology and posterior probability to further determine consensus among analyses of each of the three data sets analyzed. Nodal support was determined using Bayesian posterior probabilities (PP) > 0.95 as the threshold criterion for strong support.

Incongruence—Congruence of the ITS and cpDNA data sets was tested using the incongruence length difference (ILD) test as implemented in PAUP*. The nrDNA ITS and cpDNA data sets were analyzed using 1000 homogeneity replicates with heuristic searches as described earlier under parsimony analysis. Incongruence also was determined visually for trees with incongruent topologies between different data sets (nrDNA vs. cpDNA). If incongruence was detected, the conflicting branches were evaluated individually for relative support given parsimony bootstrap and Bayesian posterior probabilities. Eventually, the data were combined regardless of the outcome of the ILD test (see Discussion).

Analysis of character evolution—The habit and merosity data were compiled from several literature sources (Schindler, 1905; Orchard, 1975, 1979, 1981, 1985, 1990). Floral merosity was scored as 2, 3, 4, or 5 and habit as terrestrial, aquatic, or semiaquatic. The five most likely trees recovered from the combined data Bayesian analysis were evaluated for topology most closely matching that of the majority rule consensus tree. Character states were then optimized on the best-fit phylogenetic hypothesis so that relative branch lengths, as determined by Bayesian analysis, could be incorporated for ML analysis of ancestral states. Ancestral state optimization was performed using both parsimony and likelihood methodologies implemented in the program Mesquite version 1.04 (Maddison and Maddison, 2003). We choose to use a one-rate model following the observations of Mooers and Schluter (1999). The maximum likelihood model used for the analysis of the morphological data was Mk1 (Lewis, 2001).

RESULTS

The nrDNA ITS data set consisted of 681 bp of aligned sequence, with 84 sites removed because of ambiguous alignment. There were 274 variable characters, with 229 being potentially parsimony informative (including outgroups). The

cpDNA *trnK* 5' intron comprised 665 bp of aligned sequence data of which 290 sites were variable and 173 potentially parsimony informative; the *trnK* 5' intron was not recovered for *Tetracarpaea tasmanica* Hook.f. The *matK* data set included 1506 bp of aligned sequence data of which 694 sites were variable and 425 parsimony informative. The *trnK* 3' data set had 127 bp of aligned sequence data of which 72 sites were variable and 53 potentially parsimony informative; the *trnK* 3' intron was not recovered for *Haloragis acutangula* F.Muell., *H. serra* Brongn., *Myriophyllum* sp., or *Tetracarpaea tasmanica*.

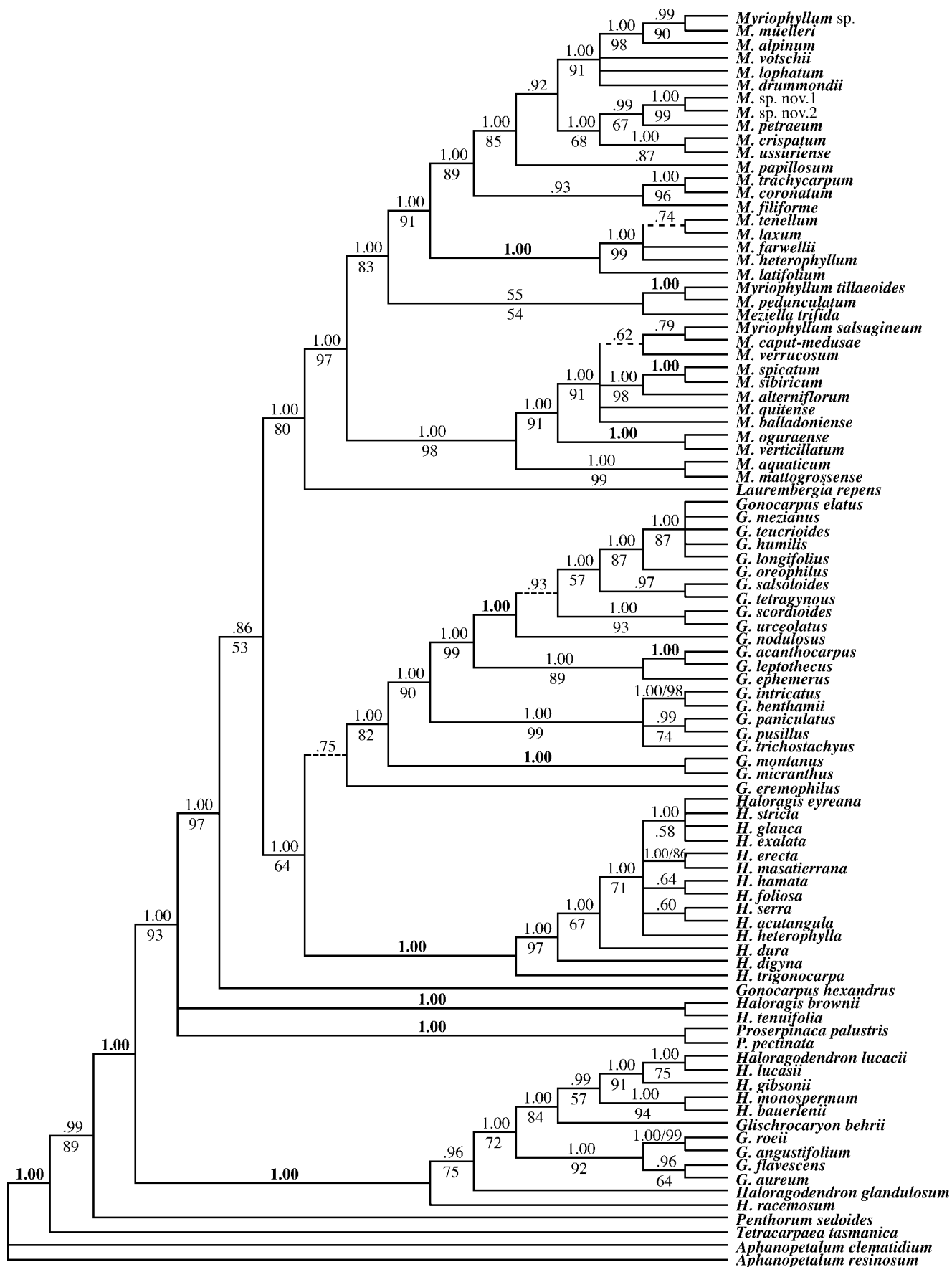
Parsimony results—Parsimony analysis of ITS recovered five islands with a total of 12 182 most parsimonious trees with a score of 1746 steps (CI = 0.37, RI = 0.76, RC = 0.28). Parsimony analysis of cpDNA data (*trnK* + *matK*) resulted in 87 255 equally parsimonious trees of 1860 steps (CI = 0.61, RI = 0.86, RC = 0.52). Parsimony analysis of the combined data resulted in 28 812 equally parsimonious trees of 3706 steps (CI = 0.48, RI = 0.80, RC = 0.36). Parsimony bootstrap scores are displayed on the results of Bayesian analyses of the respective data sets (Figs. 1–3).

Bayesian results—Posterior probability distributions of 3000 sampled trees were obtained for each Bayesian analysis using best-fit ML models (Table 2). In all cases, the two separate analysis runs converged on similar likelihood scores for each of the four data sets examined after less than 1 million generations. Visual comparison of the majority consensus trees from the two separate runs for each data set disclosed no major discrepancies between tree topology or posterior probability nodal support. Final trees represented the majority rule consensus trees of 2800 trees, conservatively discarding the first 200 (2.0×10^6 generations) as burn-in for each individual data set (Figs. 1–3) with average, maximum, and minimum likelihood scores listed in Table 2. Bayes branch lengths are represented on phylograms based on the trees with the best likelihood score obtained from analysis of each data set (Fig. 4a–c).

Incongruence—The ILD tests indicated significant differences between the nrDNA ITS and cpDNA data partitions ($P < 0.005$). ITS and cpDNA trees also were visually evaluated for incongruent relationships that were well supported in both parsimony and Bayesian analyses between ITS and cpDNA results. Strong support was provided for some nodes that were incongruent between phylogenetic hypotheses based on ITS and cpDNA data sets. The strong support for conflicting results was sometimes provided by posterior probabilities supporting short branches (Fig. 4a–c) or with relatively low parsimony bootstrap support (Figs. 1, 2). These examples are discussed further in the next section.

Phylogenetic results—There was some conflict in phylogenetic hypotheses generated using the different data sets. Bayesian analysis of nrDNA (Fig. 1) resulted in a polytomy

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Fig. 1. Phylogenetic relationships in Haloragaceae as indicated by a majority rule consensus tree of 28 000 trees (after discarding burn-in) from Bayesian analysis of ITS sequence data analyzed under a GTR+I+Γ model. Numbers above branches refer to posterior probabilities, and numbers below branches are bootstrap support from parsimony analyses. Branches represented by dashed lines are not found in the strict consensus trees from the parsimony analysis of the same data set. Numbers in bold above branches have the same values in both parsimony and Bayesian analyses. When numbers are lacking below solid branches, parsimony had bootstrap values < 50 supporting that node.



of three clades, including *Proserpinaca*, *Haloragis brownii*-*H. tenuifolia*, and all other Haloragaceae, whereas parsimony analyses resolved with weak support (BS < 50) *Proserpinaca* sister to all other Haloragaceae followed by *Haloragis brownii*-*H. tenuifolia* sister to the rest of the family (not shown). The cpDNA (Fig. 2) supported *Glischrocaryon-Haloragodendron* as sister to the rest of Haloragaceae (PP = 1.0; BS = 93). The combined data also resolved the latter hypothesis (PP = 1.0; BS = 52; Fig. 3). In all cases (Figs. 1–3), *Haloragodendron* and *Glischrocaryon* formed a well-supported clade. All data sets supported the placement of *Haloragis brownii* and *H. tenuifolia* as a clade that was not allied with other *Haloragis* but was part of a basal or near basal grade containing *Glischrocaryon-Haloragodendron* and *Proserpinaca*. *Gonocarpus hexandrus* was not allied with other *Gonocarpus* in any analysis. Although its position was not well supported in any analyses, combined Bayesian analyses resolved a weakly supported sister relationship to the *Haloragis-Gonocarpus* + *Myriophyllum* clade (Fig. 3).

Haloragis (here forward excluding *H. brownii*-*H. tenuifolia*) and *Gonocarpus* (here forward excluding *G. hexandrus*) formed a well-supported clade in all analyses and were sister to a *Laurembergia-Meziella-Myriophyllum* clade in the cpDNA and combined analyses. Analyses of ITS resolved these same relationships but weakly supported *G. hexandrus* (Fig. 1) sister to *Laurembergia-Meziella-Myriophyllum*. *Haloragis* was monophyletic in all analyses (Figs. 1–3). *Gonocarpus* was monophyletic in cpDNA and combined analyses. Analyses of ITS identified a paraphyletic *Gonocarpus*. *Gonocarpus eremophilus* Orchard, *G. micranthus* Thunb., and *G. montanus* (Hook.f.) Orchard formed a poorly supported basal grade to *Haloragis*. A clade including *Laurembergia*, *Meziella*, and *Myriophyllum* was present in all analyses, with *Laurembergia* sister to *Myriophyllum-Meziella*. ITS resolved a weakly supported sister group relationship of *Meziella* to *Myriophyllum* in the Bayesian analysis (PP = 0.89), a hypothesis not resolved by parsimony (Fig. 1). Analyses of combined data and cpDNA supported *Meziella* as part of a monophyletic *Myriophyllum*. Within *Myriophyllum*, two major clades were supported in the combined data analyses: (1) mostly Australian and North American endemics including *Meziella* and (2) South American taxa and a geographically disparate alliance of *Myriophyllum* (Fig. 3).

Within *Haloragis*, *H. trigonocarpa* was sister to the rest of the genus as supported by cpDNA and combined data, whereas ITS results were equivocal in the Bayesian analysis. Short branches and weak support were resolved for other relationships within this genus, although combined analyses did provide phylogenetic support for some groupings (Fig. 3). In all analyses, *H. erecta* (Banks ex. Murray) Oken and *H. masatierrana* resolved as a well-supported clade nested within *Haloragis*. Several clades within *Gonocarpus* were recovered consistently using different data sets. A sister group relationship between *G. montanus* and *G. micranthus* was well

supported, and they were sister to the rest of *Gonocarpus* (less *G. eremophilus*) using the cpDNA and combined data. Bayesian analysis of ITS depicted these taxa basal to *Gonocarpus-Haloragis* with weak support (Fig. 1). Within *Gonocarpus*, branching after *G. micranthus*-*G. montanus*, was the “benthamii clade” (see Fig. 3) containing five taxa endemic to Western Australia. Branching next was the “acanthocarpus clade” (*G. acanthocarpus* (Brongn.) Orchard, *G. ephemerus* Orchard, *G. leptothecus* (F.Muell.) Orchard; Fig. 3) using the cpDNA and combined data. *Gonocarpus acanthocarpus* and *G. leptothecus* were well supported as sister taxa in all data sets, but were placed basal to the “benthamii clade” with weak support using the Bayesian analyses of ITS (Fig. 1). *Gonocarpus ephemerus* was part of the “acanthocarpus clade” using the cpDNA and combined data, but this relationship was not supported using ITS alone. The “tetragynus clade” (see Fig. 3) was resolved in all analyses, although relationships within this clade were inconsistent.

Character analyses—The parsimony reconstruction of ancestral character states was equivocal for aquatic habit (Fig. 5). There were either three independent origins of the aquatic habit at nodes 2 (*Haloragis brownii*-*H. tenuifolia* [= *Meionectes*]), 3 (*Proserpinaca*), and 6 (*Laurembergia-Meziella-Myriophyllum*) or two independent origins of the aquatic habit at nodes 1 (*Haloragis brownii*-*H. tenuifolia*) and 6 (*Laurembergia-Myriophyllum*) with an intermediate reversal back to the terrestrial habit beginning with node 3 (*Gonocarpus hexandrus* [= *Trihaloragis*]). The first hypothesis was favored by the ML reconstruction of ancestral characters, but not significantly (Fig. 5).

Parsimony reconstruction of ancestral character states favored a hypothesis of two independent origins of trimerous flowers from a tetramerous ancestor (Fig. 6), with the ancestral character trimerous at node 1 and with a reversal to tetramery at node 3 and a second origin of trimery at node 4. The ML reconstructions of ancestral characters also favored this hypothesis, although the possibility of four independent origins of trimerous flowers was not statistically rejected ($P < 0.05$; Fig. 6). Multiple reductions to dimerous flowers were resolved by both parsimony and ML reconstructions (Fig. 6).

DISCUSSION

Data and phylogenetic analyses—A primary concern in choosing data for these analyses was to incorporate genes that were informative at multiple taxonomic levels. In this case, relationships of the family Haloragaceae at the intergeneric and intrageneric level required genes with different evolutionary rates. The nrDNA ITS region is highly variable, and its use has been recommended when examining interspecific relationships while its utility for examining intergeneric relationships has varied widely depending on lineage (Baldwin et al., 1995). Its

←

Fig. 2. Phylogenetic relationships in Haloragaceae as indicated by a majority rule consensus tree of 28 000 trees (after discarding burn-in) from Bayesian analysis based on cpDNA *trnK* and *matK* data analyzed using individual DNA substitution models appropriate to each data partition (Table 2). Numbers above branches refer to posterior probabilities, and numbers below branches are bootstrap support from parsimony analyses. Branches represented by dashed lines are not found in the strict consensus trees from the parsimony analysis of the same data set. Numbers in bold above branches have the same values in both parsimony and Bayesian analyses. When numbers are lacking below solid branches, parsimony had bootstrap values <50 supporting that node.

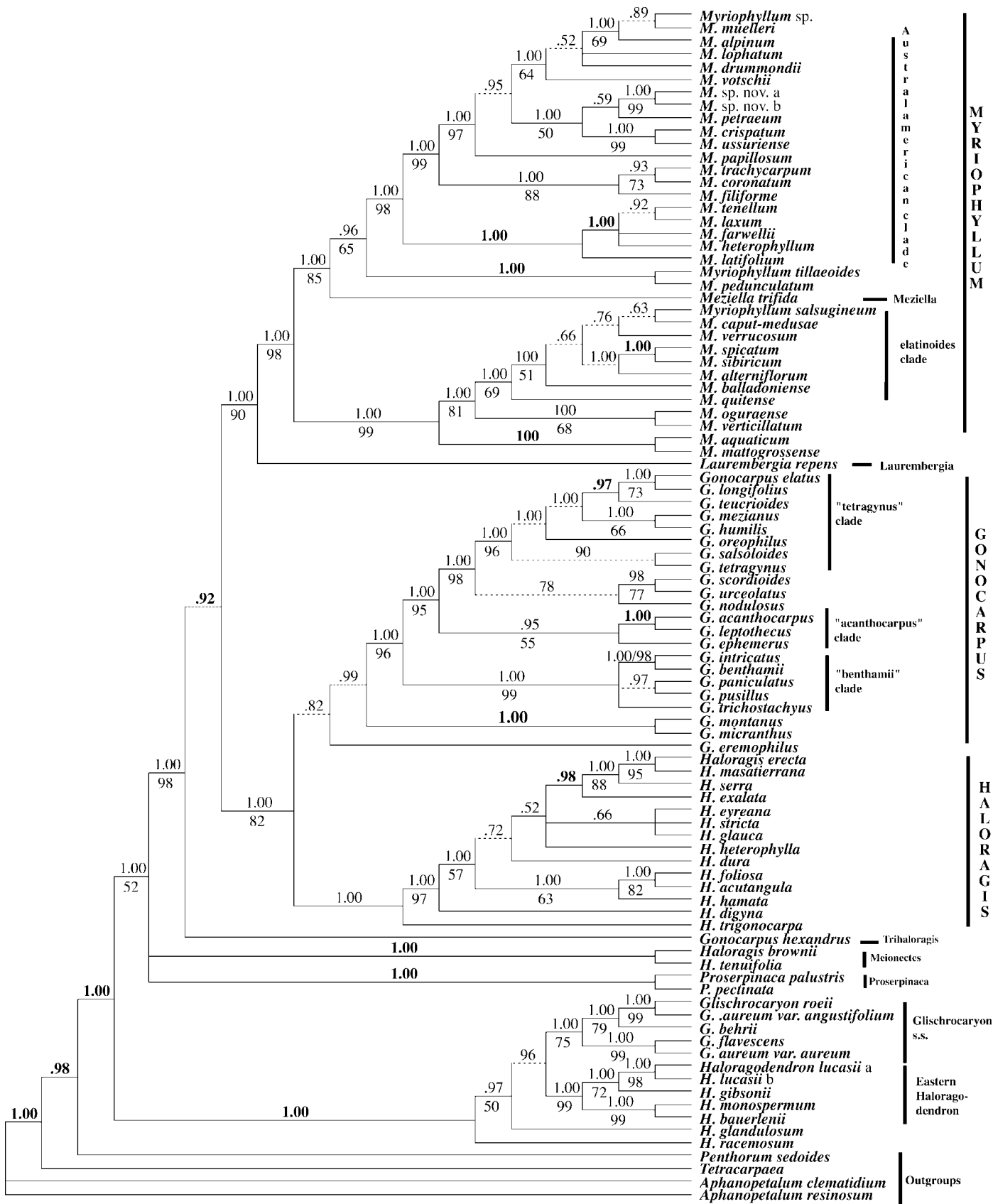


TABLE 2. Average likelihood parameters estimated for each of the data partitions in the Bayesian combined analysis (columns 2–7), cpDNA analysis (columns 8–12), and ITS analysis (column 13) after the first 2000 trees were discarded as burn-in. Average, maximum, and minimum $-\ln$ likelihood values are given in the final three rows corresponding to the data set analyzed (sequentially, combined data, cpDNA data, ITS data). The *matK* data were partitioned into first, second, and third codon positions (pos1, pos2, pos3).

	Combined data						cpDNA data alone					ITS data alone
	<i>trnK5</i>	<i>trnK3</i>	<i>matK</i> pos1	<i>matK</i> pos2	<i>matK</i> pos3	ITS	<i>trnK5</i>	<i>trnK3</i>	<i>matK</i> pos1	<i>matK</i> pos2	<i>matK</i> pos3	ITS
	GTR+I+ Γ	GTR+I+ Γ	GTR+ Γ	GTR+I+ Γ	GTR+ Γ	GTR+I+ Γ	GTR+I+ Γ	GTR+I+ Γ	GTR+ Γ	GTR+I+ Γ	GTR+ Γ	GTR+I+ Γ
No. sites	665	127	502	502	502	538	665	127	502	502	502	538
C>T	1.349	0.710	1.266	1.300	1.887	3.670	1.467	0.667	1.338	1.345	1.804	4.521
C>G	0.536	0.786	0.915	1.152	1.486	0.535	0.594	0.849	0.965	1.058	1.530	0.375
A>T	0.442	0.243	0.278	0.097	0.223	1.677	0.451	0.233	0.304	0.118	0.223	2.057
A>G	1.000	1.299	1.535	1.270	2.065	2.066	1.022	1.397	1.602	1.291	2.103	1.795
A>C	0.762	1.179	1.148	0.649	1.631	0.922	0.845	1.227	1.221	0.694	1.617	0.890
A	0.346	0.353	0.278	0.323	0.306	0.203	0.349	0.351	0.277	0.317	0.307	0.196
C	0.154	0.169	0.208	0.186	0.139	0.306	0.147	0.163	0.204	0.191	0.131	0.319
G	0.185	0.169	0.182	0.148	0.143	0.270	0.185	0.154	0.182	0.154	0.134	0.283
T	0.315	0.309	0.333	0.343	0.412	0.220	0.319	0.332	0.337	0.338	0.428	0.203
α	0.742	8.982	0.659	0.559	1.405	1.084	1.071	11.13	0.924	1.006	1.573	1.037
p(inv)	0.181	0.182	—	0.196	—	0.661	0.214	0.252	—	0.202	—	0.297
Avg.	—ln 25216.700						—ln 15144.805					—ln 9345.921
Min.	—ln 25282.749						—ln 15188.134					—ln 9405.853
Max	—ln 25167.067						—ln 15126.596					—ln 9300.528

high variability may be problematic when the data are applied singly at higher phylogenetic levels (e.g., Baldwin et al., 1995; Soltis and Kuzoff, 1995; Kim and Jansen, 1996), especially when there has been little divergence time between multiple lineages at deep nodes (Donoghue and Sanderson, 1995; Seelanen et al., 1997). Because the cpDNA *matK*-coding region evolves at a slower rate, this locus was expected to provide a more accurate picture of higher-level relationships, especially intrageneric relationships, although we also expected that some informative characters would facilitate elucidation of lower-level relationships, especially when including the *trnK* introns. Not surprisingly, the ITS data set and cpDNA data set provided some incongruent results when treated separately at levels less appropriate for each given data set, and they have very different likelihood model parameters (Table 2). At the family level, ITS displayed high homoplasy (CI = 0.37), a result that proved, as would be expected, to be much more moderate at lower phylogenetic levels (see Table 3). Also, branches along the backbone of the phylogeny were relatively short and had little support, especially under parsimony bootstrap (Figs. 1 and 4c). The cpDNA analyses also resolved short branches along the backbone of the phylogeny, but homoplasy was much lower (CI = 0.67), and nodal support was strong under both parsimony and Bayesian analyses for the resolved relationships.

Incongruence length difference (ILD) tests found incongruence between the ITS and cpDNA data. Both empirical and simulated data (Dolphin et al., 2000; Yoder et al., 2001; Barker and Lutzoni, 2002) have demonstrated that this test has recommended noncombinability of data even where data

performed better when partitions were combined. The ILD test does appear to be conservative with low susceptibility to type II error, thus it is a simple way to examine data partitions initially if congruence is not rejected. Of course, when congruence is rejected, a number of reasons involving variation in rates of molecular evolution between data sets may lead to a rejection of combinability (Dolphin et al., 2000; Darlu and Lecointre, 2002; Barker and Lutzoni, 2002). Variable evolutionary rates among data sets can be problematic when combining data, but Bayesian analyses with case appropriate ML models fit to individual partitions of data can help alleviate many of these problems (Nylander et al., 2004). Thus, a compromise is not needed to decide whether to combine data based on differing models of evolution among partitions (Bull et al., 1993; Chippindale and Wiens, 1994). We used this approach in all combined data analyses.

As discussed earlier, incongruence may also exist between the nuclear and plastid genomes as a consequence of hybridization or lineage sorting. In the case of incongruence between data sets, each case was examined individually, and interpretation of the combined data analyses includes discussion of incongruence where appropriate.

Phylogenetic hypotheses—*Penthorum* and *Tetracarpaea*—The position of *Penthorum* and *Tetracarpaea* as part of Haloragaceae s.l. has been suggested based solely on results of recent large-scale phylogenetic analyses in which these disparate genera were found to ally as a grade in a clade including Haloragaceae and Aphanopetalaceae (Fishbein et al., 2001; APG II, 2003). Morphological similarities were not

Fig. 3. Phylogenetic relationships in Haloragaceae as indicated by a majority rule consensus tree of 28 000 trees (after discarding burn-in) from Bayesian analysis based on ITS and cpDNA combined data analyzed using individual DNA substitution models appropriate to each data partition (Table 2). Numbers above branches refer to posterior probabilities, and numbers below branches are bootstrap support from parsimony analyses. Branches represented by dashed lines are not found in the strict consensus trees from the parsimony analysis of the same data set. Numbers in bold above branches have the same values in both parsimony and Bayesian analyses. When numbers are lacking below solid branches, parsimony had bootstrap values <50 supporting that node.

C. ITS DATA



Fig. 4. Continued.

was a high level of homoplasy in ITS (CI = 0.37; Table 3) at this deep phylogenetic level along with the weak parsimony bootstrap support (<0.50) along the backbone of the phylogenetic hypothesis (Fig. 1). Additionally, strict consensus of parsimony trees one step longer than the best trees (1747 steps) results in a polytomy of four clades (*Haloragodendron-Glischrocaryon*, *Haloragis brownii-H. tenuifolia*, *Proserpinaca*, and all other Haloragaceae). In contrast, the cpDNA provided strong support for *Haloragodendron-Glischrocaryon* as sister to all other Haloragaceae (BS = 93; PP = 1.0) with much lower homoplasy (CI = 0.67).

It is noteworthy that when the data sets are combined, Bayesian analysis resolves *Glischrocaryon-Haloragodendron* sister to all other Haloragaceae with a strong posterior probability (PP = 1.0) but weak parsimony bootstrap (BS = 52; Fig. 3), thus resolving the same phylogenetic hypothesis as the cpDNA taken alone (Fig. 2). Posterior probability has been demonstrated to represent a less biased estimator of confidence than BS (Wilcox et al., 2002; Erixon et al., 2003), even if the support provided by PPs may represent a more liberal estimate of branch support, leading to the potential for disproportionately high support for some branches (Suzuki, 2002). Recent evidence also has shown that PP scan assign high confidence to

extremely short, incorrectly inferred branches (Alfaro et al., 2003); thus, branch length is important to consider in interpreting the high PP support provided for the contrasting results between the combined and ITS analyses for the basal nodes (Fig. 4a, c). Given the higher quality of the cpDNA data for this level of phylogeny (based on measures of homoplasy; see Table 3), we consider that the resolution and branch support provided by cpDNA in the parsimony and Bayesian analyses and the strongly supported resolution of *Haloragodendron-Glischrocaryon* as sister to all Haloragaceae in the Bayesian analysis of the combined data analyses is the most likely (Figs. 2, 3). Perhaps the incorporation of additional, more slowly evolving, single-copy nuclear markers would provide a better understanding of higher-level relationships in Haloragaceae.

Glischrocaryon-Haloragodendron—The monophyly of *Haloragodendron-Glischrocaryon* is well supported, but monophyly of two distinct genera is not supported in all data sets (Figs. 1–3). ITS and cpDNA analyses yield conflicting phylogenetic hypotheses. ITS strongly supports both a monophyletic *Glischrocaryon* and *Haloragodendron*, whereas the cpDNA supports paraphyly of *Haloragodendron* in respect to *Glischrocaryon*. The incongruence between data sets cannot be easily reconciled. Shallow basal branches are resolved for this clade (Fig. 4a, b) by both data sets, perhaps suggesting a rapid radiation (Fishbein et al., 2001; Donoghue and Sander-son, 1992). The combined data also suggest that *Haloragodendron* is paraphyletic in relation to *Glischrocaryon* (Fig. 3), but nodal support, especially parsimony bootstrap values, is reduced for basal relationships in this clade indicating the incongruence may have a biological basis (i.e., ancient hybridization, lineage sorting) and needs to be explored further.

Morphology also supports the close relationship of *Haloragodendron* and *Glischrocaryon* but does not provide a clear view of generic limits. Orchard (1975) pointed out the strong resemblance of fruit characters between the two, both having a single seed develop (from four ovules) and occupy the entire fruit. Praglowski (1970) emphasized that the pollen morphology of *Haloragodendron* (= *Haloragis* sect. *Pleianthus* subsect. *Spongiocarpus* in part) and *Glischrocaryon* was nearly identical and differed from that of all other Haloragaceae, leading him to suggest their merger. *Glischrocaryon* species are distinct from *Haloragodendron* based on vegetative morphology. They have annual green, round stems and often highly reduced, alternate leaves (i.e., characteristics associated with the arid habitats [excluding alternate leaves] in which these taxa are found). In contrast, species assigned to *Haloragodendron* have perennial brown-red, four-angled stems with relatively large opposite leaves. Other Haloragaceae genera (i.e., *Haloragis* and *Gonocarpus*) have both alternate- and opposite-leaved members, suggesting the ambiguity of this characteristic in defining generic limits in the family. Given the conflict between molecular analyses, further sequence data are needed to evaluate these inconsistencies before these generic limits can be confidently resolved; thus, the circumscription of these genera remains tentative.

Incongruence between cpDNA and ITS phylogenetic hypotheses is also found regarding the dimerous *Glischrocaryon behrii*. ITS supports its position as part of a clade including *G. angustifolium-G. roei*, whereas cpDNA places it as paraphyletic to other *Glischrocaryon* and sister to the “eastern *Haloragodendron*” (Figs. 1–3). *Glischrocaryon behrii*

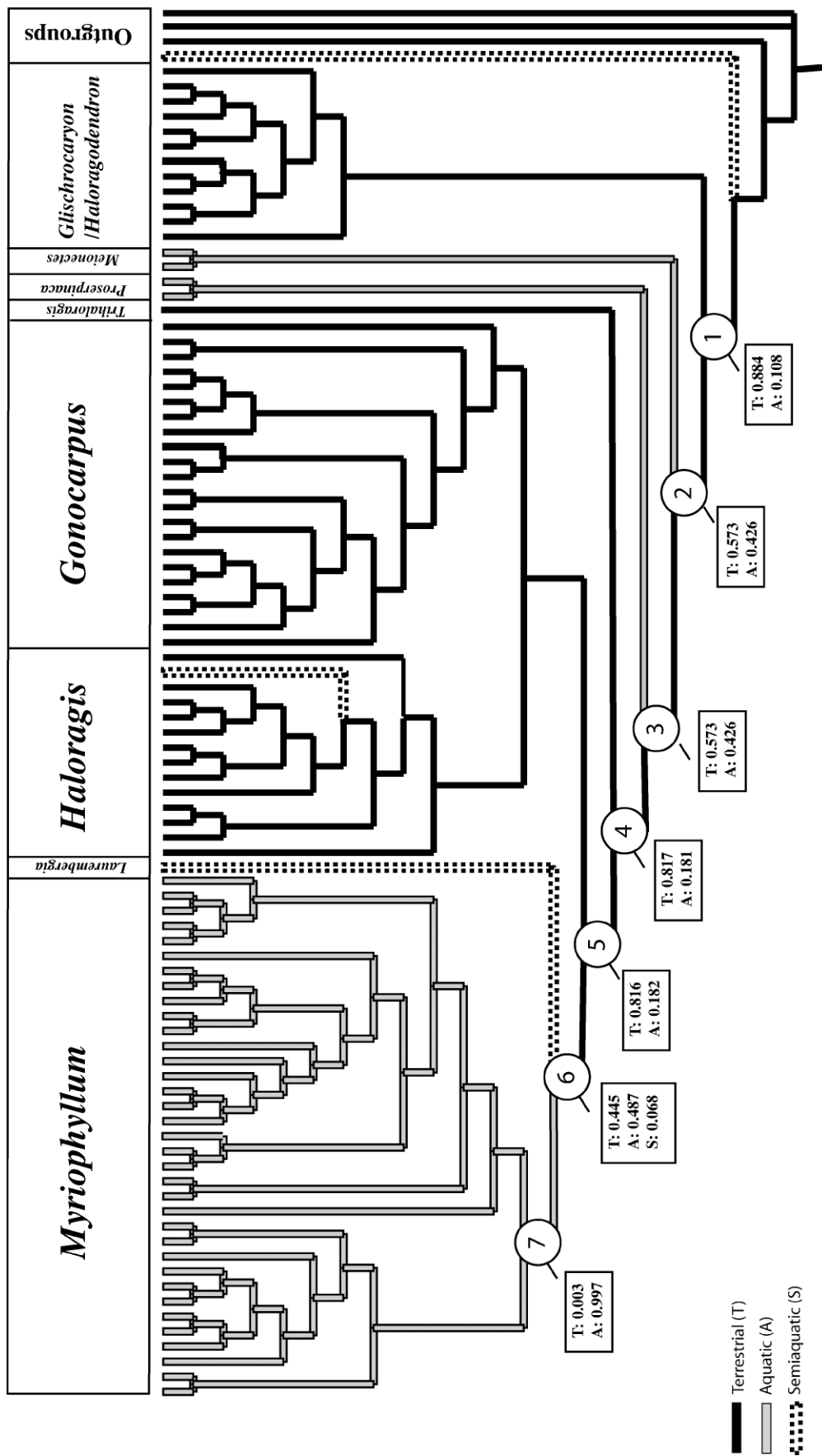


Fig. 5. Maximum likelihood reconstructions of ancestral habit character states in Haloragaceae. Semiaquatic (S), aquatic (A), and terrestrial (T) habits were treated separately on the best-fit (second best likelihood score) tree from the combined ITS and cpDNA data analyses to account for branch lengths. Branches are: solid black (terrestrial), black outlined (aquatic), dashed (semiaquatic). Ambiguous ancestral character states at the nodes of interest are indicated by a circle. Aquatic, semiaquatic, and terrestrial habit transitions are indicated in the boxes by each numbered node according to the maximum likelihood estimations of ancestral states.

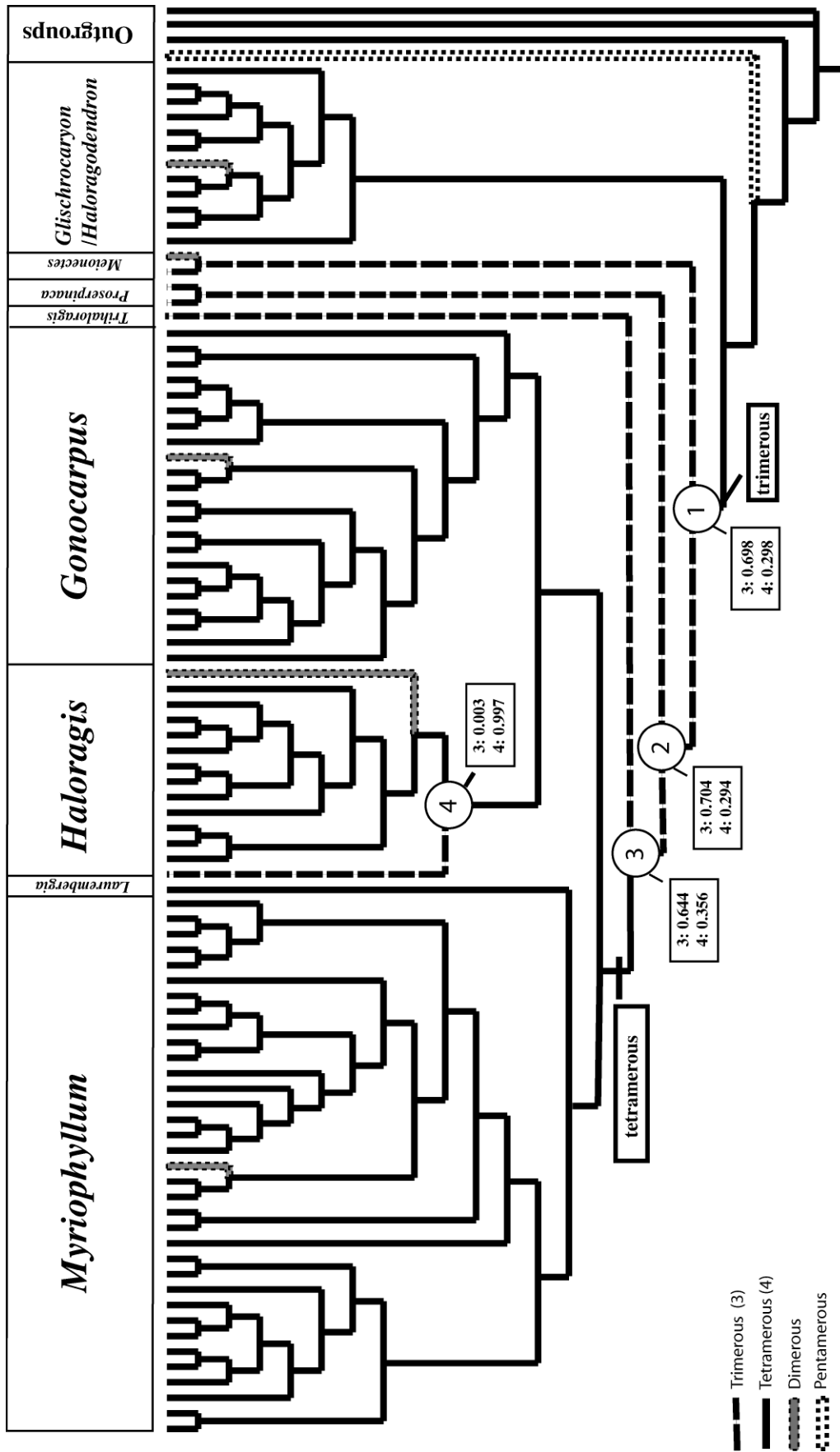


Fig. 6. Maximum likelihood reconstructions of ancestral floral character states in Haloragaceae. Dimerous, trimerous, tetramerous, and pentamerous flowers were treated separately on the best-fit (second best likelihood score) tree from the combined ITS and cpDNA data analyses to account for branch lengths. Branches are represented as black dashed-outline (dimerous), long solid-dashed (trimerous), solid (tetramerous), and short-dashed (pentamerous). Ancestral character states are indicated in the boxes by each pertinent numbered node along with the maximum likelihood probabilities for ancestral states denoted as trimerous (3) or tetramerous (4).

TABLE 3. Consistency indices (CI) as a measure of homoplasy for nrDNA ITS and cpDNA *trnK* + *matK* data at different levels of phylogenetic inference. All data were included initially; then, branches were pruned gradually to include fewer taxa at varying levels of the phylogeny (taxa referred to as clades identified in Fig. 3).

Taxonomic comparisons	ITS (CI)	cpDNA (CI)
All data	0.37	0.60
Without outgroups	0.39	0.61
Without outgroups + <i>Proserpinaca</i> + <i>Meionectes</i>	0.40	0.61
Without above + <i>Glischrocaryon</i> + <i>Haloragodendron</i>	0.43	0.63
Without above + <i>Myriophyllum</i> + <i>Laurembergia</i>	0.62	0.86
<i>Gonocarpus</i> only	0.71	0.90
<i>Haloragis</i> only	0.88	0.93
<i>Glischrocaryon</i> + <i>Haloragodendron</i> only	0.82	0.94

is the only member of *Glischrocaryon* that ranges into eastern Australia. Its sister group relationship to the “eastern *Haloragodendron*” (Fig. 2) may reflect an evolutionary split between this clade and *Glischrocaryon*. The combined analysis supports placement of *G. behrii* with *Glischrocaryon*, perhaps reflecting the strong support given by ITS and the relatively short branches resolved for the cpDNA hypothesis (Fig. 4b).

Given our inclusive sampling of *Glischrocaryon* and *Haloragodendron*, some discussion of intergeneric relationships is warranted. All data sets resolve a strongly supported “eastern *Haloragodendron*” (Figs. 1–3), which are geographically disjunct from the western species (*H. glandulosum* and *H. racemosum*). A close relationship between *Glischrocaryon flavescens* (Drumm.) Orchard and *G. aureum* is also well supported by all data sets, a relationship predicted by Orchard (1975). We collected multiple samples of the two varieties of *Glischrocaryon aureum* (Lindl.) Orchard as part of this study. The more widespread *G. aureum* var. *angustifolium* (Nees) Orchard is distinct from *G. aureum* var. *aureum* at the molecular level (Figs. 1–4) and is strongly supported as the sister to *G. roei* (cpDNA and combined data) or *G. roei* + *G. behrii* (ITS). Orchard (1975) had considered *G. aureum* var. *angustifolium* as a link between *G. aureum* var. *aureum* and *G. roei* Endl. It shares vegetative and habitat features with *G. roei* but lacks its highly inflated fruits. Conversely, *G. aureum* var. *angustifolium* possesses the winged fruits found in *G. aureum* var. *aureum*. Orchard suggested *G. aureum* var. *angustifolium* may have arisen through hybridization between *G. aureum* and *G. roei*, and our data do not preclude this possibility. Given the morphological and phylogenetic evidence, we propose that *Glischrocaryon angustifolium* (= *G. aureum* var. *angustifolium*: Table 5) should be recognized as a distinct species.

We also have detected natural hybridization between *Glischrocaryon roei* and *G. angustifolium*. A *Glischrocaryon* accession (Moody 395, CONN; Appendix 1) from Western Australia had the distinctly inflated fruits of *G. roei* but the

distinctly winged fruits of *G. angustifolium*. ITS evidence showed polymorphisms at all nucleotide sites that varied between *G. roei* and *G. angustifolium*. Because ITS is inherited biparentally, the ITS copies of *G. roei* and *G. angustifolium* appearing together in this single specimen confirmed its hybrid status, perhaps explaining some of the taxonomic uncertainty described by Orchard (1975). This result provided further genetic evidence of hybridization in the family outside of *Myriophyllum* (Moody and Les, 2002). Putative *Glischrocaryon* hybrids also have been reported on Kangaroo Island off the coast of South Australia (Orchard, 1975, 1990) where *Glischrocaryon* with trimerous flowers were growing in sympatry with the dimerous *G. behrii* and tetramerous *G. angustifolium*, providing circumstantial evidence of hybridization that should be examined further using molecular markers.

Proserpinaca and *Meionectes*—*Proserpinaca* and *Haloragis brownii*-*H. tenuifolia* are closely related yet distinct from each other and retain a basal branching position relative to most other Haloragaceae in all phylogenetic hypotheses (Figs. 1–3). These taxa are disjunct; *Proserpinaca* is found only in the New World (South America to Canada), whereas *Haloragis brownii* and *H. tenuifolia* are Australian endemics. The fossil record shows that *Proserpinaca* once had a much wider range, possibly having a circumboreal distribution as early as the Pliocene (Katz et al., 1965; Praglowski, 1970; Huckerby and Oldfield, 1976). Although the precise placement of *Haloragis brownii*-*H. tenuifolia* in relation to *Proserpinaca* and *Glischrocaryon*-*Haloragodendron* remains uncertain, the monophyly of *Haloragis brownii*-*H. tenuifolia* and the placement of this clade outside of *Haloragis* is clearly supported by all analyses (Figs. 1–3).

The aquatic habit of *H. brownii* and *H. tenuifolia* is uncommon in *Haloragis* (except for the helophyte *H. heterophylla*) as are the dimerous flowers of *H. brownii* and the trimerous flowers of *H. tenuifolia*. The trimerous (sometimes dimerous) perfect flowers and pinnatifid, submerged leaves of *H. tenuifolia* are similar to those in *Proserpinaca*. The most evident morphological differences between *Haloragis brownii*-*H. tenuifolia* and *Proserpinaca* are found in other floral characters. Whereas *Haloragis brownii* and *H. tenuifolia* have conspicuous, hooded petals and two whorls of stamens, *Proserpinaca* has only rudimentary petals and a single whorl of stamens, reductions that are also common elsewhere in the family. Brown (1814) recognized a monotypic *Meionectes* (= *M. brownii*), but most subsequent authors have synonymized *Meionectes* with *Haloragis* (Candolle, 1828; Schindler, 1905; Orchard, 1975, 1990). Based on the molecular evidence (Figs. 1–3) and the unique morphology of *Haloragis brownii*-*H. tenuifolia* in relation to other Haloragaceae, we reinstate the genus *Meionectes* H.Br., which we circumscribe as comprising solely these two species (see Table 5).

TABLE 4. Pairwise distances for combined ITS and cpDNA data, comparing *Penthorum* (the most closely related outgroup with sequence similarity as the criterion) to two basal branching Haloragaceae (*Haloragodendron*, *Proserpinaca*) and to other representative taxa in various phylogenetic positions in the family (see Fig. 3). Gh = *Gonocarpus hexandrus*, Gm = *Gonocarpus montanus*, Hb = *Haloragis brownii*, He = *Haloragis erecta*, Hr = *Haloragodendron racemosum*, Lr = *Laurembergia repens*, Ma = *Myriophyllum aquaticum*, Ps = *Penthorum sedoides*, Pp = *Proserpinaca palustris*.

Taxa	Ps	Hb	Pp	Hb	Gh	Gm	He	Lr	Ma
Ps	—	0.1087	0.1047	0.1064	0.1062	0.1191	0.1200	0.1305	0.1244
Hr	0.1087	—	0.0584	0.0459	0.0470	0.0722	0.0662	0.0793	0.0822
Pp	0.1047	0.0584	—	0.0491	0.0554	0.0629	0.0621	0.0734	0.0720

Gonocarpus—Various discrepancies exist in respect to the relationships proposed for *Gonocarpus* species in the treatments of Orchard (1975) and Schindler (1905). Notably, Schindler (1905) placed *Gonocarpus* in synonymy with *Haloragis*, yet all known species at the time (now included in *Gonocarpus*) were in section *Monanthus* of *Haloragis*, except *G. hexandrus*, *G. nodulosus* Nees, and *G. paniculatus* (R.Br. ex Benth.) Orchard (Appendix S1, see Supplemental Data in online version of this article). *Gonocarpus nodulosus* was placed within its own subgenus (*Pseudohaloragis*), and *G. hexandrus* was included in subsection *Trihalorrhagis* along with the aquatic *H. tenuifolia*, which has already been discussed. Orchard (1975) split *Gonocarpus* from *Haloragis* but did not interpret the genus in a phylogenetic context. Our combined data support a monophyletic *Gonocarpus* (excluding *G. hexandrus*) sister to *Haloragis*.

Although Orchard (1975, 1990) placed *G. hexandrus* within *Gonocarpus*, this was the only species for which he found no affiliation with other taxa in the genus. This species is unique among *Gonocarpus* with its trimerous flowers and racemose inflorescences (the last found otherwise only in *G. paniculatus*). In the final combined data analysis (Fig. 3), this taxon is sister to a clade of *Haloragis-Gonocarpus* + *Laurembergia-Meziella-Myriophyllum*. This relationship is inconsistent between cpDNA and ITS. Bayesian analysis of ITS weakly supported *G. hexandrus* as sister to *Laurembergia-Meziella-Myriophyllum* (PP < 0.95), whereas parsimony did not resolve this relationship (Fig. 1). Alternatively, cpDNA weakly resolved *G. hexandrus* as sister to *Haloragis-Gonocarpus* + *Laurembergia-Meziella-Myriophyllum* (also representative of the combined data). This taxon is clearly distinct phylogenetically from *Gonocarpus* and all other *Haloragaceae* genera (Figs. 1–3) and unique from all *Gonocarpus* taxa with trimerous flowers, thus we recognize it as the distinct, monotypic genus *Trihaloragis* (see Table 5). Three subspecies of *G. hexandrus* have been recognized (Orchard, 1975, 1990), and further studies of this poorly known taxon are warranted.

Although incomplete, our representative sampling of *Gonocarpus* allows for some discussion of relationships in the genus. All analyses resolve the sister relationship of *G. montanus* (Hook.f.) Orchard (= *H. depressa* var. *montana*) and *G. micranthus* (PP = 1.0). Although Orchard (1975) suggested that these taxa were only distantly related, both were included in subsection *Lamprocalyx* by Schindler (1905) because they share similar fruit types and large, deltoid calyx lobes. The “benthamii clade” is well supported (Figs. 1–3) and corresponds with Orchard’s (1975) hypothesis of a closely related Western Australia group (Appendix S1: node 1, group D). This clade includes *G. paniculatus* (the only racemose *Gonocarpus*), which Schindler (1905) placed in subsection *Spongiocarpus* among the taxa now included in *Haloragodendron* (Appendix S1). A sister relationship of *G. acanthocarpus* and *G. leptothecus* is well supported in all analyses, an expected result given their synonymous treatment as *G. acanthocarpus* by most authors prior to Orchard (1975).

A clade of several closely related taxa (here referred to as the tetragynus clade; Fig. 3) is also well supported in all analyses, but relationships among the species remain dubious. Before the description of *G. humilis* and *G. oreophilus* by Orchard (1975), herbarium specimens of the taxa from this clade typically were identified either as *G. tetragynus* or *G. teucioides*. Although these taxa appear to be closely related (as would be expected from morphology), they also are divergent at the molecular

level (Fig. 4). However, incongruence between phylogenetic hypotheses based on nuclear and chloroplast markers among these taxa may indicate a history of hybridization (Figs. 1–2). Forms intermediate between *G. elatus* and either *G. oreophilus* or *G. meizianus* (see Fig. 3) have been described (Orchard, 1975). For this study, extreme morphological forms of members of the tetragynus clade were used for molecular analyses. Forms with morphological intergradations among these taxa also were collected in the field and possessed various degrees of polymorphism at ITS nucleotide sites (Appendix S1). The polymorphic states reflected differences at sites that were clearly associated with the well-defined morphological taxa, which we interpreted as a consequence of occasional, recent hybridization among these taxa. To better understand the relationships among members of the tetragynus clade and their propensity for hybridization, additional studies with more intensive population-level sampling are needed.

Haloragis—Although species richness in *Haloragis* was reduced by splitting *Gonocarpus* and *Haloragodendron* Orchard (1975), the genus still displays wide morphological diversity among the >26 species remaining. Characters within *Haloragis* include alternate or opposite leaves, terrestrial or semiaquatic habit, two-, three- or four-merous flowers, and 2–4-loculed ovaries. All our data strongly support the monophyly of *Haloragis* (excluding *H. brownii-H. tenuifolia*). *Haloragis* can be distinguished from *Gonocarpus* by a host of morphological characters involving carpel septation, locule number, and many-flowered dichasia (Orchard, 1975).

There is little resolution and/or weak support for most intrageneric relationships in *Haloragis*, yet some are notably well supported and congruent among data sets. Combined data and cpDNA analyses agreed in resolving *H. trigonocarpa* as sister to all other *Haloragis*, whereas ITS analyses resulted in a basal polytomy (Fig. 1). *Haloragis trigonocarpa* is one of two trimerous species in *Haloragis*. The other species, *H. gossei*, was not sampled but recently has been considered closely related to *H. trigonocarpa*, the two differing only in minor fruit characteristics (Orchard et al., 2005). Also notable is the position of the helophytic *H. heterophylla* as nested within *Haloragis*, clearly demonstrating its independent origin from the aquatic *Meionectes* (= *H. brownii-H. tenuifolia*; Fig. 3).

Laurembergia-Meziella-Myriophyllum—*Laurembergia* is sister to *Meziella-Myriophyllum* in all analyses, and this relationship is well supported (Figs. 1–3). The helophytic *Laurembergia* differs from other aquatic taxa in the family by lacking a submerged form that is distinct from the terrestrial form. In its fruit and ovary structure, *Laurembergia* is strikingly similar to *Gonocarpus*, which was the affiliation favored by Orchard (1975). However, it is the only genus in the family besides *Myriophyllum* in which truly unisexual flowers are found, although functional monoecy has been described for several *Gonocarpus*.

A nested position of *Meziella* within *Myriophyllum* is well supported in the cpDNA and combined analyses. Its position is resolved as sister to all *Myriophyllum* in ITS Bayesian analyses with weak support (PP = 0.89), whereas parsimony did not resolve the relationship but included *Meziella* instead as part of a polytomy with two *Myriophyllum* clades. The strong support in the combined and cpDNA analysis for the inclusion of *Meziella* within *Myriophyllum* and lack of support for the ITS results brings into question its generic status.

TABLE 5. Phylogenetic classification proposed for Haloragaceae R. Br.

Haloragaceae R. Br.
<i>Gonocarpus</i> Thunb. (excluding <i>G. hexandrus</i>)
<i>Glischrocaryon</i> Endl. (including new species <i>G. angustifolium</i> (Nees) Moody & Les)
<i>Haloragis</i> J.R.Forst. & G.Forst. (excluding <i>Haloragis brownii</i> and <i>H. tenuifolia</i>)
<i>Haloragodendron</i> Orchard
<i>Laurembergia</i> P.J.Bergius
<i>Meionectes</i> R.Br. (= <i>Haloragis brownii</i> and <i>H. tenuifolia</i>)
<i>Meziella</i> Schindl.
<i>Myriophyllum</i> L.
<i>Proserpinaca</i> L.
<i>Trihaloragis</i> Moody & Les (= <i>Gonocarpus hexandrus</i>)

Meziella has been regarded as distinct from *Myriophyllum* based on a host of characters, but most conspicuously its four nutlets do not separate because of a persistent spiny calyx, whereas in *Myriophyllum* the nutlets lack the spiny calyx and separate at maturity (Orchard and Keighery, 1993). Within Haloragaceae, four woody nutlets form only within *Myriophyllum* and *Meziella*. Other characters used as evidence to support the unique generic status of *Meziella* (e.g., single whorl of stamens and apiculate stamens [Orchard and Keighery, 1993]) also occur in some *Myriophyllum* species (e.g., *M. mattogrossense* Hoehne and *M. decussatum* Orchard [not included in these analyses]). Molecular evidence indicates that its status as a distinct genus deserves further consideration. Given that the nrDNA data resolved *Meziella* sister to *Myriophyllum* (although with weak support), we suggest a conservative approach, to retain *Meziella*, pending the outcome of further analyses using more informative genomic DNA data.

Two well-supported clades are resolved within *Myriophyllum*, one comprising most of the endemic Australian and North American species and another comprising the South American species, circumboreal taxa (e.g., *M. sibiricum* Kom., *M. verticillatum* L.), and a group of several predominately southern hemisphere species once placed in synonymy with *M. elatinoides* (now part of the "elatinoides clade"; Fig. 3). Although our sampling of *Myriophyllum* provided a thorough coverage of the major lineages, which was sufficient to address the questions explored in this study, a more comprehensive survey of taxa has been conducted specifically to examine the systematics of the genus (Moody, 2004; M. Moody and D. Les, unpublished data) and will be discussed in detail in a subsequent treatment.

Character evolution—Inherent to the accuracy of our ancestral state reconstruction is the accuracy of our combined phylogenetic analyses. Some of the relationships resolved by the combined data are incongruent with our phylogenetic hypothesis based on ITS data alone, most importantly regarding the phylogenetic position of *Meionectes*, *Proserpinaca* and *Trihaloragis*. The well-supported resolution and agreement between cpDNA and combined phylogenetic hypotheses regarding deep nodes suggests that the hypotheses based on these data sets are the most reliable regarding ancestral character-state reconstruction for Haloragaceae. In some cases, relatively short branches were recovered regarding crucial relationships (i.e., *Trihaloragis*) for our ancestral character-state analyses (Fig. 4a) because of this ML ancestral

character state reconstructions, which take into account branch length, are particularly insightful (Figs. 5, 6).

Aquatic habit—The evolution of the aquatic habit is suspected to have at least 50 and perhaps upward of 100 separate origins among angiosperms (Cook, 1996). Although the advent of the aquatic habit is suspected to have evolved multiple times within core eudicot families (Cook, 1996), evidence supporting multiple origins of the submerged aquatic habit within a single core eudicot family has not yet been presented to the best of our knowledge. The transition from a terrestrial to aquatic habit, followed by a reversal to a terrestrial habit within angiosperms also appears not to have been addressed previously. A recent, extensive literature search uncovered only a single reference in which such reversals were proposed (Cook, 1999); however, no such hypothesis has yet been tested using a comparative phylogenetic approach.

Unfortunately, our results are equivocal as to whether a reversal from an aquatic to a terrestrial habit has occurred in Haloragaceae (Fig. 5). The ML analyses of ancestral characters yielded a higher probability in support of three origins of the aquatic habit (rather than two independent origins to aquatic habit with an intermediate reversal; Fig. 5), but the level of probability was not significant. Notably, species of *Proserpinaca* and *Meionectes* share the ability to survive terrestrial conditions for prolonged periods of time. Their retention of terrestrial characteristics such as rigid stems and leaves with cuticles and stomata could facilitate a transition back to land. It must be noted that a transition to an aquatic habit directly from woody, perennial *Haloragodendron-Glischrocaryon* ancestors (Fig. 5) may represent a more extreme shift, but whether transitional taxa may have existed but become extinct is unknown.

Our phylogenetic evidence does suggest at least two transitions to an aquatic habit in Haloragaceae (Fig. 5). One of the notable aspects concerning multiple aquatic origins in the family is the convergence of similar vegetative characteristics (adaptations to submersed conditions) among the aquatic genera. Most notable are the pinnate leaves in *Myriophyllum* and *Proserpinaca*. Although highly dissected leaves are common among aquatic taxa (Sculthorpe, 1967; Cronk and Fennessy, 2002), the morphology of a linear, central rachis with linear, parallel, lateral pinnae borne from the rachis is uncommon, yet it is a feature that appears to have evolved independently within Haloragaceae. *Meionectes brownii* and *M. tenuifolia* have a similar pinnate pattern in their submerged leaves but differ in having either multifid pinnae (*M. brownii*) or a flattened laminate rachis (*M. tenuifolia*). The convergent characteristic of an amphibious habit among the aquatic Haloragaceae genera also is noteworthy; an amphibious habit may well have facilitated the adaptation of *Myriophyllum* and *Meionectes* to Australia's severe climatic fluctuations.

Merosity—Recent phylogenetic analyses of the angiosperms have defined a well-resolved core eudicot clade (APG II, 2003). The early diverging eudicots have been recognized as having a high level of lability in floral merosity and floral form (Endress, 1994; Drinnan et al., 1994), a characterzation largely supported by ancestral character state reconstructions on phylogenetic hypotheses (Albert et al., 1998). In turn, the core eudicots appear to have a much less labile merosity with pentamery prominent (Endress, 1990; Drinnan et al., 1994;

Albert et al., 1998). Recent phylogenetic analyses of core eudicots suggest a sister-group relationship of Gunnerales to the core eudicots, leading to the hypothesis that the dimerous flowers found among Gunnerales and other eudicots represent a transitional merosity leading to pentamery in the core eudicots (Soltis et al., 2003).

Although tetramerous flowers are common among core eudicot families (e.g., Brassicaceae, Crassulaceae, Haloragaceae, etc.), dimerous and trimerous flowers are unusual. Haloragaceae are nested well within Saxifragales in the core eudicots, yet vary extremely in merosity. Dimerous flowers are found in *Glischrocaryon behrii*, *Meionectes brownii*, *Haloragis digyna*, and *Myriophyllum coronatum* Meijden. Soltis et al. (2003, p. 466) state, "In core eudicots there is sometimes variation between a pentamerous and tetramerous perianth merosity, but *there is not a dimerous perianth* (in contrast to the early-branching eudicots)" [emphasis ours], a statement that needs modifying. Although dimerous flowers may be uncommon in the core eudicots, evidence suggests that they have evolved by way of reductions multiple times within Haloragaceae (Fig. 6).

Trimerous flowers are uncommon among the core eudicots, although they are found among the unisexual flowers of Viscaceae and Fagaceae. Perfect trimerous flowers such as those found in Haloragaceae are even more uncommon (Judd et al., 2002). Both parsimony and ML ancestral character state reconstructions support a transitional pathway to trimerous flowers from tetramerous flowers with a reversal to tetramery (Fig. 6) rather than multiple reductions within *Haloragis* and *Gonocarpus* (Schindler, 1905; Orchard, 1975). Although it is indisputable that labile merosity characterizes the early-branching eudicots (Endress, 1994; Drinnan et al., 1994), lability of merosity among core eudicots may deserve further evaluation at the family level. Our results have shown Haloragaceae to be a clear example of highly labile merosity within the core eudicots with multiple origins of dimerous and trimerous flowers (Fig. 6).

Conclusions—A molecular phylogenetic approach toward the systematics of Haloragaceae has helped to resolve relationships within the family that previously were difficult to elucidate. Reinstatement of *Meionectes* and recognition of *Trihaloragis* provide new perspectives in the taxonomy and evolution of Haloragaceae. Genetic distances based on DNA sequence data and patterns of morphological character distributions combine to show a distant sister group relationship of Haloragaceae to *Penthorum* and *Tetracarpaea* and support a reevaluation of the option of merger of these genera with the family. Phylogenetic analysis of DNA sequence data indicates that the aquatic habit has evolved multiple times within Haloragaceae; however, a possible reversal from an aquatic to a terrestrial habit within the family cannot be rejected. A further assessment of the evolution of aquatic habit that couples phylogenetic information with developmental studies would be beneficial and might help to uncover patterns in the evolution of characteristics adaptive to the aquatic habit. Merosity is labile among Haloragaceae with dimerous and trimerous flowers, apparently having evolved multiple times within this angiosperm family, a pattern not predicted by the distribution of floral characteristics within other core eudicots. Comprehensive phylogenetic studies continue to be a vital tool in assessing character evolution.

Taxonomy—

Trihaloragis—M. L. Moody & D. H. Les, gen. nov. TYPE: *Haloragis hexandra* F. Muell., *Fragm.* 3. 1862. ≡ *Trihaloragis hexandra* (F. Muell.) M. L. Moody & D. H. Les.

Herba perennis vel suffrutex, caules 4-costati. Folia in partibus inferioribus opposita, superis alterna, lanceolata ad oblanceolata. Flores 3-meri, perfecti; sepala 3, deltoidea, viridia; petala 3, cucullata, viridia ad cremea; stamina 6; styli 3; ovarium viride, ovoideum, 3-costatum, incomplete 3-loculatum, septis solum in partibus inferioribus, loculis petalis oppositis, uniovulatis.

Perennial herb or subshrub, stems 4-ribbed. Leaves opposite basally becoming alternate in upper parts, lanceolate to oblanceolate. Flowers trimerous, perfect; sepals 3, deltoid, green; petals 3, hooded, green to cream; stamens 6; styles 3; ovary green, ovoid, 3-ribbed, incompletely 3-locular, septae present only in lower part, locules opposite the petals, one ovule per locule.

Trihaloragis hexandra—(F. Muell.) M. L. Moody & D. H. Les, comb. nov. Basionym: *Haloragis hexandra* F. Muell., *Fragm.* 3, p. 31. 1862. *Haloragis lanceolata* R. Br. ex Benth., *Fl. Aust.* 2. 1864. *Gonocarpus hexandrus* (F. Muell.) Orchard, *Tax. Rev. Fam. Halorag.* 1975. TYPE: Australia: "In locis uliginosis ad sinum Wilson's Inlet Novae Hollandiae austro-occidentalis." *Oldfield*, s.n. (holotype MEL!; isotype MEL!).

Glischrocaryon angustifolium—(Nees) M. L. Moody & D. H. Les, comb. et stat. nov. Basionym: *Loudonia aurea* var. *angustifolia* Nees in Lehm., *Pl. Preiss.* 1. p. 159. 1844. *Glischrocaryon aureum* var. *angustifolium* (Nees) Orchard, *Tax. Rev. Fam. Halorag.* 1975. TYPE: "In glareosis sterilibus districtus Hay m. Novembri a. 1840. Herb." *Preiss* 2079 (holotype B?: not located; isotype P!).

Meionectes tenuifolia—(Benth.) M. L. Moody & D. H. Les, comb. nov. Basionym: *Haloragis tenuifolia* Benth., *Fl. Aust.* 2, p. 477. 1864. Type: Australia, Drummond, 4th Coll. n. 86" (isotype MEL!).

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APPENDIX 1. Taxa used for molecular analyses. When multiple taxa are listed, these multiple specimens were sampled and sequenced for ITS. GenBank numbers follow taxonomic authority. An em dash (—) in place of data for a gene indicates missing data. Taxa for which DNA was extracted directly from herbarium specimens are represented by an asterisk (*) before the collector's name. ANBG—Australian National Botanic Gardens. HPG—Harold Porter Botanical Garden. Herbarium abbreviation as per Index Herbariorum.

Taxon—GenBank accession nos.: ITS, *trnK* 5' intron, *matK*, *trnK* 3'; *Voucher specimen* Herbarium, Source.

- Glischrocaryon angustifolium** (Nees) Moody & Les—EF178776, EF178958, EF179050, EF178866. *Moody* 393 CONN, WA, Australia; *Moody* 416 CONN, WA, Australia. **G. aureum** (Lindl.) Orchard—EF178779, EF178961, EF179053, EF178869. *Moody* 386 CONN, WA, Australia; *Moody* 422 CONN, WA, Australia. **G. behrii** (Schindl.) Orchard—EF178777, EF178959, EF179051, EF178867. *Moody* 436 CONN, Vic., Australia; ANBG, ACT, Australia. **G. flavescens** (Drumm.) Orchard—EF178778, EF178960, EF179052, EF178868. *Moody* 394 CONN, WA, Australia. **G. roei** Endl.—EF178775, EF178957, EF179049, EF178865. *Moody* 397 CONN, WA, Australia. **G. roei** × **G. angustifolium**, *Moody* 391 CONN, WA, Australia.
- Gonocarpus acanthocarpus** (Brongn.) Orchard—EF178757, EF178939, EF179031, EF178847. **Thompson* 187 NSW, QLD, Australia. **G. benthamii** Orchard—EF178756, EF178938, EF179030, EF178846. *Moody* 399 CONN, WA, Australia. **G. elatus** (A.Cunn. ex. Fenzl) Orchard—EF178752, EF178934, EF179026, EF178842. *Moody* 489 CONN (cult.) NSW, Australia. **G. ephemerus** Orchard—EF178754, EF178936, EF179028, EF178844. **Patrick* 3463 PERTH, WA, Australia. **G. eremophilus** Orchard—EF178769, EF178951, EF179043, EF178859. **Latz* 8808 NSW, NT, Australia. **G. hexandrus** (F. Muell.) Orchard—EF178759, EF178941, EF179033, EF178849. **Bright* 93 PERTH, WA, Australia; *Lepschi* 3360 PERTH, WA, Australia. **G. humilis** Orchard—EF178763, EF178945, EF179037, EF178853. *Moody* 429 CONN, NSW, Australia; *Moody* 487 CONN, NSW, Australia. **G. intricatus** (Benth.) Orchard—EF178753, EF178935, EF179027, EF178843. *Moody* 401 CONN, WA, Australia. **G. leptothecus** (F. Muell.) Orchard—EF178758, EF178940, EF179032, EF178848. *Martine* 803 CONN, NT, Australia. **G. longifolius** (Schindl.) Orchard—EF178772, EF178954, EF179046, EF178862. cult. ANBG, ACT, Australia; **James* s.n. NSW, Australia. **G. mezeianus** (Schindl.) Orchard—EF178760,

- EF178942, EF179034, EF178850. *Moody 442* CONN, NSW, Australia. *G. micranthus*, Thunb. —EF178771, EF178953, EF179045, EF178861. *Moody 479* CONN, NSW, Australia; *Moody 463* CONN, NSW, Australia. *G. montanus* (Hook. f.) Orchard—EF178770, EF178952, EF179044, EF178860. *Moody 448* CONN, NSW, Australia; *Moody 449b* CONN, NSW, Australia. *G. nodulosus* Nees—EF178762, EF178944, EF179036, EF178852. *Moody 396* CONN, WA, Australia. *G. oreophilus* Orchard—EF178751, EF178933, EF179025, EF178841. *Moody 490* CONN, cult. ACT, Australia. *G. paniculatus* (R. Br. ex Benth.) Orchard—EF178765, EF178947, EF179039, EF178855. *Moody 414* CONN, WA, Australia; *Moody 406* CONN, WA, Australia. *G. pusillus* (R. Br. ex Benth.) Orchard—EF178766, EF178948, EF179040, EF178856. *Moody 401* CONN, WA, Australia; *Middleton 101 PERTH, WA, Australia. *G. salsoloides* Rchb. ex Spreng. —EF178755, EF178937, EF179029, EF178845. *Coveny 17342 CANB, NSW, Australia. *G. scordioides* (Benth.) Orchard—EF178750, EF178932, EF179024, EF178840. *Moody 392* CONN, WA, Australia; *Hislop 1233 PERTH, WA, Australia. *G. tetragynus* Labill. —EF178764, EF178946, EF179038, EF178854. *Moody 468* CONN, NSW, Australia; *Moody 474* CONN, NSW, Australia. *G. teucroides* DC. —EF178761, EF178943, EF179035, EF178851. *Moody 462* CONN, NSW, Australia; cult. ANBG, ACT, Australia. *G. trichostachyus* (Benth.) Orchard—EF178767, EF178949, EF179041, EF178857. *Moody 398* CONN, WA, Australia. *G. urceolatus* Orchard—EF178768, EF178950, EF179042, EF178858. *Bean 14450 NSW, QLD, Australia. *Gonocarpus (hybrid?)*. *Moody 459* CONN, Vic., Australia; *Moody 472* CONN, NSW, Australia; *Moody 478* CONN, NSW, Australia.
- Haloragis acutangula* F. Muell.—EF178749, EF178931, EF179023, —. *Bates 33500 PERTH, SA, Australia. *H. digyna* Labill.—EF178747, EF178929, EF179021, EF178838. *Moody 411* CONN, WA, Australia. *H. dura* Orchard—EF178745, EF178927, EF179019, EF178837. *Moody 385* CONN, WA, Australia; *Moody 390* CONN, WA, Australia. *H. erecta* (Banks ex Murray) Oken—EF178736, EF178918, EF179010, EF178828. *Rixon 20 NSW, Auckland, NZ. *H. exalata* F. Muell. —EF178741, EF178923, EF179015, EF178833. *Miles s.n. PERTH, NSW, Australia; Miles s.n. PERTH, NSW, Australia. *H. eyreana* Orchard—EF178740, EF178922, EF179014, EF178832. *Jusaitis A78 PERTH, SA, Australia. *H. foliosa*, Benth. —EF178748, EF178930, EF179022, EF178839. *Moody 420* CONN, WA, Australia. *H. glauca* Lindl. EF178744, EF178926, EF179018, EF178836. *Moody 426* CONN, NSW, Australia. *H. hamata* Orchard—EF178738, EF178920, EF179012, EF178830. *Cranfield 10585 PERTH, WA, Australia. *H. heterophylla* Brongn.—EF178743, EF178925, EF179017, EF178835. *Moody 458* CONN, NSW, Australia; *Moody 471* CONN, WA, Australia. *H. masatierrana* Skottsbo.—EF178737, EF178919, EF179011, EF178829. (cult.) CONN. *H. serra* Brongn. —EF178746, EF178928, EF179020, —. *Moody 488* CONN (cult.) NSW, Australia. *H. stricta* R. Br.—EF178742, EF178924, EF179016, EF178834. *Williams 88074 PERTH, QLD, Australia. *H. trigonocarpa* F. Muell.—EF178739, EF178921, EF179013, EF178831. *DJE 3100 PERTH, WA, Australia.
- Haloragodendron bauerlenii* (F. Muell.) Orchard—EF178783, EF178965, EF179057, EF178873. *Moody 483* CONN (cult.) NSW, Australia. *H. gibsonii* Wilson & Moody—EF178785, EF178967, EF179059, EF178875. *Moody 480* CONN (cult.) NSW, Australia. *H. glandulosum* Orchard—EF178780, EF178962, EF179054, EF178870. (cult.) ANBG 2000, ACT, Australia. *H. lucasii* (Maiden & Betche) Orchard—EF178784, EF178966, EF179058, EF178874. *Moody 481* CONN (cult.) NSW, Australia; EF178786, EF178968, EF179060, EF178876. *Moody 482* CONN (cult.) NSW, Australia; *Moody 485* CONN, NSW, Australia; *Moody 486* CONN, NSW, Australia. *H. monospermum* (F. Muell.) Orchard—EF178781, EF178963, EF179055, EF178871. *Moody 475* CONN, NSW, Australia; *Moody 491* CONN (cult.) ANBG, ACT, Australia. *H. racemosum* (Labill.) Orchard—EF178782, EF178964, EF179056, EF178872. *Moody 400* CONN, WA, Australia; *Moody 402* CONN, WA, Australia; *Moody 403* CONN, WA, Australia.
- Laurembergia repens* (L.) P. J. Berguis—EF178735, EF178917, EF179009, EF178827. *J. P. Rourke* (cult.) HPBG, Cape Town, South Africa; Williams 113 C, South Africa.
- Meionectes brownii* Hook. f. —EF178773, EF178955, EF179047, EF178863. *Moody 438* CONN, Vic, Australia; *Moody 408* CONN, WA, Australia. *M. tenuifolia* (Benth.) Moody & Les—EF178774, EF178956, EF179048, EF178864. *Wright s.n. PERTH, WA, Australia; Cranfield 15976 PERTH, WA, Australia.
- Meziella trifida* (Nees) Schindl. —EF178734, EF178916, EF179008, EF178826. *Moody 410* CONN, WA, Australia; *Moody 405* CONN, WA, Australia; *Moody 404* CONN, WA, Australia.
- Myriophyllum alpinum* Orchard—EF178720, EF178902, EF178994, EF178812. *Moody 449* CONN, NSW, Australia; *Moody 453* CONN, NSW, Australia. *M. alterniflorum* DC.—EF178704, EF178886, EF178978, EF178797. *Moody 109a* CONN, WI, USA; *Moody 111* CONN, WI, USA. *M. aquaticum* (Vellozo) Verd.—EF178727, EF178909, EF179001, EF178819. *Moody 51* CONN, CA, USA; *Moody 56* CONN, FL, USA. *M. balladoniense* Orchard—EF178708, EF178890, EF178982, EF178801. *Moody 389* CONN, WA, Australia. *M. caput-medusae* Orchard—EF178703, EF178885, EF178977, EF178796. *Moody 443* CONN, NSW, Australia; *Moody 446* CONN, NSW, Australia. *M. coronatum* Meijden—EF178717, EF178899, EF178991, EF178809. *Pajmans 3039 CANB, QLD, Australia. *M. crispatum* Orchard—EF178721, EF178903, EF178995, EF178813. *Moody 445* CONN, NSW, Australia; *Moody 437* CONN, NSW, Australia. *M. drummondii* Orchard—EF178725, EF178907, EF178999, EF178817. *Moody 409* CONN, WA, Australia; *Moody 417* CONN, WA, Australia. *M. farwellii* Morong—EF178731, EF178913, EF179005, EF178823. *Moody 97* CONN, MN, USA; *Moody 106* CONN, WI, USA. *M. filiforme* Benth.—EF178716, EF178898, EF178990, EF178808. *Wilson 1810 NSW, NT, Australia. *M. heterophyllum* Michx. —EF178733, EF178915, EF179007, EF178825. *Moody 101* CONN, MN, USA; *Moody 143* CONN, MA, USA. *M. latifolium* F. Muell.—EF178729, EF178911, EF179003, EF178821. *Orchard 4793 NSW, NSW, Australia; Jacobs 6706 NSW, NSW, Australia. *M. laxum* Shuttlew.—EF178732, EF178914, EF179006, EF178824. *Moody 170* CONN, SC, USA; *Moody 77* CONN, FL, USA. *M. lophatum* Orchard—EF178718, EF178900, EF178992, EF178810. *Moody 456* CONN, NSW, Australia; *Moody 455* CONN, NSW, Australia. *M. mattogrossense* Hoehne—EF178728, EF178910, EF179002, EF178820. Ritter 2314 LPB, Carrasco, Bolivia. *M. muelleri* Sond.—EF178719, EF178901, EF178993, EF178811. *Jacobs 6597 NSW, NSW, Australia. *M. oguraense* Miiki—EF178705, EF178887, EF178979, EF178798. Kadono s.n. HYO, Hyogo, Japan. *M. papillosum* Orchard—EF178724, EF178906, EF178998, EF178816. *Moody 424* CONN, NSW, Australia; Les 614 CONN, NSW, Australia. *M. pedunculatum* Hook. f.—EF178711, EF178893, EF178985, EF178804. *Moody 452* CONN, NSW, Australia; *Moody 467* CONN, NSW, Australia. *M. petraeum* Orchard—EF178712, EF178894, EF178986, EF178805. *Archer 1564 NSW, WA, Australia; *Brown 1123 PERTH, WA, Australia. *M. quitense* Kunth.—EF178700, EF178882, EF178974, EF178793. *Moody 183* CONN, OR, USA. *M. salsugineum* Orchard—EF178701, EF178883, EF178975, EF178794, *Moody 412* CONN, Vic, Australia; Les s.n. (cult.) U of Tasmania. *M. sibiricum* Kom.—EF178706, EF178888, EF178980, EF178799. *Moody 82* CONN, CA, USA; *Moody 99* CONN, MN, USA. *M. spicatum* L.—EF178702, EF178884, EF178976, EF178795. *Moody 86* CONN, CA, USA; *Moody 117* CONN, WI, USA. *M. tenellum* Bigelow—EF178730, EF178912, EF179004, EF178822. *Moody 110* CONN, WI, USA; *Moody 93* CONN, MN, USA. *M. tillaeoides* Diels—EF178710, EF178892, EF178984, EF178803. *Moody 415* CONN, WA, Australia. *M. trachycarpum* F. Muell.—EF178715, EF178897, EF178989, EF178807. Jacobs 8843 NSW, NT, Australia; Martine 863 CONN, WA, Australia. *M. ussuriense* Maxim.—EF178726, EF178908, EF179000, EF178818. Kadono “Kasai-1” HYO, Hyogo, Japan; Kadono “Kasai-2” HYO, Hyogo, Japan. *M. verrucosum* Lindl.—EF178707, EF178889, EF178981, EF178800. *Moody 427* CONN, NSW, Australia; Les 601 CONN, NSW, Australia. *M. verticillatum* L.—EF178709, EF178891, EF178983, EF178802. *Moody 177* CONN,

- OR, USA; *Moody 147* CONN, ME, USA. *M. votschii* Schindl.—EF178714, EF178896, EF178988, EF178806. **Rixon 31* NSW, Australia. *M. sp.* EF178713, EF178895, EF178987, —. *Les 542* CONN, NSW, Australia. *M. sp. nov. a*, EF178722, EF178904, EF178996, EF178814. *Les 622* CONN, NSW, Australia. *M. sp. nov. b*, EF178723, EF178905, EF178997, EF178815. *Moody 476* CONN, NSW, Australia.
- Proserpinaca palustris* L.—EF178787, EF178969, EF179061, EF178877. *Moody 59* CONN, FL, USA; *Moody s.n.* CONN, CT, USA. *P. pectinata* Lam.—EF178788, EF178970, EF179062, EF178878. **Nelson 19432* USCH, SC, USA; **Horn 4610* USCH, SC, USA.
- Outgroups:
- Penthorum sedoides* L.—EF178789, EF178971, EF179063, EF178879. *Moody 515* CONN, CT, USA. *Tetracarpaea tasmanica* Hook. f.—EF178790, —, EF179064, — **Wieck 577* NSW, TAS, Australia. *Aphanopetalum clematideum* (Harv.) Domin—EF178792, EF178972, EF179065, EF178880. *Moody 421* CONN, WA, Australia. *A. resinsum* (Harv.) Domin—EF178791, EF178973, EF179066, EF178881. *Moody 484* CONN (cult.) NSW, Australia.
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