PHYLOGENETIC SYSTEMATICS AND CHARACTER EVOLUTION IN THE ANGIOSPERM FAMILY HALORAGACEAE

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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, Glischrocarpon, Haloragis, Haloragodendron) 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 (Triboralagis) 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 (Glischrocarpon, 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 (Crinquot, 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).

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

<table>
<thead>
<tr>
<th>Genus</th>
<th>Distribution</th>
<th>Habit</th>
<th>No. species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glischrocaryon</td>
<td>Australia</td>
<td>Terrestrial</td>
<td>4</td>
</tr>
<tr>
<td>Gonocarpus</td>
<td>Australia, New Zealand, S. E. Asia</td>
<td>Terrestrial</td>
<td>(\approx)36</td>
</tr>
<tr>
<td>Haloragis</td>
<td>Australia, New Zealand, S. Pacific</td>
<td>Terrestrial</td>
<td>(\approx)26</td>
</tr>
<tr>
<td>Haloragodendron</td>
<td>Australia</td>
<td>Terrestrial</td>
<td>5</td>
</tr>
<tr>
<td>Laurembergia</td>
<td>Panropical</td>
<td>Semiaquatic</td>
<td>4</td>
</tr>
<tr>
<td>Meziella</td>
<td>S. W. Australia</td>
<td>Aquatic</td>
<td>1</td>
</tr>
<tr>
<td>Myriophyllum</td>
<td>Cosmopolitan</td>
<td>Aquatic</td>
<td>(\approx)60</td>
</tr>
<tr>
<td>Proserpinaca</td>
<td>New World</td>
<td>Aquatic</td>
<td>3</td>
</tr>
</tbody>
</table>

* Three species are aquatic.

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 (Praglowski, 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 Proserpinaca are similar to those of Myriophyllum but also occur in the aquatic/semiaquatic members of Haloragis (H. brownii (Hook.f.) Schindler., H. heterophylla Brongn., and H. tenuifolia Bentham.). The trimerous, perfect flowers of Proserpinaca rarely occur elsewhere in the family and are found only in Gonocarpus hexandrus (F.Muell.) Orchard, Haloragis gosssei 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 submersed 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 aquatic taxa 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 Fennesy, 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. tenuifolia 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 laciniate 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. tenella*). In contrast, the helophytic *Laurenbergia* 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 (*Glischrocarpon*, *Gonocarpus*, *Haloragis*, *Proserpinaca*). Moreover, dimerous flowers, another unusual condition among core eudicots, are present in *Glischrocarpon behnrii* (Schindl.) Orchard, *Haloragis brownii*, *Haloragis digynia* (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, Penharaeaceae, and Tetracarpaeaceae (Fishbein et al., 2001) were sampled. All known species of *Glischrocarpon*, *Haloragodendron*, 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%) *Laurenbergia* 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 ITS2 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 trnK5R (5'-TCTTGGTTATCTAAATGATA) and matK70R (5'-GTGTTGGTGGAC 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, trnK360F, tmK2R, matK70R, tmKR, and a newly developed Haloragaceae specific primer trnK3F (5'-CGTGGATTCTAAATGATA) (CTAGA). 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 Sequencer 4.1.2 (Gene Code Corp., Ann Arbor, Michigan, USA) and manually aligned using the program MacClade 4.01 (Maddison and Maddison, 2000). 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 replications. 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 10,000.

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 $3.0 \times 10^8$ 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—in 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 semi-aquatic. 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 28,000 trees, conservatively discarding the first 200 (2.0 $\times 10^6$ 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...
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-Halorgadodendron* 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-Halorgadodendron* and *Proserpinaca*. *Gono- carpus 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 *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. masatierana* 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* (Brong.) Orchard, *G. epemerus* 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* Orchard was part of the “acanthocarpus clade” using the cpDNA and combined data, but this relationship was not supported using ITS alone. The “tetragnus 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
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).

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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; Seelan 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 combinatorial (Dolphin et al., 2000; Darlu and Lecointre, 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
considered in this disposition. Until recently, *Penthorum* had been included in Saxifragaceae, whereas *Tetracarpaea* comprised a monotypic Tetracarpaeaceae. Based on the phylogenetic results of Fishbein et al. (2001), we used *Aphanopetalum* as an outgroup in these analyses to consider the position of *Penthorum* and *Tetracarpaea* within a Haloragaceae s.l. Examination of the phylograms (Fig. 4a–c) and comparisons of pairwise distances of combined data (Table 4) indicated that *Penthorum* and *Tetracarpaea* are much more distinct from all Haloragaceae taxa than any Haloragaceae are to each other at the molecular level.

Circumscription of Haloragaceae has been based on common floral characters including tetramerous flowers (sometimes two- or three-merous), keeled or hooded petals, and an inferior ovary of 2–4 carpels (1–4 locules) with 1–2 ovules per locule. *Tetracarpaea* has some similarities to Haloragaceae. As its name suggests, it is four-carpellate and has four- or five-merous flowers, but its hypanthium and superior ovary with numerous ovules contrast markedly with Haloragaceae. The pentamerous flowers, hypanthium, superior ovary, and many-seeded fruits of *Penthorum* also differ markedly from the typical characteristics found in Haloragaceae. Arguably, the floral character states of *Penthorum* could be regarded as more similar to those found in Crassulaceae, another closely allied family. Although the molecular evidence supports the monophyly of Penthoraceae, Tetracarpaeaceae, and Haloragaceae in a broad sense, the extensive molecular divergence and discordant morphological characters indicate that *Penthorum* and *Tetracarpaea* are distinct from Haloragaceae. While it is important to recognize the relationship of these taxa to Haloragaceae in a phylogenetic context, we suggest that they retain their unique status in relation to Haloragaceae to emphasize their distinctive features, a disposition recommended earlier by the APG (1998) and as currently recommended for Aphanopetalaceae (APG II, 2003).

**Basal nodes of Haloragaceae**—The combined and cpDNA analyses resolved Glischrocaryon-Haloragodendron as sister to the rest of the family (PP = 1.0; BS = 52), a result that conflicted with results from ITS analyses (Figs. 1–3). There

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**Fig. 4.** Phylogenetic relationships in Haloragaceae as indicated by phylograms of best likelihood trees selected from 28 000 trees recovered from Bayesian analyses of (A) combined ITS and cpDNA data (second most likely tree also used for ancestral state analysis); (B) cpDNA data; (C) ITS data. Branch lengths are proportional.
C. ITS DATA

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 Sanderson, 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. Spongicarpus 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 Gomocarpus) 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 (semaqauatic). 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).
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 sympathy with the dimerous *G. behrii* and tetramerous *G. angustifolium*, providing circumstantial evidence of hybridization that should be examined further using molecular markers.

Proserpinicae and Meionectes—Proserpinicae 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; *Proserpinicae* 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 *Proserpinicae* 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 *Proserpinicae* 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 *Proserpinicae*. The most evident morphological differences between *Haloragis brownii*—*H. tenuifolia* and *Proserpinicae* are found in other floral characters. Whereas *Haloragis brownii* and *H. tenuifolia* have conspicuous, hooded petals and two whorls of stamens, *Proserpinicae* 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.B.R., 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*, *Proserpinicae*) 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 = *Proserpinicae palustris*.

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**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. hexandra*, *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. hexandra* was included in subsection *Trilhorraghis* 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. hexandra*) sister to *Haloragis*.

Although Orchard (1975, 1990) placed *G. hexandra* 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. hexandra* as sister to *Laurembergia-Meziella-Myriophyllum* (PP < 0.95), whereas parsimony did not resolve this relationship (Fig. 1). Alternatively, cpDNA weakly resolved *G. hexandra* as sister to *Haloragis-Gonocarpus* + *Laurembergia-Meziella-Myriophyllum* (also representative of the combined data). This clade is distinctly 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 *Trilhorraghis* (see Table 5). Three subspecies of *G. hexandra* 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 *Lampworkalax* 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 *Spongicarpus* among the taxa now included in *Haloragodendron* (Appendix S1). A sister relationship of *G. acanthocarpus* and *G. leptotheccus* 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 tetragnus 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. tetragonus* or *G. teucroides*. 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 plastid markers among these taxa may indicate a history of hybridization (Figs. 1–2). Forms intermediate between *G. elatus* and either *G. oreophilus* or *G. mezeianus* (see Fig. 3) have been described (Orchard, 1975). For this study, extreme morphological forms of members of the tetragnus 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 tetragnus 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 *Haloragodon* 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 seption, 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 *Meziella* (= *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.

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<td>Gonocarpus Thumb. (excluding G. hexandrus)</td>
</tr>
<tr>
<td>Gloschrocarpon Endl. (including new species G. angustifolium (Nees) Moody &amp; Les)</td>
</tr>
<tr>
<td>Haloragis J.R. Forst. &amp; G. Forst. (excluding Haloragis brownii and H. tenuifolia)</td>
</tr>
<tr>
<td>Haloragodendron Orchard</td>
</tr>
<tr>
<td>Laurembertia P.J. Bergius</td>
</tr>
<tr>
<td>Meionectes R. Br. (= Haloragis brownii and H. tenuifolia)</td>
</tr>
<tr>
<td>Meziella Schindl.</td>
</tr>
<tr>
<td>Myriophyllum L.</td>
</tr>
<tr>
<td>Proserpinaca L.</td>
</tr>
<tr>
<td>Tribhaloragis Moody &amp; Les (= Gonocarpus hexandrus)</td>
</tr>
</tbody>
</table>

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 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. matthiessenii* 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 Tribhaloragis. 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., Tribhaloragis) 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*–Gloschrocarpon 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 submerged 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, lateralpinnae 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 characterization 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 pentameroy 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 (Solitès 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. Solitès et al. (2003, p. 466) state, “In core eudicots there is sometimes variation between a pentameros 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 distinct sister group relationship of Haloragaceae to *Penthorum* and *Tetracarpae* 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**


Herba perennis vel suffrutex, caules 4-costati. Folia in partibus inferioribus opposita, superis alternà, lanceolata ad ob lanceolata. Flores 3-meri, perfecti; sepala 3, deltoidea, viridia; petala 3, cucullata, viridia ad cremna; 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.


**LITERATURE CITED**


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Haloragis acutangula —EF178770, EF178949, EF179041, EF178857. ANBG, ACT, Australia. H. lucasii —EF178767, EF178947, EF179039, EF178855.

G. micranthus —EF178741, EF178891, EF178983, EF178802.


M. farwellii Moody—EF178733, EF178915, EF179007, EF178825. *Morong—EF178741, EF178923, EF179015, EF178833. *Miles s.n. PERTH, NSW, Australia; Miles s.n. PERTH, NSW, Australia.


Outgroups: