

Phylogenetic studies in *Callitriche*: implications for interpretation of ecological, karyological and pollination system evolution

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Abstract

Species of *Callitriche* (Callitrichaceae) exhibit considerable diversity in chromosome number ($n=3-20$), ecology (submersed, amphibious, semiamphibious, terrestrial) and pollination systems (anemophily, hypohydrophily). *Callitriche* is the only genus of angiosperms in which both aerial pollination and hypohydrophily have been documented. Phylogenetic analyses were conducted using morphologic and *rbcL* sequence characters for 20 taxa that represent the range of chromosome number, ecological and pollination system diversity in the genus. Parsimony analyses reveal that aneuploid reduction ($n=5$ to 4) and polyploidy ($n=5$ to 10) have evolved at least twice in the genus; whereas the obligately submersed growth habit and hypohydrophily have evolved once. Although internal geitonogamy (a unique self-fertilization system known only in *Callitriche*) occurs in at least seven species in the genus, the analyses reveal that it likely evolved only once. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Callitriche*; Phylogeny; Hydrophily; Evolution

1. Introduction

Callitriche is a widespread genus of about 50 species of aquatic and terrestrial herbs with reduced and unisexual flowers (Cronquist, 1981). *Callitriche* species vary widely in their ecology, karyology, and pollination systems; however, evolutionary questions regarding variation in these attributes have not been tested explicitly due to a limited understanding

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of phylogenetic relationships in the genus. Phylogenetic analyses are also needed to test the soundness of the infrageneric classifications currently in use.

Species of *Callitriche* exhibit diverse growth habits which, as in many aquatics (Sculthorpe, 1967), reflect local environmental conditions. Most species are amphibious. When occurring in the water, plants of these species typically possess floating rosettes of spatulate leaves that develop when stems reach the water surface. Some amphibious species (e.g. *C. heterophylla*, *C. verna* [= *C. palustris*]), can also grow as submersed plants with limp, linear leaves. Terrestrial ecads of amphibious *Callitriche* species can produce intermediate leaf forms when plants are stranded on wet soil. Several species (e.g. *C. cribrosa*) are apparently semiamphibious; they possess the submersed and floating-leaved ecads, but lack the ability to grow on land. About 10 species (e.g. *C. peploides*, *C. nuttallii*) are ‘terrestrial’ (i.e. helophytic) and occur in seasonally wet habitats. Plants of terrestrial species appear similar to terrestrial ecads of amphibious species. Lastly, some species (e.g. *C. hermaphroditica*, *C. truncata*) are obligately submersed.

Chromosome numbers in *Callitriche* vary from $n=3-20$ (Philbrick, 1994 and Refs. therein). Associations have been noted between chromosome number, growth habit, pollen structure and pollination system (Philbrick and Jansen, 1991; Philbrick and Anderson, 1992; Martinsson, 1993; Philbrick, 1994; Cooper et al., 2000), although the lack of explicit phylogenetic hypotheses has limited meaningful evolutionary interpretations to date.

Pollination systems in *Callitriche* are diverse (Philbrick and Anderson, 1992; Philbrick, 1993), given its seemingly uniform floral structure among species. Pollen transport can occur by wind or underwater in various species; however, reports of pollen transport across the water surface (epihydrophily) remain unsubstantiated (see Discussion in Philbrick and Anderson, 1992). Indeed, *Callitriche* is the only genus known to possess both aerial (e.g. anemophily) and underwater (hypohydrophily) pollination mechanisms. As in other hydrophilous genera (Les, 1988), anemophily is likely the ancestral state in *Callitriche*; yet it is uncertain whether hypohydrophily has a single origin in the genus.

Geitonogamy (pollination between flowers on a plant) is also widespread in *Callitriche* (Philbrick and Anderson, 1992). Geitonogamy may be the primary pollination system of many species, although empirical genetic data are lacking. Internal geitonogamy, a system that involves the growth of pollen tubes through nonfloral vegetative tissues, is highly unusual in angiosperms, but has been documented in seven *Callitriche* species (Philbrick and Anderson, 1992; Philbrick and Bernardello, 1992). Clarification of how often internal geitonogamy evolved in *Callitriche* has awaited testing with reference to a phylogenetic analysis of the genus.

Two infrageneric classifications of *Callitriche* have been proposed (Fig. 1). The first monograph of *Callitriche* was published by Hegelmaier (1864), who subdivided the genus into two sections largely defined on the basis of growth habit. Section *Callitriche* (= *Eucallitriche*) Hegelm. contained terrestrial and amphibious species; whereas, section *Pseudocallitriche* Hegelm. contained two obligately submersed species (*C. hermaphroditica*, *C. truncata*).

Subsequent systematic studies of *Callitriche* have resulted in the description of ≈ 40 additional species. Although all of these species essentially correspond to Hegelmaier’s section *Callitriche*, some (e.g. *C. cribrosa*, *C. lusitanica*) have a combination of features

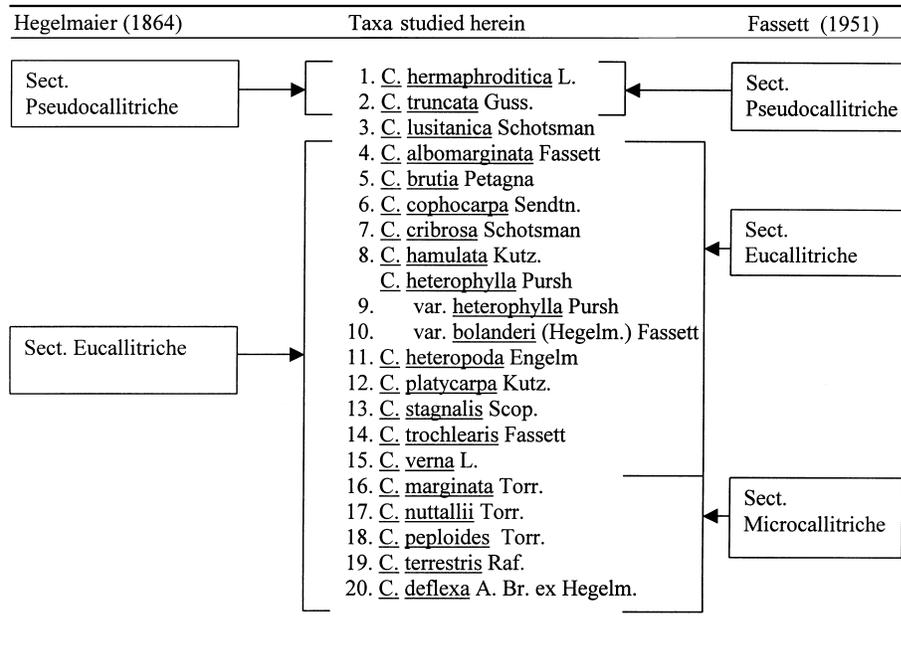


Fig. 1. Application of the classifications of Hegelmaier (1864) and Fassett (1951) to the species studied. Note: *Callitriche lusitanica* cannot be placed confidently in any section, because it combines features of both Hegelmaier's and Fassett's sectional delimitations (see text for discussion).

that was unknown to Hegelmaier, i.e. 'amphibious' species that lack the terrestrial ecad. Since Hegelmaier's time, only one additional species (*C. pulchra*) has been described that corresponds to section *Pseudocallitriche*, which currently comprises three species: *C. hermaphroditica*, *C. truncata* and *C. pulchra*.

Hegelmaier's classification was modified by Fassett (1951) who focused on species in the New World. Fassett's section *Pseudocallitriche* paralleled that of Hegelmaier; however, he removed the terrestrial species that lacked floral bracts from section *Callitriche* and placed them into a third section (*Microcallitriche* Fassett).

Schotsman (1967, 1972) contemplated the division of the genus into five groups, but did not publish a formal classification. Her groups I and V corresponded to Hegelmaier's section *Pseudocallitriche* and Fassett's section *Microcallitriche*, respectively. However, Schotsman found it difficult to place some species into either classification scheme suggested by Hegelmaier or Fassett. Her newly described *C. lusitanica* (Schotsman, 1961) possessed submersed linear-leaved ecads similar to *C. hermaphroditica* (section *Pseudocallitriche*); whereas, it also produced spatulate leaves with stomates above the water surface, a feature corresponding to section *Callitriche* of both Hegelmaier and Fassett. With the inadequacy of both former classifications indicated by the discovery of *Callitriche lusitanica*. (Schotsman, 1967, 1972) incorporated chromosome numbers to assist with her delimitation of groups. For example, group II (*C. lusitanica*) was characterized by $n=4$, and was thus intermediate

between groups I ($n=3$) and III ($n=5, 10$). Group IV contained the only two species of the genus that apparently were aneuploid derivatives of higher euploids (*C. brutia*, $n=14$; *C. hamulata*, $n=19$).

Explicit phylogenetic hypotheses are needed to test the naturalness of groups proposed in past classifications and floristic treatments, and to evaluate the reliability of the characteristics upon which they have been based. Here, we hypothesize phylogenetic relationships among 20 taxa of *Callitriche* based upon parsimony analyses of morphological characters and *rbcL* gene sequences. The species studied encompass the range of ecological, karyological and pollination system diversity in the genus, and represent all three sections recognized in current taxonomic treatments.

2. Materials and methods

Twenty species and varieties of *Callitriche* were studied (Fig. 1). We selected 17 morphological features, including characters that were featured prominently in past systematic treatments of *Callitriche*, such as in the descriptions of Hegelmaier (1864), Fassett (1951) and Schotsman (1967, 1972). Character states were compiled for each of the 22 taxa (20 *Callitriche* taxa; two *Hippuris* species as outgroups; Appendix A) included in our analysis and are summarized in Table 1. Character states were treated as unordered in all analyses. *Hippuris* was selected as the outgroup for phylogenetic analyses, because it is arguably the closest extant sister genus to *Callitriche* (Olmstead and Reeves, 1995).

DNA sequences of the *rbcL* gene were obtained from the same 20 *Callitriche* taxa and two *Hippuris* species. Manual sequencing methods were employed following procedures for DNA isolation, amplification and sequencing as described in Les et al. (1993). A total of 1348 nucleotides was examined for each of the 17 taxa with fewer sites surveyed for *H. vulgaris* (1324 positions), *C. verna* (1310 positions), *C. stagnalis* (1306 positions), *C. trochlearis* (1291 positions) and *C. heterophylla* var. *bolanderi* (1272 positions). Voucher specimens are listed in Appendix B. All sequences have been submitted to the GenBank database under the following accession numbers: AF248008=*Callitriche albomarginata*; F248009=*Callitriche brutia*; AF248010=*Callitriche cophocarpa*; AF248011=*Callitriche cribrosa*; AF248012=*Callitriche deflexa*; AF248013=*Callitriche hamulata*; AF248014=*Callitriche hermaphroditica*; AF248015=*Callitriche heterophylla* var. *heterophylla*; AF248016=*Callitriche heterophylla* var. *bolanderi*; AF248017=*Callitriche heteropoda*; AF248018=*Callitriche lusitanica*; AF248019=*Callitriche marginata*; AF248020=*Callitriche nuttallii*; AF248021=*Callitriche peploides*; AF248022=*Callitriche platycarpa*; AF248023=*Callitriche stagnalis*; AF248024=*Callitriche terrestris*; AF248025=*Callitriche truncata*; AF248026=*Callitriche trochlearis*; AF248027=*Callitriche verna*; AF248028=*Hippuris vulgaris*; AF248029=*Hippuris lanceolata*.

Phylogenetic analyses were conducted using PAUP* 4.0d59 software (Swofford, 1998; by permission) to perform unweighted parsimony analyses. Data were partitioned to allow the analysis of each separate dataset as well as their combination. Heuristic search (TBR swapping) was used for the morphological data where several thousand equally minimal length trees were obtained. The branch and bound algorithm was used for the *rbcL* and combined datasets to ensure the recovery of minimal length trees. The ‘furthest’ addition

Table 1
Character analysis of the morphological features analyzed herein^a

1	Endothelial thickenings (presence/absence). Cell wall thickenings occur in the endothecium of the anther walls of some species.
2	Inflated floral bracts (present/absent). Delicate inflated bracts subtend the unisexual flowers in some species.
3	Internal geitonogamy (present/absent). A unique self-fertilization system occurs in some species of <i>Callitriche</i> (cf., Philbrick and Anderson, 1992; Philbrick and Bernardello, 1992).
4	Fruit wing (present/absent). Some species possess a thin wing-like extension of the carpel wall.
5	Fruit pedicel (present/absent). Species either have a sessile fruit (pedicel < 0.5 mm) or a pedicel that ranges from ≈ 2 to 5 cm long.
6	Fruit orientation (upright/reflexed). Fruits of most species are upright at maturity, while others are distinctly reflexed.
7	Style orientation (upright/reflexed). Styles are either upright or reflexed.
8	Peltate scales on leaves (present/absent). Glandular peltate scales either occur on the leaves or are lacking.
9	Exine thickness. Exine thickness was scored as three states: well developed (>0.5 μm), reduced (0.0–0.3 μm), or absent. For detailed discussion of exine thickness in <i>Callitriche</i> ; see Cooper et al. (2000).
10	Gemmae shape on sexine. The gemmae that occur on the sexine are either pointed, rounded or absent (exineless or nearly exineless pollen). For detailed discussion of gemmae shape in <i>Callitriche</i> ; see Cooper et al. (2000).
11	Sexine surface pattern. Three states were scored: polygonal, granular or absent (exine lacking). For detailed discussion of sexine patterning in <i>Callitriche</i> ; see Cooper et al. (2000).
12	Pollen apertures. Three states were scored: inaperturate, but with an exine; aperture-like regions; inaperturate (exine lacking).
13	Fruit position (aerial/geocarpic). Fruits either mature aerially or are pushed into the ground during development (geocarpic).
14	Bisexual vs. unisexual flowers. All species of <i>Callitriche</i> have unisexual flowers, whereas flowers in <i>Hippuris</i> are predominantly bisexual.
15	Calyx. A calyx is lacking in <i>Callitriche</i> , but is represented by a rim around the top of the ovary in <i>Hippuris</i> .
16	Carpel number. The ovary is bicarpellate in <i>Callitriche</i> and unicarpellate in <i>Hippuris</i> .
17	Leaf arrangement. Leaves are opposite in <i>Callitriche</i> and whorled in <i>Hippuris</i> .

^a Characters 1–13 provide resolution within *Callitriche*, whereas 14–17 support the monophyly of *Callitriche* relative to *Hippuris*.

sequence and ‘MulTrees’ option were used in all analyses. The level of internal support for each resolved clade was determined using bootstrap analysis (500 replicates; ‘full heuristic’ search). Trees were rooted using the *Hippuris* sequences as outgroups. Strict consensus was used to depict results where more than one minimal length tree topology was recovered.

As a means of gaining insight into character evolution, ACCTRAN and DELTRAN optimizations were conducted using PAUP. The *rbcL* tree was considered to be the best approximation of the phylogeny, and the morphological characters most relevant to the issues discussed in the paper were mapped onto it.

3. Results

3.1. Morphological data

The strict consensus of 4997 equally minimal length trees (32 steps) is shown in Fig. 2. The consistency index (CI, with, and without, autapomorphies) was 0.6562, and the retention index (RI) was 0.8070.

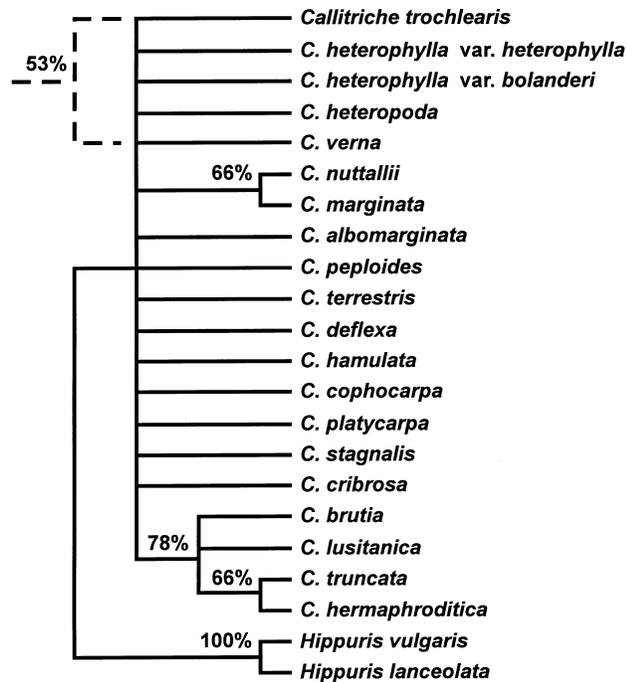


Fig. 2. The strict consensus tree (32 steps) based on morphological data (branch lengths not proportional). Dashed lines indicate a clade that is not present in the strict consensus tree, but occurs with moderate internal support in the majority rule consensus tree (see text for discussion). Bootstrap values are given above each node.

Four nonhomoplasious synapomorphies support the monophyly of *Callitriche* (unisexual flowers, lack of a calyx, bicarpellate ovary, opposite leaves) relative to the outgroup *Hippuris* (bisexual flowers, calyx present, unicarpellate ovary, whorled leaves).

The strict consensus tree produced from the morphological data set (Fig. 2) is largely unresolved. Within *Callitriche*, only three moderately supported clades (bootstrap values 66–78%) are indicated. Long fruit pedicels (also in *C. brutia*) and geocarpic fruits are synapomorphic for *C. nuttallii* and *C. marginata*. The monophyly of *C. truncata* and *C. hermaphroditica* is indicated singly by their loss of peltate scales. Four characters support the inclusion of *C. brutia* and *C. lusitanica* in a clade with *C. truncata* and *C. hermaphroditica*: sexine gemmae shape, pollen aperture type, lack of an exine, and absence of inflated floral bracts.

A number of additional clades are resolved by a majority-rule consensus tree, but only one of them with any appreciable internal support. This clade (with 53% bootstrap support; Fig. 2) includes both varieties of *C. heterophylla*, *C. heteropoda*, *C. trochlearis*, and *C. verna*, and is supported by one nonhomoplasious synapomorphy (internal geitonogamy). Within this clade, *C. heterophylla* (both varieties) and *C. heteropoda* occur as a subclade, but only with low internal support (32% bootstrap).

3.2. Molecular (*rbcL*) data

A single maximum parsimony tree (93 steps; CI=0.871; CI excluding autapomorphies =0.844; RI=0.934) was recovered from the *rbcL* data (Fig. 3). The *rbcL* data produced a total of 63 parsimony informative characters and 16 parsimony noninformative characters in the analysis. A pairwise comparison of uncorrected ('*p*') distances indicated low sequence divergence across all taxa, ranging from 0% (several comparisons within *Callitriche*) to a maximum of only 3.6% (*Hippuris vulgaris* × *Callitriche albomarginata*). This low level of sequence divergence indicated that complications due to multiple substitutions in the molecular data set should be minimal.

Nevertheless, a separate analysis using step-matrix weighting to accommodate transition/transversion bias and codon position bias was made following the methods described by Les et al. (1997). This weighting scheme produced a tree topology that was identical to that in Fig. 3, except that *C. deflexa*, *C. peploides*, *C. terrestris* and *C. verna* collapsed into a polytomy; a result not surprising given the weak level of internal support (29–55% bootstrap) in the unweighted analysis for phylogenetic resolution in this portion of the *rbcL* cladogram. With the low observed level of sequence divergence, weighting is probably unnecessary and serves only to reduce the maximum level of resolution extractable from the *rbcL* dataset. Consequently, we selected the cladogram from the unweighted analysis as the best depiction of phylogenetic signal contained in the *rbcL* data.

The *rbcL* tree (Fig. 3) is considerably more resolved than the tree obtained using morphological data. The *rbcL* data indicate a basal split between members of clade A (*C. hermaphroditica*, *C. lusitanica*, *C. truncata*; 100% bootstrap), all with aneuploid chromosome numbers ($n=3, 4$), and the rest of the genus (clade B; 58% bootstrap). A fourth aneuploid species (*C. cribrosa*, $n=4$), occurs in clade D and is well separated from the aneuploids in clade A (see in the following).

The majority of the species occur in clade B, which is further resolved as two more inclusive clades (clades C and D). Clade D (81% bootstrap) contains six amphibious species (*C. brutia*, *C. cophocarpa*, *C. hamulata*, *C. cribrosa*, *C. platycarpa*, *C. stagnalis*) all of which occur in Europe or have a largely Mediterranean distribution. Both euploid and aneuploid chromosome numbers occur in clade D. The monophyly of *C. platycarpa*, *C. stagnalis*, and *C. cribrosa* is indicated as a subclade (92% bootstrap support) within clade D. Finer resolution of relationships among these species is not possible from *rbcL* data alone. The monophyly of *C. hamulata*, *C. brutia*, and *C. cophocarpa* is also indicated (89% bootstrap). *C. hamulata* and *C. brutia* are resolved as monophyletic (98% bootstrap).

Clade C (92% bootstrap) comprises 11 New World taxa, except for *C. verna*, which also occurs in the Old World. Two terrestrial species, *C. deflexa* and *C. terrestris*, are basal in this clade (55 and 29% bootstraps, respectively). Relationships among the remaining nine taxa are only partially resolved. Three monophyletic clades are indicated: (a) *C. trochlearis*, *C. heterophylla* var. *heterophylla*, *C. heterophylla* var. *bolanderi*, and *C. heteropoda* (58% bootstrap); (b) *C. trochlearis* and *C. heterophylla* var. *heterophylla* (62% bootstrap); and (c) *C. nuttallii*, *C. marginata*, *C. albomarginata* (50% bootstrap). Interrelationships of *C. verna* and *C. peploides* remain ambiguous.

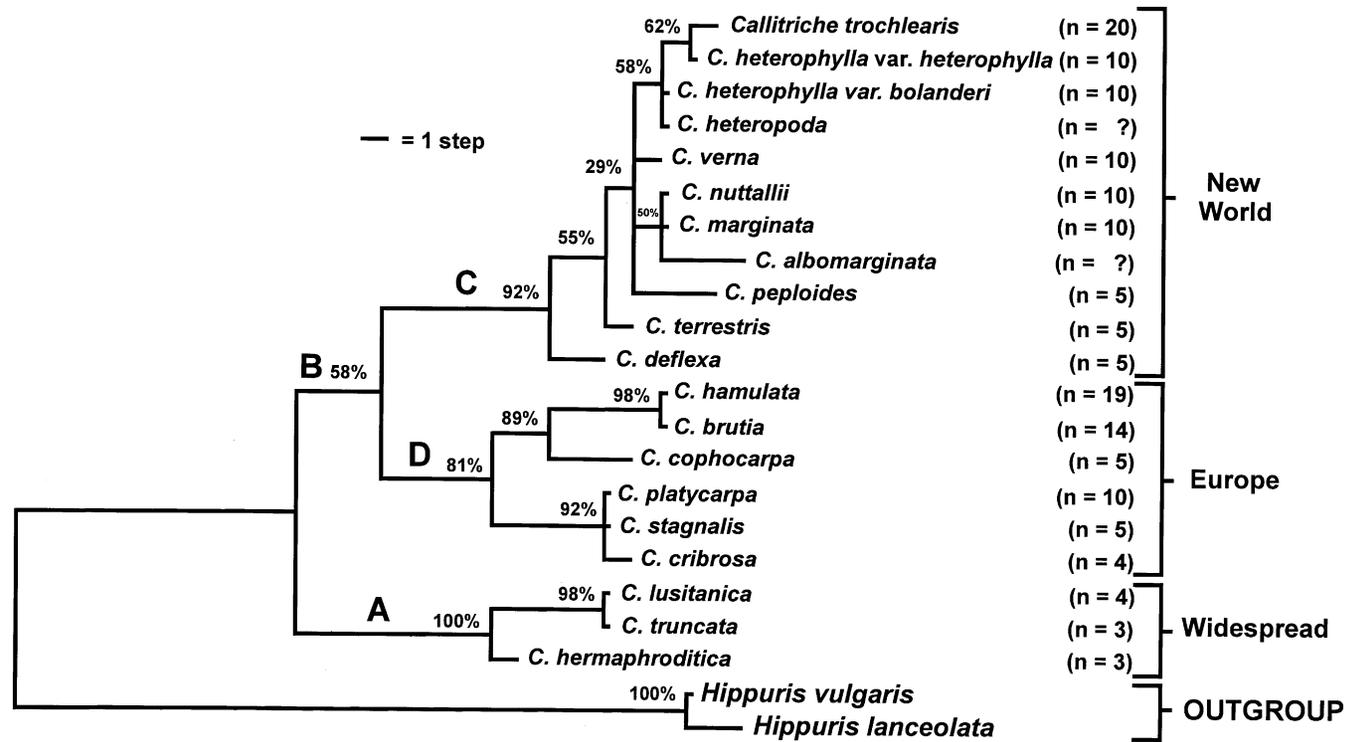


Fig. 3. The 93-step maximum parsimony tree based on molecular (*rbcl*) data (see text for discussion). The scale indicates a branch length of one step. Uppercase letters (A–D) refer to major clades discussed in the text. Bootstrap values are given above each node. Chromosome numbers and geographical affinities are indicated for each taxon.

3.3. Combined molecular and morphological data

A combined data set consisting of 17 morphological and 1348 *rbcL* characters produced a single minimal length tree of 138 steps. This tree (Fig. 4) has a CI=0.739 (0.705 excluding autapomorphies) and RI=0.850.

Although their bootstrap values differ somewhat, the basic topology of the combined *rbcL*/morphology tree (Fig. 4) is similar to the *rbcL* tree (Fig. 3). All four major clades (A–D) are retained and the topologies for clades A and D are identical in both analyses. Clade C differs only by slight differences in weakly supported portions of the *rbcL* tree. These include the grouping of *C. verna* with *C. trochlearis*, *C. heteropoda* and *C. heterophylla*, and by resolution of a weakly supported clade that places *C. albomarginata*, *C. terrestris* and *C. peploides* together with *C. nuttallii*, *C. marginata* and *C. deflexa*. One notable difference is the position of *C. deflexa* which is basal in clade C of the *rbcL* tree, but is nested higher in the tree produced by the combined data. A slight decrease in bootstrap support observed for clades resolved in both analyses indicated disagreement between the morphological and molecular data sets.

The inclusion of *C. verna* in a clade with *C. trochlearis*, *C. heteropoda*, and the two varieties of *C. heterophylla* was moderately supported (61% bootstrap) in the combined analysis, but the position of *C. verna* was unresolved by *rbcL* data alone. The placement of *C. trochlearis* also differed between the analyses. The *rbcL* data resolved *C. trochlearis* as sister to *C. heterophylla* var. *heterophylla*, whereas the combined morphological/*rbcL* data placed *C. trochlearis* in a position basal to a clade that included *C. heteropoda* and the two varieties of *C. heterophylla*. However, none of the bootstrap values for these competing topologies were particularly high.

3.4. Character optimization

Optimizations of character state changes for three characters most relevant to the issues discussed herein (internal geitonogamy, exine thickness, pollen apertures) are shown in Fig. 5. The locations of character state changes for internal geitonogamy and pollen apertures were the same for ACCTRAN and DELTRAN optimizations (Fig. 5A and B). Optimizations for state changes regarding exine thickness were different in ACCTRAN and DELTRAN analyses (Fig. 5C and D, respectively).

4. Discussion

4.1. Infrageneric classification of *Callitriche*

The phylogenetic relationships recovered in our analyses are incongruent with the sectional classifications of Hegelmaier (1864) and Fassett (1951), as well as the groups proposed by Schotsman (1967). Even so, our results correspond more to Hegelmaier (1864) than to Fassett (1951) or Schotsman (1967). Hegelmaier's two sections largely reflect the basal split of clades A (section *Pseudocallitriche*) and B (section *Callitriche*) that were

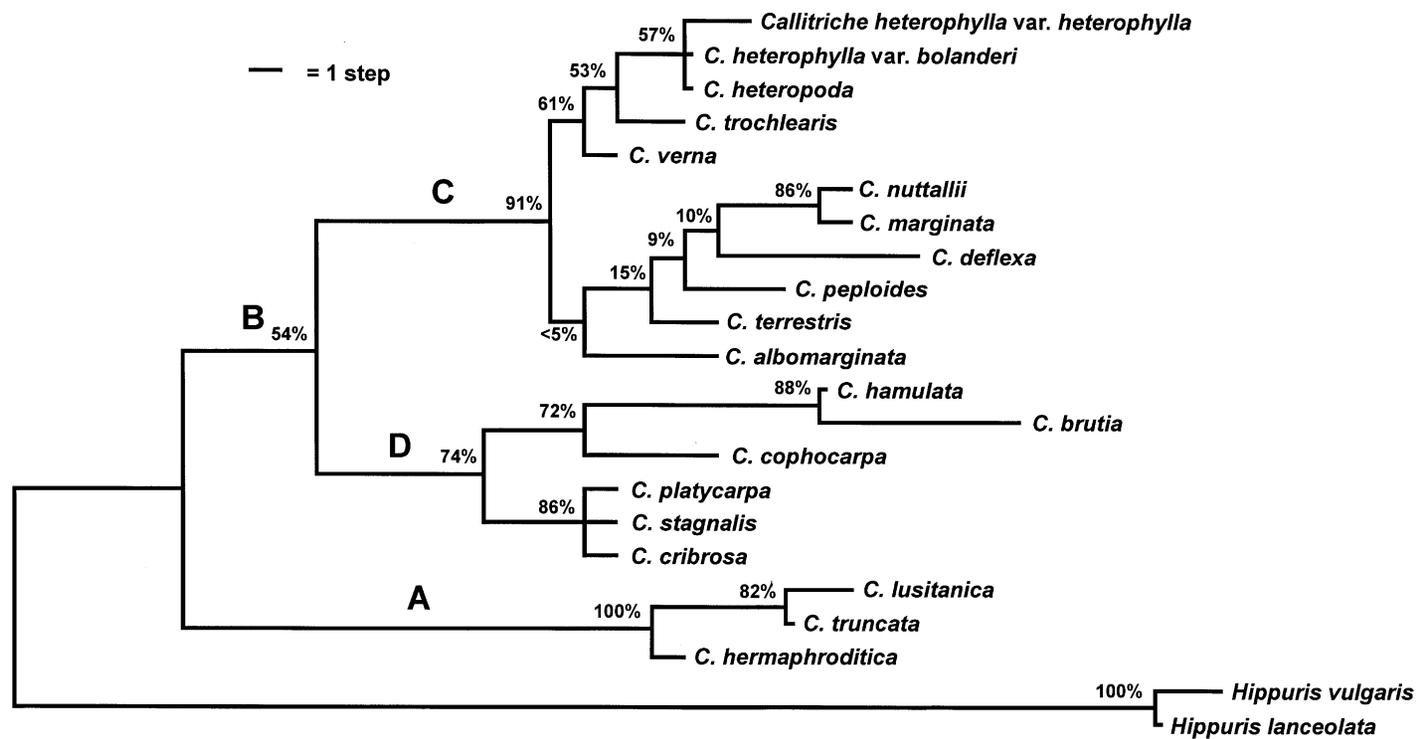
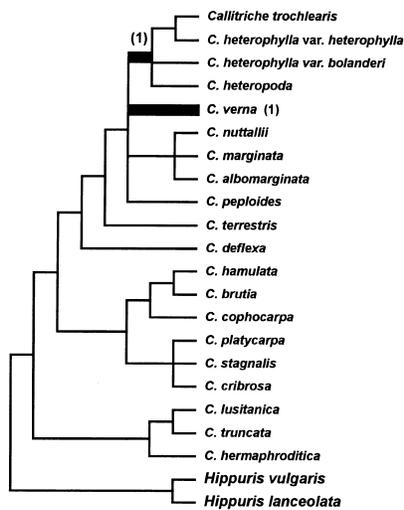
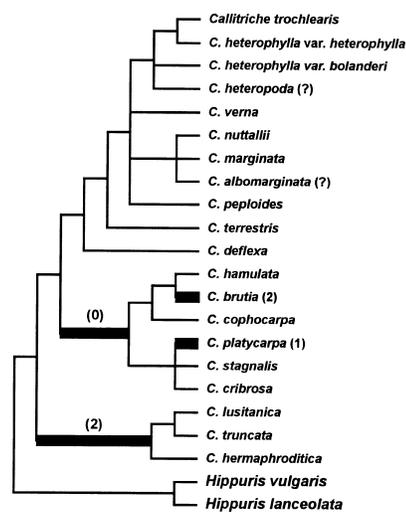


Fig. 4. The maximum parsimony tree (138 steps) based on combined morphological and molecular (*rbcL*) data (see text for discussion). The scale indicates a branch length of one step. Uppercase letters (A–D) refer to major clades discussed in the text. Bootstrap values are given for each node.

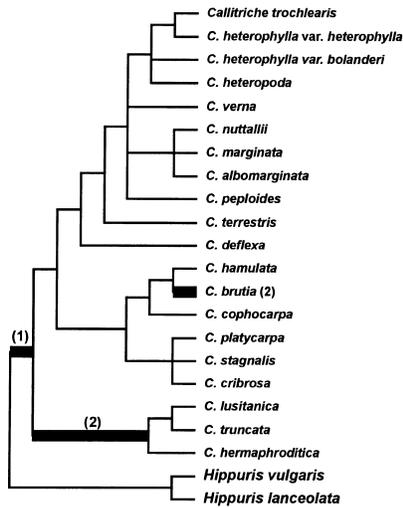
A. internal geitonogamy (ACCTRAN/DELTRAN)



B. pollen aperture (ACCTRAN/DELTRAN)



C. exine thickness (ACCTRAN)



D. exine thickness (DELTRAN)

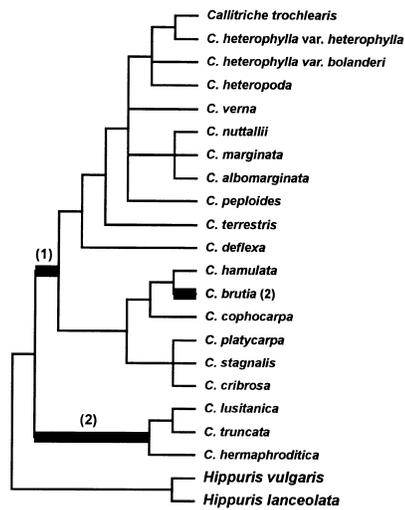


Fig. 5. A–D. States of three selected morphological characters optimized on the *rbcl* cladogram (branch lengths not proportional). State assignments (in parentheses) are shown either above branches or at terminals. ACCTRAN and DELTRAN optimizations were identical for A (internal geitonogamy) and B (pollen aperture). ACCTRAN and DELTRAN optimizations are shown for exine thickness in C and D, respectively. Thick bars indicate character state changes.

resolved by our analyses (Figs. 3 and 4). The placement of the anomalous *C. lusitanica* as monophyletic with the two species of section *Pseudocallitriche* (*C. hermaphroditica*, *C. truncata*) is the single inconsistency between our results and those of Hegelmaier (1864). Whether clades C and D (Fig. 3) should be recognized as separate sections requires analyses of the remaining species in the genus, especially species from Africa, Australia, and South America.

Schotsman (1961) recognized the anomalous features of *C. lusitanica*, which combined characters of both sections *Pseudocallitriche* and *Callitriche*. *Callitriche lusitanica* has submersed and floating rosette forms (section *Callitriche*), but lacks a terrestrial form (Schotsman, 1967). In contrast, fruits and submersed leaves of *C. lusitanica* are most like *C. hermaphroditica* (section *Pseudocallitriche*). Our results place *C. lusitanica* firmly within a monophyletic clade along with *C. hermaphroditica* and *C. truncata*.

Our results do not support recognition of Fassett's (Fassett, 1951) section *Microcallitriche*, which requires the addition of 22 steps to the shortest *rbcL* tree to constrain the section topologically as a monophyletic group.

Philbrick and Jansen (1991) conducted a study of chloroplast DNA restriction fragments of 11 primarily North American *Callitriche* taxa and rooted their cladogram using *Callitriche stagnalis* as a 'functional outgroup'. The topology generated in this study is similar to that of Philbrick and Jansen (1991), however, *Callitriche stagnalis* does not occur at the base of the phylogeny when the root is determined using members of Hippuridaceae as an outgroup.

4.2. Chromosome number evolution

A wide range of chromosome numbers occur in *Callitriche*: $n=3, 4, 5, 10, 14, 19, 20$ (see, e.g. Schotsman, 1967; Philbrick, 1994). Based on the hypothesized base number of $x=5$, there are two phyletic chromosome number series: euploid ($n=5, 10, 20$; including $n=14, 19$) and aneuploid reduction ($n=4, 3$). The aneuploid reduction series is composed of *C. hermaphroditica* ($n=3$), *C. truncata* ($n=3$), *C. cribrata* ($n=4$), *C. lusitanica* ($n=4$) and *C. pulchra* ($n=4$; not included in this study). Two species have odd-polyploid chromosome numbers: *C. brutia* ($n=14$) and *C. hamulata* ($n=19$). Hedberg and Hedberg (1977) suggested that these species are of allopolyploid origin, while Schotsman (1982) suggested autopolyploidy.

Both the *rbcL* and *rbcL*/morphology analyses indicate that polyploidy has occurred several times in *Callitriche*. Clade B possesses species that exhibit $n=4-20$. Tetraploidy has occurred at least twice in the genus; once in *C. platycarpa* and once in the putative common ancestor of *C. heterophylla*, *C. trochlearis*, *C. heteropoda*, *C. verna*, *C. nuttallii*, and *C. marginata*. Polyploidy is also associated with the clade comprising *C. brutia* and *C. hamulata* (see above). Two octoploid species occur in *Callitriche* (*C. trochlearis* and *C. antarctica*). Because it was possible to include only *C. trochlearis* in this study, the question of whether octoploidy has evolved once or twice remains unresolved.

Aneuploid reduction from $x=5$ occurred at least twice, once each in clades A and D. Clade A is characterized by aneuploid reduction and the topology in Figs. 3 and 4 indicates that aneuploid reduction from $n=5$ to 4 occurred once in the ancestor to these three species, with a subsequent reduction to $n=3$ occurring twice; i.e. independently in *C. hermaphroditica*

and *C. truncata*. Aneuploidy also occurs in clade D, as evidenced by *C. cribrosa* ($n=4$), which is monophyletic with *C. stagnalis* ($n=5$) and *C. platycarpa* ($n=10$).

4.3. Pollen structure

The phylogenetic hypotheses forwarded in this study also provide insight into the evolution of pollen characteristics in *Callitriche*. Several studies have described the pollen morphology and ultrastructure of *Callitriche* (e.g. Martinsson, 1993; Cooper et al., 2000). For example, interspecific variation in exine thickness (or absence) and the presence or absence of aperture-like regions have been observed. Pollen characteristics appear to vary with respect to growth habit (e.g. terrestrial vs. amphibious) and pollination type (e.g. anemophily vs. hypohydrophily; cf. Cooper et al., 2000). Herein, we will focus on phylogenetic patterns.

Most *Callitriche* species possess a well-developed exine (Cooper et al., 2000), although there are notable differences in exine thickness among terrestrial (thicker exines) and amphibious (thinner exines) species (Cooper et al., 2000). The terrestrial species are monophyletic (Figs. 3 and 4, clade C) with amphibious species from North and South America that possess 'reduced' exines (Cooper et al., 2000). It is evident that the terrestrial species retain the ancestral (thick) exine condition in *Callitriche*, as is indicated by the presence of a thick exine in *Hippuris* (Erdtman, 1952; Moore and Webb, 1978). Terrestrial species of *Callitriche* also occur in Australia and New Zealand (Mason, 1959). It is yet unclear whether these terrestrial species also possess thick exines or whether they share any close evolutionary relationship with the terrestrial species of North America.

Several *Callitriche* species possess a rudimentary exine (*C. hamulata*, annual ecad of *C. hermaphroditica*) or lack an exine altogether (*C. brutia*, perennial ecad of *C. hermaphroditica*, *C. lusitanica*, *C. truncata*) (Cooper et al., 2000). The reduction or loss of the exine is associated with growth habit and pollination system of the species (Cooper et al., 2000). Our analyses indicate that exine reduction has evolved at least twice in *Callitriche*; once each in clades A and D (Figs. 3 and 4). All three species in clade A either lack or possess but a rudimentary, exine. Exine loss (or extreme reduction) is also synapomorphic for *C. brutia* and *C. hamulata*.

Some authors (see, e.g. Clapham et al., 1962) have considered *C. brutia* and *C. hamulata* to be subspecies of *C. intermedia* Hoffm. We have followed the taxonomy of (Schotsman, 1967) who considered them as distinct species, but emphasize that our analyses did not reveal any molecular (*rbcL*) apomorphies that would distinguish them. Further study is necessary to clarify whether *C. brutia* and *C. hamulata* are distinct species or simply represent different ecads of the same species, with *C. brutia* exhibiting an exineless form, similar to *C. hermaphroditica*.

The presence of aperture-like areas in the pollen (Cooper et al., 2000) does not follow a clear phylogenetic pattern. It is reasonable to consider that aperturate pollen is the plesiomorphic condition in *Callitriche*, because species of *Hippuris* (the outgroup) have distinctly aperturate (zonocolpate) pollen (Moore and Webb, 1978). Terrestrial species, such as *C. deflexa* and *C. nuttallii*, possess the thickest exines and aperture-like areas (Cooper et al., 2000). Amphibious species have reduced exines, but their possession of aperture-like regions varies. All species in clade C (Fig. 3) possess aperture-like regions (note that *C. albomarginata* was not studied by Cooper et al., 2000). In contrast, only one species

(*C. platycarpa*) in clade D has aperture-like regions with the remainder being inaperturate. Both ACCTRAN and DELTRAN analyses herein (Fig. 5B) indicate the most parsimonious explanation is that aperture-like regions were lost in the ancestor of clade D, with *C. platycarpa* either retaining the ancestral state or exhibiting an evolutionary reversal back to it.

4.4. Pollination system evolution

Anemophily, which is common in angiosperms, occurs in Hippuridaceae and is likely the ancestral pollination system in *Callitriche*. In contrast, hydrophily occurs in only eight angiosperm families, the majority being monocots (Les et al., 1997). Only two dicot families possess hypohydrophilous species: Ceratophyllaceae (exclusively hypohydrophilous) and Callitrichaceae. *Callitriche* is the only genus with both aerial and hydrophilous pollination systems (see discussion in Philbrick and Anderson, 1992; Philbrick, 1993; Les et al., 1997).

The phylogenetic hypotheses proposed here provide a framework for exploring pollination system evolution in *Callitriche*. *Callitriche* includes both anemophilous and hypohydrophilous pollination systems (Philbrick and Anderson, 1992; Philbrick, 1993). Most species produce aerial flowers and are assumed to be anemophilous. Two species (*C. hermaphroditica*, *C. truncata*) produce obligately submersed flowers. Although both are reportedly hypohydrophilous, data indicating underwater outcrossing are available only for *C. hermaphroditica* (Philbrick, 1993). Nonetheless, circumstantial evidence (e.g. similar floral developmental patterns, style orientation, pollen structure) suggests that hypohydrophily also occurs in *C. truncata* and perhaps *C. lusitanica*, although the latter can also produce aerial flowers.

Phylogenetic analyses of *rbcL* data (Fig. 3) and combined *rbcL*/morphological data (Fig. 4) conclusively place *C. hermaphroditica*, *C. truncata* and *C. lusitanica* in the same clade. The mechanisms of pollination in *C. truncata* and *C. lusitanica* are uncertain, although their occurrence in this clade with *C. hermaphroditica* could indicate similarities in pollination type as well (yet the mixture of submersed and aerial flowers in *C. lusitanica* is problematic in this regard). Regardless, it is most likely that hypohydrophily evolved only once in the genus, either strictly in *C. hermaphroditica* or in the ancestor of clade A.

4.5. Geitonogamy and internal geitonogamy

Geitonogamy is predominant in *Callitriche* (e.g. Schotsman, 1985; Philbrick and Anderson, 1992) and is promoted by the proximity of stamen and style in adjacent flowers, either aerial or submersed. A unique form of geitonogamy ('internal geitonogamy', IG) occurs in aerial and submersed flowers of seven *Callitriche* species (Philbrick and Anderson, 1992; Philbrick and Bernardello, 1992). IG occurs in two North American species (*C. heterophylla*, *C. trochlearis*), four South American species (*C. nubigena*, *C. heteropoda*, *C. lechleri*, *C. rimosa*), and in the geographically widespread *C. verna* (Philbrick and Bernardello, 1992). Morphological and combined *rbcL*/morphological data analyses suggest monophyly for the four IG species included in this study (*C. heterophylla*, *C. heteropoda*, *C. trochlearis* and *C. verna*; Figs. 3 and 4). The phylogenetic position of *C. verna* is unresolved by *rbcL* data alone. The only apparent feature supporting the monophyly of these four IG species

in the combined morphological/*rbcL* data analyses, is the occurrence of IG. The removal of this character (avoiding circularity) results in the topology indicated in Fig. 3. Philbrick and Jansen (1991) reported the monophyly of three IG species (*C. heterophylla*, *C. verna*, *C. trochlearis*), based on RFLP analyses of chloroplast DNA. Although the selection of taxa in that study was limited, our combination of the RFLP and *rbcL* data (both surveying variation in the chloroplast genome) resulted in a tree which was completely congruent with the *rbcL* tree (Fig. 4), but showed no resolution for any of the non-IG species of clade C (results not shown). The addition of the chloroplast RFLP data resulted in the monophyly (84% bootstrap support) of the four IG species (including *C. verna*); hence, we conclude that the system has evolved only once in *Callitriche*, at least with respect to the four species that we have surveyed.

4.6. Growth habit evolution in *Callitriche*

There is no doubt that the aquatic growth habit in angiosperms is derived from terrestrial ancestors (e.g. Arber, 1920; Sculthorpe, 1967; Cook, 1996). Cook (1996) proposed that the extant aquatic flora reflects 100+ separate invasions of the aquatic environment by terrestrial plant lineages. The widespread nature of derived aquatic habits, however, does not preclude the possibility of reversals; i.e. an aquatic lineage giving rise to terrestrial descendants.

The occurrence of terrestrial *Callitriche* species in phylogenetically derived positions, and given that the outgroup (Hippuridaceae) is completely aquatic, indicates the possibility that terrestrial species of *Callitriche* have aquatic ancestors. However, an interpretation of growth habit evolution in *Callitriche* is complicated by several factors. First, growth habits are diverse in the genus (see above). Secondly, 'terrestrial' entails a complex suite of structural (e.g. stomate and cuticle formation, attributes of producing rigid stems and leaves) and physiological characters (e.g. ability to withstand desiccation and high-ultraviolet radiation). How these features relate to the various ecads in these polymorphic plants are yet poorly understood. Consequently, even in light of our phylogenetic insight, we cannot assess definitively the polarity of growth habit evolution in *Callitriche*.

5. Conclusions

Callitriche exhibits remarkable diversity of growth habit, chromosome number and pollination system variation. Herein, we present the most comprehensive phylogenetic analysis of the genus yet conducted and propose that the submersed growth habit, hypohydrophily and internal geitonogamy have each evolved once. In contrast, aneuploid reduction, polyploidy and reduction in pollen exine thickness have evolved at least twice in the genus.

This study focused on species from the Mediterranean region, North America and, to a lesser degree, South America. Species from Africa, Asia and Australia need to be added. Of particular interest are the relationships among terrestrial species in Australia and the Americas, which remain to be resolved. Even so, this study provides general support for a two-section classification (Section *Callitriche*, Section *Pseudocallitriche*) in *Callitriche*.

The natural history of pollination and its relationship with aneuploidy in some species of *Callitriche* is intriguing. Both aerial and underwater pollination occur, yet floral structures are qualitatively similar throughout the genus. Phylogenetic insight serves as a framework with which to interpret evolutionary patterns, e.g. hypotheses of evolutionary polarity of character change. Even so, the details of how pollination occurs and how seemingly similar floral features accommodate both aerial and submersed pollen transport and capture remain poorly understood. Further understanding of character state change as it relates to floral function is needed before a satisfying view of pollination system evolution in *Callitriche* can be achieved.

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Appendix A

Data matrix for the 17 morphological characters analyzed in this study for species of *Callitriche* and *Hippuris*. ?=character state not known.

		Character number
		12345678911111111
		01234567
1.	<i>C. albomarginata</i>	?1000011??01111
2.	<i>C. brutia</i>	0001111122?201111
3.	<i>C. cophocarpa</i>	11000001110001111
4.	<i>C. cribrosa</i>	11010001110001111
5.	<i>C. deflexa</i>	10010001100101111
6.	<i>C. hamulata</i>	01010011112001111
7.	<i>C. hermaphroditica</i>	0001001022?201111
	<i>C. heterophylla</i>	

8.	<i>var. heterophylla</i>	11100001111101111
9.	<i>var. bolanderi</i>	11100001111101111
10.	<i>C. heteropoda</i>	1110000111??01111
11.	<i>C. lusitanica</i>	1001001122?201111
12.	<i>C. marginata</i>	11011101100111111
13.	<i>C. nuttallii</i>	10011111100111111
14.	<i>C. peploides</i>	10000101100101111
15.	<i>C. platycarpa</i>	11010001110101111
16.	<i>C. stagnalis</i>	11010001100001111
17.	<i>C. terrestris</i>	1000010111?101111
18.	<i>C. truncata</i>	0001001022?201111
19.	<i>C. trochlearis</i>	11110001110101111
20.	<i>C. verna</i>	11110001110101111
21.	<i>H. vulgaris</i>	1?0000010??100000
22.	<i>H. lanceolata</i>	1?0000010??100000

Appendix B

Voucher specimens for the taxa studied for *rbcL* analyses. One voucher specimen for each species is listed. All voucher specimens are deposited in the herbarium of Western Connecticut State University unless otherwise noted

1. *Callitriche albomarginata* Fassett: Bolivia, Dept. Cochabamba, 15 May 1995, Ritter 2098 (NHA).
2. *Callitriche brutia* Petagn.: Spain, Madrid Parish, 17 June 1994, Philbrick 4568.
3. *Callitriche cophocarpa* Sendtn.: Sweden, Uppsala, 12 June 1994, Philbrick 4563.
4. *Callitriche cribrata* Schotsman: Spain, Badajoz Province, 24 June 1994, Philbrick 4599.
5. *Callitriche deflexa* A. Br. ex Hegelm.: Mexico, Jalisco, 21 March 1993, Philbrick 3223.
6. *Callitriche hamulata* Kutz.: England, Derby Co., 8 June 1994, Philbrick 4550.
7. *Callitriche hermaphroditica* L.: Russia, region of Novosibirsk (Karasuk River), 7 July 1993, Philbrick 4503.
8. *Callitriche heterophylla* var. *heterophylla* Pursh.: United States of America, Washington, Gray's Co., 22 June 1987, Philbrick 2099.
9. *Callitriche heterophylla* var. *bolanderi* (Hegelm.) Fassett: United States of America, California, Modoc Co., 10 June 1990, Philbrick 2255.
10. *Callitriche heteropoda* Engelm.: Bolivia, Dept. Cochabamba, 5 July 1994, Ritter 1220 (NHA).
11. *Callitriche lusitanica* Schotsman: Spain, boundary of Huelva and Badajoz Provinces, 24 June 1994, Philbrick 4597.
12. *Callitriche marginata* Torr.: United States of America, California, Riverside Co., 28 March 1990, Philbrick 2204.

13. *Callitriche nuttallii* Torr.: United States of America, Alabama, Mobile Co., 13 March 1988, Philbrick & Haynes 2128.
14. *Callitriche peploides* Torr.: United States of America, Alabama, Mobile Co., 13 March 1988, Philbrick & Haynes 2127.
15. *Callitriche platycarpa* Kutz.: England, Devon, 6 June 1994, Philbrick 4534.
16. *Callitriche stagnalis* Scop.: United States of America, Washington, Coos Co., 11 June 1987, Philbrick 2057.
17. *Callitriche terrestris* Raf.: United States of America, Mississippi, Lowndes Co., Philbrick, McDaniel & Haynes 2143.
18. *Callitriche trochlearis* Fassett: United States of America, California, Mendocino Co., 8 June 1987, Philbrick 2043.
19. *Callitriche truncata* Guss.: Spain, boundary of Huelva and Badajoz Provinces, 24 June 1994, Philbrick 4595.
20. *Callitriche verna* L.: Russia, region of Novosibirsk (Lake Berchikuh), 14 July 1993, Philbrick 4507.
21. *Hippuris vulgaris* L.: United States of America, California, Mono Co., 4 June 1990, Philbrick 2226.
22. *Hippuris lanceolata* Retz.: Sweden, Uppsala (cultivated), 13 June 1994, Philbrick 4567.

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