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Systematics of Two Imperiled Pondweeds (*Potamogeton vaseyi*, *P. gemmiparus*) and Taxonomic Ramifications for Subsection *Pusilli* (Potamogetonaceae)

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Abstract*—Potamogeton* is a taxonomically problematic genus of aquatic monocotyledons, which has received limited phylogenetic study using molecular data. The group is known for extensive vegetative plasticity, confusing patterns of morphological variation and propensity for hybridization. *Potamogeton gemmiparus* and *P. vaseyi* are of conservation concern in North America where both are listed as imperiled. These vegetatively similar species are particularly difficult to distinguish in the absence of floating leaves. We studied both species and putatively related taxa in subsection *Pusilli* (e.g. *P. clystocarpus*, *P. foliosus*, and *P. pusillus*) to elucidate relationships and to develop an identification method using molecular markers. Phylogenetic analyses of nrITS and *trnK* 5′ intron sequence data clearly endorse the recognition of *P. gemmiparus* and *P. vaseyi* as distinct species but call into question the subspecific circumscription of *P. pusillus* currently followed in North America. Our data resolved *P. pusillus* in a clade with *P. foliosus*, separated substantially from *P. berchtoldii* (= *P. pusillus* subsp. *tenuissimus*), thus supporting the recognition of *P. berchtoldii* as a distinct species. Using molecular cloning techniques, we documented three clear examples of interspecific hybridization (*P. foliosus* × *P. pusillus*, *P. berchtoldii* × *P. vaseyi*, and *P. gemmiparus* × *P. vaseyi*). Simple DNA polymorphisms also indicated several *P. berchtoldii* × *P. gemmiparus* hybrids. The narrowly distributed *P. gemmiparus* and *P. clystocarpus* are similar morphologically and genetically to the wide-ranging *P. berchtoldii*, with which they both hybridize. We recommend either the recognition of *P. gemmiparus* and *P. clystocarpus* as distinct species, or more suitably as subspecies of *P. berchtoldii*, for which two new combinations are provided: ***P. berchtoldii*** subsp. ***gemmiparus*** and ***P. berchtoldii*** subsp. ***clystocarpus***.

***Keywords*—**aquatic plants, concerted evolution, hybridization, internal transcribed spacer, North American flora, *trnK* intron.

Many aquatic plants are difficult to identify accurately because of their frequent lack of flowers or fruits, high degree of phenotypic plasticity, extensive morphological reduction, and convergence in features adapted for life in water. In particular, two northeastern North American aquatic plant species *Potamogeton vaseyi* J. W. Robbins and *P. gemmiparus* (J. W. Robbins) J. W. Robbins ex Morong are quite similar vegetatively (see Figs. 20–21 in Hellquist and Crow 1980; Fig. 379 in Crow and Hellquist 2000) and identifications based solely upon morphological characteristics often are inconclusive. Because both taxa are imperiled throughout much of their range, their precise identification is of critical importance to various conservation programs.

Potamogeton gemmiparus (G5T3; vulnerable globally) is listed as critically imperiled (S1) in Connecticut, Rhode Island, and Quebec, vulnerable (S3) in Massachusetts, and possibly extirpated (SH) in New Hampshire (NatureServe 2008). It occurs also in Maine, where its imperilment status remains unranked. *Potamogeton vaseyi* (G4; apparently secure globally) is secure only in two states/provinces (New York, Ontario) but is imperiled elsewhere throughout its range, i.e.: S1 (Connecticut, Indiana, Iowa, Massachusetts, New Hampshire, and Pennsylvania), S2 (Minnesota), S3 (Maine, Quebec, Vermont, and Wisconsin); it is presumed to be extirpated (SX) from Illinois and possibly extirpated (SH) from Michigan, New Brunswick, New Jersey, and Ohio (NatureServe 2008).

Both Haynes and Hellquist (2000) and Crow and Hellquist (2000) recognized *P. vaseyi* and *P. gemmiparus* as distinct taxa in their most recent taxonomic and nomenclatural overviews of the genus *Potamogeton* in North America, although they assigned the latter to *P. pusillus* L. subsp. *gemmiparus* (J. W. Robbins) R. R. Haynes & C. B. Hellquist. Those treatments separated the taxa by their presence (*P. vaseyi*) or absence (*P. gemmiparus*) of floating leaves, despite the fact that "plants without floating leaves are easily confused" (Hellquist and Crow 1980; p. 36). Haynes and Hellquist (2000; p. 63) also remarked that "sterile collections of either taxon can easily be mistaken for the other." Moreover, their taxonomic keys contain caveats with respect to floating leaves, which in *P. vaseyi* are described as "present, at least in some plants in population" as opposed to *P. pusillus* (all subspecies; including *P. gemmiparus*) with "floating leaves absent from all plants in population" (Haynes and Hellquist 2000; p. 51). Similar sentiments were expressed by Crow and Hellquist (2000; pp. 42–43), who characterized *P. pusillus* (all subspecies; including *P. gemmiparus*) as having "floating leaves absent" as compared to *P. vaseyi* with "floating leaves present, at least on some plants." Thus, plants lacking floating leaves cannot reasonably be assigned to either taxon on the basis of this character alone. Such discrepancies are frustrating to researchers who typically cannot conduct large-scale population surveys at the time of identification or those who wish to identify single herbarium specimens. Furthermore, the lack of floating leaves in *P. vaseyi* is neither exceptional nor necessarily atypical. Morong (1893) observed that floating leaves were produced only on the fertile stems of *P. vaseyi* (in shallow water near the shore) but that plants were more common in deeper waters (> 2 m), where they lacked floating leaves and remained sterile.

Most secondary vegetative characters offer no clarification. Fernald (1932; pp. 68, 95) noted that paired nodal glands occurred "usually" in *P. gemmiparus* and "sometimes" in *P. vaseyi*. In *P. gemmiparus*, the submersed leaves are described as 0.2–0.7 mm wide and one-veined; whereas, those of *P. vaseyi* are 0.1–1.0 mm wide and 1(-3)-veined (Crow and Hellquist 2000; Haynes and Hellquist 2000). The nearly complete overlap of such characteristics makes it difficult to apply them taxonomically to these taxa in the absence of floating leaves; in that case, there are no other reliable distinguishing features (R. R. Haynes, personal communication). Fernald (1932) suggested that sterile material of *P. vaseyi* was distinguishable from *P. gemmiparus* by the position of the turions, which occur

on short, diverging axillary branchlets in the former and on more elongate, ascending branchlets in the latter. However, even this potentially informative character is of little use when turions are absent on a specimen.

The difficulty of identifying these pondweeds is not only problematic but also raises some questions regarding their taxonomic affinity. One possibility is that these taxa are not distinct but represent conspecific variants that differ primarily by the presence or absence of floating leaves. Reports that the two taxa often grow intermixed within a single locality (e.g. Haynes and Hellquist 2000) have heightened suspicions in this regard. Hagström (1916) viewed *Potamogeton gemmiparus* and *P. vaseyi* as dissimilar enough to warrant their assignment to different subsections within section *Axillares* Hagstr. (subsection *Pusilli* Graebner and subsection *Javanici* Graebner respectively). Yet, because of their "great resemblance", Hagström (1916; p. 94) regarded *P. gemmiparus* as having originated through hybridization with *P. vaseyi*, a possibility that has been dismissed by most researchers, but one that deserves further evaluation.

In such instances, analysis of DNA sequence data can provide an effective method for testing hypotheses and has been used successfully to clarify taxonomic questions in other groups of morphologically problematic aquatic plants such as *Aponogeton* (Les et al. 2005), *Callitriche* (Philbrick and Les 2000), *Glossostigma* (Les et al. 2006), *Vallisneria* (Les et al. 2008), and even the minute Lemnaceae (Les et al. 2002). Molecular data also have been used effectively to detect and document hybrids in various aquatic plants (Les et al. 2004, 2005; Moody and Les 2002) including *Potamogeton* (Du et al. 2009).

Although several molecular studies of *Potamogeton* already have been carried out (Iida et al. 2004; Whittall et al. 2004; Lindqvist et al. 2006; Zhang et al. 2008), only that by Lindqvist et al. (2006) included material of *P. vaseyi* and *P. gemmiparus*. In that study, data from the 5S-NTS locus indicated that *P. vaseyi* and *P. gemmiparus* were distinct, but potentially closely related. However, because Lindqvist et al. (2006) analyzed only one locus and a single accession for each of these taxa, the incorporation of additional molecular sequences along with a broader survey of populations is necessary for corroboration. Furthermore, it is likely that much material of these taxa has been misidentified in the past, which underscores the need for more critical evaluation and broader surveys using molecular data analysis.

In this study we have analyzed DNA sequence data for an expanded survey of North American populations, to evaluate further the distinctness of *P. vaseyi* and *P. gemmiparus*, and to establish effective molecular markers to facilitate their identification. This project was initiated to provide the State of Connecticut Endangered Species Program with a means of clarifying the taxonomic disposition of these two state endangered species. The resulting information should assist agencies in Connecticut and elsewhere with their efforts to conserve these imperiled taxa, update endangered species lists, and make more informed management decisions in issues where either taxon is involved.

Materials and Methods

We analyzed a total of 65 accessions of plants that were collected by us, sent to us by colleagues, or retrieved from GenBank (Appendix 1). From subsection *Pusilli* we evaluated 26 accessions of *P. berchtoldii*, two accessions of *P. clystocarpus* (closely related to *P. foliosus* and *P. pusillus*; Whittall et al. 2004), five accessions of *P. foliosus*, seven accessions of *P. gemmiparus*, and five accessions of *P. pusillus*. From subsection *Javanici* we evaluated 16 accessions of *P. vaseyi* and one accession each of *P. cristatus* and *P. octandrus*. *Potamogeton zosteriformis* and the closely related *P. compressus* (one accession each; both section *Axillares* subsection *Compressi*) comprised the outgroup for phylogenetic analyses, guided by the results of Lindqvist et al. (2006). Three accessions (identified initially as either *P. pusillus* or *P. vaseyi*) later were determined to represent several interspecific hybrids. Aside from our own accessions, sequences of closely related taxa were not available from GenBank for *trnK* 5′ intron data, so we rooted the trees in the combined data analysis in accordance with the topology generated by the nrITS data. Voucher specimens for all newly acquired collections were deposited at the University of Connecticut (CONN) herbarium, and associated sequences were accessioned in GenBank under the following series: GQ247388-GQ247521.

We extracted total genomic DNA from all accessions (fresh plant material) using the CTAB method of Doyle and Doyle (1987) modified as described by Les et al. (2008). The polymerase chain reaction (PCR) was used to amplify the nuclear ribosomal internal transcribed spacer region (nrITS) and the chloroplast (cpDNA) *trnK* 5′ intron for subsequent DNA sequence analysis. Amplification and sequencing followed the methods described in Les et al. (2008), using the following primer sequences for *trnK*: 0801R: 5′-TACTCCTGAAATAGAAGCGG-3′; DL19: 5′-AGTACTCGGCTTTTAAGTGC-3′. For nrITS we used ITS5 and ITS4 as described in Baldwin (1992). All sequences were aligned manually using MacClade 4.06 (Maddison and Maddison 2000). Simple indel coding (Simmons and Ochoterena 2000) was used to codify sequence "gaps" or "indels" (insertions/deletions) in the aligned nrITS DNA data matrix. Aligned sequence and indel data were deposited in TreeBASE (study number S2400).

The nrITS sequences were carefully screened for polymorphisms and signal degradation as potential indicators of hybridization. Two problematic polymorphic accessions (Mudge Pond, Connecticut; Wilson Pond, Maine) were subcloned using the TOPO TA Cloning® Kit with pCR®2.1-TOPO® Vector (Invitrogen Corporation, Carlsbad, California), and then amplified and sequenced as already described. Another polymorphic sequence (Stark Pond, Minnesota) was "pseudo-cloned", i.e. a large and consistent difference in signal strength between the two competing sets of chromatogram peaks easily allowed us to distinguish each allelic sequence visually, precluding the need for molecular cloning. The cloned nrITS sequences for each accession were grouped according to their similarity to putative parental taxa, and a consensus sequence for each group was included in phylogenetic analyses (of nrITS data). In cases where "chimeric" alleles were retrieved (Álvarez and Wendel 2003), they were assigned to the parental sequence providing the largest number of matches among sites at which the parental species differed. Six accessions (Quaddick Reservoir, Connecticut; Wilson Pond, Maine; Long Lake, Minnesota; Bearcamp Pond, Pemigewasset Lake, Kanasatka Lake, New Hampshire) each exhibited a nucleotide polymorphism at a single site. Because assignment of the two allelic sequences was unambiguous in these instances, these polymorphic accessions were not cloned.

The nrITS and *trnK* 5′ intron sequences were partitioned separately to facilitate their analysis as both independent and combined data. We first conducted a maximum parsimony (MP) analysis of each data partition using PAUP* v4.0b10 (Swofford 2002). The analyses implemented a heuristic search (100 replicates of random stepwise addition; multrees option; maxtrees increased automatically) and used *Potamogeton compressus* and *P. zosteriformis* as outgroups. Using this program we conducted a partition-homogeneity test (incongruence length-difference test or ILD; Farris et al. 1994) to evaluate the homogeneity of the two molecular data partitions (threshold = $p < 0.001$) after removing invariant and uninformative sites as recommended by Lee (2001). Bootstrap values (one random sequence addition per replicate; full heuristic search; TBR branch swapping; 1,000 replicates; maxtrees = 1,000) were used to evaluate the level of internal branch support.

Data also were evaluated by Bayesian analysis using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Appropriate evolutionary models (under the AIC criterion) were selected for each data partition using the program Modeltest (Posada and Crandall 1998; Posada and Buckley 2004). We used models that were closest to (*trnK*: GTR) or the same as (nrITS: GTR + Γ) those indicated by this analysis. All combined analyses were conducted while retaining the appropriate model for each data partition, with nonmolecular data (indels) analyzed as a "standard" datatype in MrBayes. Markov Chain Monte Carlo was implemented with four separate runs with four heated chains each, and trees were sampled every 1,000th generation for 2,000,000 generations. The first 500,000 generations were discarded as burn-in.

Results were depicted as phylogenetic tree diagrams (maximum parsimony cladograms or phylograms) showing relationships of taxa based on their DNA sequences (both nuclear and combined nuclear/chloroplast data) with nodal support indicated by both bootstrap and Bayesian posterior probability values.

Specimens in each of the major clades recovered were evaluated taxonomically using the morphological features emphasized by Fernald (1932), Haynes and Hellquist (2000), and Crow and Hellquist (2000).

Results

Analysis of the nrITS data (71 OTUs; 733 characters; 24 parsimony-informative; 2.6% missing data cells excluding gap characters) resulted in 44 equally most-parsimonious trees (49 steps) with the following statistics: consistency index (CI) = 0.94, CI excluding uninformative sites (CI_{exc}) = 0.90, retention index (RI) = 0.99. The *trnK* data included 745 characters (eight parsimony-informative; 3.6% missing data cells excluding gap characters). Results of the ILD test ($p = 1.000$) indicated complete agreement between the nrITS and cpDNA data, which justified their analysis as combined data. The combined analysis (52 OTUs, 1,477 characters; 27 parsimony-informative) resulted in 226 equally most-parsimonious trees (31 steps) with CI = 1.00, CI_{exc} = 1.00, RI = 1.00 (Figs. 1–2).

Potamogeton octandrus resolved as the sister to *P. vaseyi* in our nrITS analyses. The *P. pusillus*/*P. foliosus* clade resolved as the sister group to the *P. octandrus* + *P. vaseyi* clade; sister to this large clade was *P. cristatus*; the *P. berchtoldii* clade (including *P. clystocarpus* and *P. gemmiparus*) resolved as the sister group to the five previously mentioned species (Fig. 1). Phylogenetic analysis confirmed the distinctness of *P. gemmiparus* and *P. vaseyi*, which were highly divergent genetically (Fig. 1). *Potamogeton gemmiparus* and *P. vaseyi* differed by five nucleotides for *trnK* and 12 nucleotides for the nrITS region (17 total differences for the combined *trnK*/nrITS data). *Potamogeton berchtoldii* differed from *P. clystocarpus* by a single indel and from *P. gemmiparus* by a single nucleotide (nrITS data).

In addition to nucleotide substitutions, several diagnostic indels were observed in the aligned nrITS sequence data. A one base-pair (bp) indel situated at position 244 of the nrITS alignment was inserted in species of the *P. berchtoldii* clade (including *P. clystocarpus* and *P. gemmiparus*) and deleted in all other taxa analyzed. A 15-bp indel (alignment position 593, ITS-2 region) was inserted in the *P. berchtoldii* clade, *P. cristatus*, and the outgroup, but was deleted in *P. foliosus*, *P. octandrus*, *P. pusillus*, and *P. vaseyi*. *Potamogeton clystocarpus* had a unique 2-bp deletion at alignment position 514 (Whittall et al. 2004), and a 4-bp indel (at alignment position 460) was uniquely inserted in the subclade of *P. foliosus* that included two plants from Minnesota and one of the cloned sequences from Mudge Pond, Connecticut. One other indel (a 1-bp insertion at position 251) was confined to the two outgroup sequences.

One GenBank nrITS sequence of material collected in Texas and reported by Whittall et al. (2004) as *P. pusillus* (AY714288) was identical to those of *P. berchtoldii* and did not fall within the clade of other *P. pusillus* sequences representing material from Connecticut, Minnesota and China. Presumably it represents a misidentified accession. However, the nrITS sequences of Texas *P. foliosus* material (AY714292, AY714293; Whittall et al. 2004) did fall within the same clade as Connecticut material of that taxon.

The major clades recovered by DNA sequence analysis were consistent morphologically (Table 1; Fernald 1932; Haynes and Hellquist 2000; Crow and Hellquist 2000). The *P. vaseyi* clade contained plants without nodal glands and with fine submersed leaves (< 1 mm wide), axillary turions and small floating leaves (in some). The *P. foliosus* clade contained plants lacking nodal glands, but with open stipular sheaths and capitate inflorescences bearing keeled fruits. The *P. pusillus* clade contained plants with paired (but sometimes inconspicuous) nodal glands, fused stipular sheaths, leaves with two rows of lacunae along each side of the midrib, and inflorescences with interrupted whorls bearing unkeeled, obovoid fruits with concave sides. The *P. berchtoldii* clade contained plants with paired nodal glands, open stipular sheaths, leaves with 2–3 rows of lacunae along each side of the midrib, and continuous inflorescences bearing unkeeled, ovoid fruits with rounded sides. The *P. berchtoldii* clade also contained specimens of *P. gemmiparus*, which differed from *P. berchtoldii* primarily by their single-veined submersed leaves. Specimens of *P. gemmiparus* resembled plants of *P. vaseyi* that lacked floating leaves, but were distinguishable from those by their terminally disposed turions and presence of nodal glands.

We did not examine the voucher specimens of GenBank sequences for *P. clystocarpus*, which also fell within the *P. berchtoldii* clade (Fig. 1). However, published descriptions of *P. clystocarpus* (e.g. Fernald 1932) indicate a high degree of similarity with *P. berchtoldii* (e.g. stems with paired nodal glands and open stipular sheaths, three-veined leaves with up to four rows of lacunae along each side of the midrib, and axillary or terminal, continuous inflorescences). Reportedly, these species differ by the presence of keeled fruits with basal tubercles in the former.

Subcloning and sequencing revealed that two polymorphic accessions (identified initially as "*P. vaseyi*" from Wilson Pond, Maine and "*P. pusillus*" from Mudge Pond, Connecticut) with severe nrITS sequence corruption represented interspecific hybrids; i.e. sequences of cloned alleles recovered from single hybrid plants matched the different alleles fixed in distinct parental taxa (Fig. 1). The first hybrid involved *P. vaseyi* and *P. gemmiparus* and the second *P. foliosus* and *P. pusillus*. In the former case the same hybrid alleles were observed from three different accessions cloned and sequenced from the same lake in two consecutive years. Pseudocloned alleles from a polymorphic accession from Stark Pond, Minnesota (identified initially as "*P. vaseyi*") indicated that these plants actually were hybrids of *P. berchtoldii* and *P. vaseyi* (Fig. 1), with the former contributing a stronger chromatogram signal. In hybrid cases involving *P. vaseyi*, the maternal parents (as indicated by the maternally-inherited *trnK* 5′ intron data) were *P. berchtoldii* or *P. gemmiparus*. The maternal parent of the *P. foliosus* × *P. pusillus* hybrid could not be elucidated by our cpDNA data, which were identical for these two species. Several intermediate "chimeric" clones were recovered from both subcloned hybrid accessions (Tables 2–3), indicating that the nrITS region had not yet undergone complete homogenization in either hybrid (Álvarez and Wendel 2003).

A few accessions of *P. berchtoldii* (Wilson Pond, Maine; Bearcamp Pond, Pemigewasset Lake, New Hampshire) exhibited a C/T polymorphism in nrITS at the single nucleotide position that differentiated *P. gemmiparus* from *P. berchtoldii*, which indicated hybridization. In one case, a single-nucleotide substitution observed in *P. berchtoldii* from Coventry Lake, Connecticut occurred as a C/G polymorphism in one accession of *P. berchtoldii* from Quaddick Reservoir, Connecticut, which indicated intraspecific recombination.

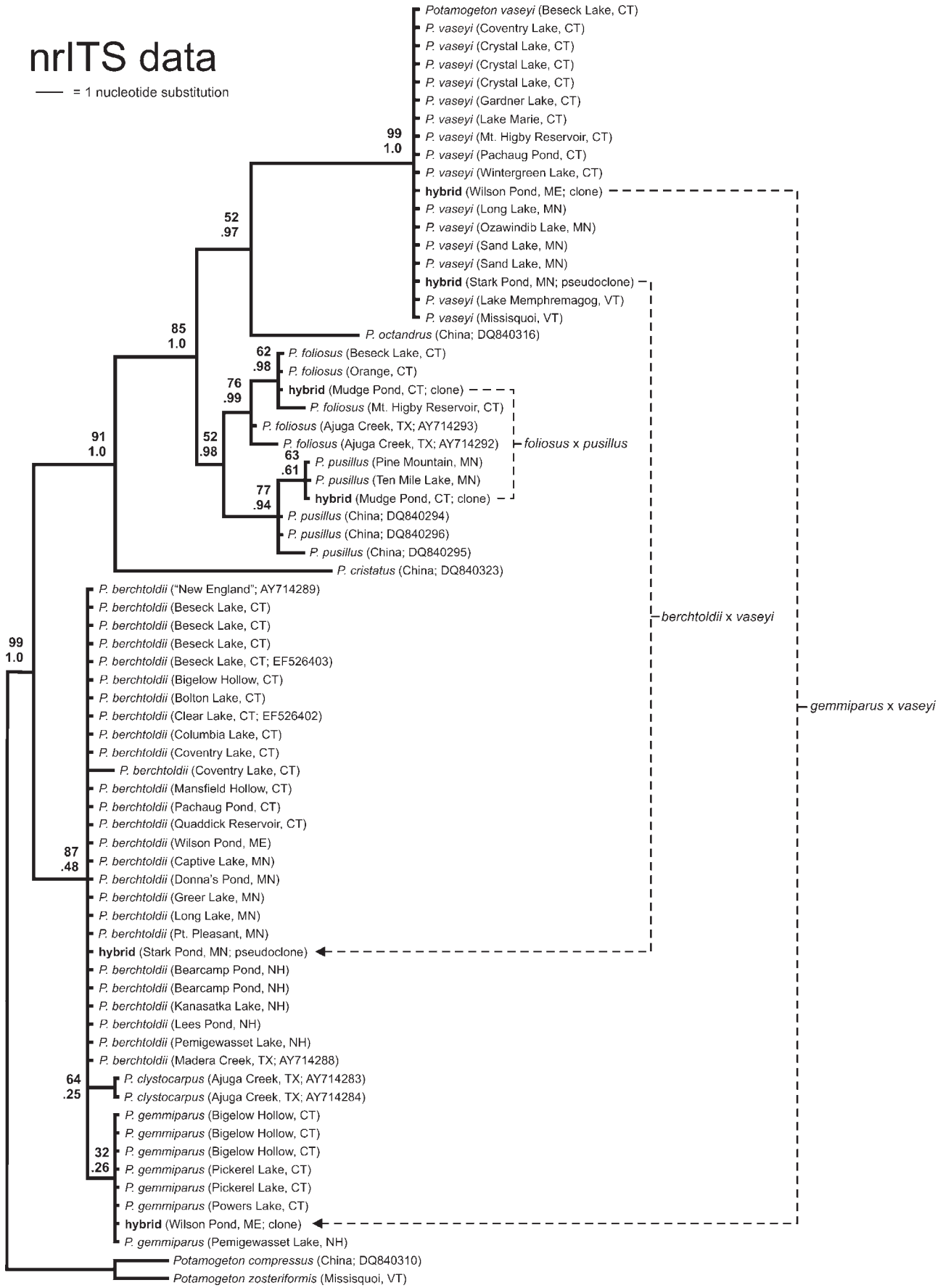

FIG. 1. One of 44 equally parsimonious trees derived from MP analysis of 71 nrITS sequences from *Potamogeton* species and hybrids (only the *P. gemmiparus* clade collapses in the strict consensus tree due to three polymorphic *P. berchtoldii* accessions). Bootstrap values (upper) and Bayesian posterior probabilities (lower) are shown for all branches. Branch lengths are proportional to the scale indicated. The parentages of three hybrids are shown by the dashed lines, with their maternal parents indicated by arrows. Lack of detectable cpDNA variation between *P. foliosus* and *P. pusillus* precluded determination of the maternal parent of their hybrid. Previously reported sequences are indicated by their GenBank accession numbers; the remainder are newly reported (See Appendix 1).

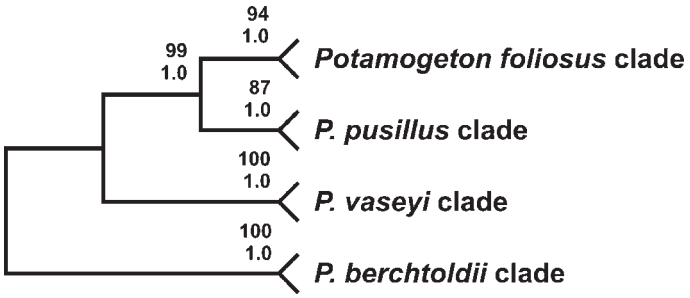

FIG. 2. Strict consensus tree derived from MP combined analysis of 52 nrITS and cpDNA sequences from *Potamogeton* species and hybrids. Bootstrap values (upper) and Bayesian posterior probabilities (lower) are shown for all branches. The cpDNA data were less variable than the nrITS data; however, they provided additional support for the clades most relevant to the present study.

DISCUSSION

Newly reported DNA sequences from the nrITS and *trnK* 5′ intron loci for *Potamogeton gemmiparus* and *P. vaseyi* indicate that these taxa are sufficiently divergent at the molecular level to readily facilitate their identification using genetic methods and they do not simply represent environmental forms of a single, polymorphic species. In this way, our data corroborate previous results obtained by Lindqvist et al. (2006), who reported a similar pattern of divergence for 5S-NTS sequences derived from single accessions of the same taxa. Our highest phylogenetic resolution came from the analysis of nrITS sequence data (Fig. 1). Data from the *trnK* 5′ intron were less variable; however, these chloroplast data generated phylogenetic results that were completely congruent with the nrITS data as indicated by the ILD value (p = 1.000). The phylogeny derived from the combination of the nrITS and cpDNA data (Fig. 2) resolved the same major clades as did the nrITS data alone. For simplicity, the remainder of our discussion will focus primarily on the results obtained from the nrITS data set.

Despite their morphological similarity, *P. gemmiparus* and *P. vaseyi* are not particularly closely related, and are not sister taxa in a phylogenetic sense. In our study *P. vaseyi* most closely associated with *P. octandrus*, another member of section *Axillares* subsection *Javanici*; however, it also showed closer affinity to *P. foliosus* and *P. pusillus* (*Axillares*, subsection *Pusilli*) as well as *P. cristatus* (subsection *Javanici*), than to *P. gemmiparus*. It is evident that the subsectional classification of *Potamogeton* needs a thorough reevaluation to determine whether infrageneric taxa (e.g. subsections) are delimited in such a way as to be meaningful phylogenetically.

Although both the nrITS data and cpDNA data indicate a fairly distant relationship between *P. gemmiparus* and *P. vaseyi* (Figs. 1–2), enough genetic similarity exists to allow their hybridization, at least with respect to the case we have documented from Wilson Pond, Maine. The existence of hybrids between these taxa may provide some explanation why Hagström (1916) believed that *P. gemmiparus* originated from hybridization with *P. vaseyi*. However, the genetic distinctness of these taxa makes it unlikely that the former specifically evolved via a hybrid origin from the latter, but rather it is more likely to have originated from a common ancestor with *P. berchtoldii*. We also documented a hybrid involving *P. vaseyi* and *P. berchtoldii*. The plants from Wilson Pond, Maine are unusual in that some displayed the C/T nucleotide polymorphism that distinguished *P. gemmiparus* from *P. berchtoldii* and others showed chimeric nrITS sequences as a consequence of hybridization between *P. gemmiparus* and *P. vaseyi*. Thus, it is evident that Wilson Pond plants represent a history of multiple hybridization events.

Given their fairly distant relationship, the convergent morphology of the submersed foliage of *P. gemmiparus* and *P. vaseyi* is impressive and creates difficulty with their identification (Table 1). As a means of facilitating the identification of these taxa when floating leaves are absent, we recommend the use of turion position whenever possible. As Fernald (1932) noted, turions of *P. vaseyi* occur on short, diverging, axillary branchlets in contrast to *P. gemmiparus* where they occur terminally on elongate, ascending branchlets. Although Fernald (1932) remarked that nodal glands sometimes occur in *P. vaseyi* they were absent in the material we examined, which is consistent with the description given by Haynes and Hellquist (2000). We also have observed that the cross-section of submersed leaves is nearly flat in *P. vaseyi*; whereas the leaves are thicker along the central axis and somewhat concave in *P. gemmiparus*. However, verification using molecular markers should always be used when a definitive identification is required.

In the course of evaluating the relationship between *P. gemmiparus* and *P. vaseyi*, our analyses provided additional taxonomic insight with respect to the affinities of several other taxa, notably *P. pusillus*. From our own experience it is clear that *P. pusillus* is difficult to identify conclusively, particularly from dried material. Because plants are commonly sterile, the distinction of *P. pusillus* and *P. berchtoldii* often has relied on the interpretation of the stipular sheath (open along the back edge vs. fully fused into a cylinder), which reportedly can vary and also requires careful dissection of rehydrated material to evaluate with confidence. Although some authors consider these stipule differences to be unreliable taxonomically (e.g. Haynes 1974), others have effectively distinguished *P. pusillus* and *P. berchtoldii* using this single character alone (e.g. Kaplan and Štěpánek 2003).

The problematic identification of narrow-leaved pondweeds is infamous (Haynes 1974). Whittall et al. (2004) remarked that even "pondweed experts" were able only to correctly identify "less than one in three samples" in their study of several narrow-leaved pondweeds. Ironically, even a specimen of

TABLE 1. Vegetative morphology of several similar *Potamogeton* species (adapted from Fernald 1932; Haynes and Hellquist 2000). All but the first character apply to submersed shoots and leaves. Fruit characters are discussed in the text.

	Floating leaves	Nodal glands	Stipule margins	Lacunal band #	Vein #	Blade width (mm)	Turions
P. berchtoldii	absent	present	free	2–10	1–3(-5)	0.2–2.5	terminal
P. clystocarpus	absent	present	free	0–8	3(-5)	0.7–1.7	unknown
P. foliosus	absent	absent	free	0–4	1–3(-5)	0.3–2.3	axillary or terminal
P. gemmiparus	absent	present	free	0–4	1	0.2–0.7	terminal
P. pusillus	absent	present	connate	0–4	1–3	0.5–1.9	axillary or terminal
P. vaseyi	absent or present	absent	free	0–4	1(-3)	0.1–1.0	axillary

TABLE 2. Genic recombination indicated by polymorphisms among clones recovered from *P. gemmiparus* × *P. vaseyi* hybrids from Wilson Pond, Maine. Positions in the nrITS alignment that were polymorphic for nucleotide identity (letters) or indels (0 or 1) are indicated by parentheses and bold type. For phylogenetic analysis, consensus sequences were used, which represented the majority nucleotide/indel configuration observed for a given taxon. Data for other taxa studied are provided for comparison. Only sites that differed between *P. gemmiparus* and *P. vaseyi* are shown.

	Alignment position												
Taxon	47	61	104	131	135	244	390	435	541	585	593	605	663
P. gemmiparus	C	A	C	A	C	0	C	C	A	C	0	C	T
clone 1.1	C	A	C	A	C	0	C	C	A	C	0	C	T
clone 1.3	C	A	C	A	C	0	C	C	A	C	0	C	T
clone 1.6	C	A	C	A	C	0	C	C	**(C)**	**(T)**	**(1)**	**(-)**	**(G)**
clone 2.1	C	A	C	A	C	**(1)**	**(T)**	**(T)**	A	C	0	C	**(G)**
clone 2.2	**(T)**	A	C	A	C	0	C	C	A	C	0	C	T
clone 2.3	**(T)**	**(T)**	**(T)**	A	C	0	C	C	A	C	0	C	T
clone 2.5	C	A	C	A	C	0	C	C	A	C	0	C	T
clone 3.2	C	A	C	A	C	0	C	C	A	C	0	C	T
clone 3.3	C	A	C	A	C	0	C	C	A	C	0	C	T
P. vaseyi	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 1.2	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 1.4	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 1.5	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 1.7	T	T	T	G	G	1	**(C)**	**(C)**	**(A)**	**(C)**	**(0)**	**(C)**	G
clone 2.4	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 2.6	T	T	T	G	A	1	T	T	**(A)**	**(C)**	**(0)**	**(C)**	**(T)**
clone 2.7	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 3.1	**(C)**	**(A)**	**(C)**	**(A)**	**(C)**	**(0)**	T	T	C	T	1	-	G
clone 3.4	T	T	T	G	A	1	T	T	C	T	1	-	G
clone 3.5	**(C)**	**(A)**	**(C)**	G	A	1	T	T	C	T	1	-	G
P. berchtoldii	C	A	C	A	C	0	C	C	A	C	0	T	T
P. clystocarpus	C	A	C	A	C	0	C	C	A	C	0	T	T
P. compressus	T	A	C	T	C	1	C	C	A	C	0	T	T
P. cristatus	T	A	C	A	C	1	C	C	T	T	0	T	T
P. foliosus	T	A	C	A	A	1	C	T	T	T	1	-	T
P. octandrus	T	A	C	G	A	1	C	T	T	T	1	-	A
P. pusillus	T	A	C	A	A	1	C	T	T	T	1	-	T
P. zosteriformis	T	A	C	T	C	1	C	C	A	C	0	T	T

"*P. pusillus*" from that study appears to have been misidentified because its nrITS sequence (GenBank AY714288) is an exact match with sequences derived from *P. berchtoldii*.

Unlike European botanists who have retained *P. pusillus* and *P. berchtoldii* as distinct species (e.g. Preston 1995), it has become common practice for North American botanists (e.g. Haynes and Hellquist 2000; Crow and Hellquist 2000) to merge these two taxa as subspecies of *P. pusillus*; i.e. *P. pusillus* L. subsp. *pusillus* and *P. pusillus* subsp. *tenuissimus* (Mertens & W. D. J. Koch) R. R. Haynes & Hellquist, respectively. However, our results clearly support the recognition of *P. pusillus* and *P. berchtoldii* as different species not only because they are distinct genetically but because their taxonomic delimitation as subspecies would be inconsistent with their phylogenetic relationships (Fig. 1). The distinctness of these taxa is not surprising given that several studies using isozyme data (Hettiarachchi and Triest 1991; Kaplan and Štěpánek 2003) have consistently indicated a relatively distant relationship. Because the *P. pusillus* clade resolved in our study contained specimens from Asia and North America, it is apparent that the genetic integrity of this species is maintained worldwide.

Potamogeton gemmiparus also has been treated as a subspecies of *P. pusillus* (e.g. Haynes and Hellquist 2000); however, that disposition is unacceptable because these taxa occur in different clades with a relatively distant phylogenetic relationship (Fig. 1). Polymorphisms observed for the single-nucleotide mutation that differentiated *P. gemmiparus* from *P. berchtoldii* indicate that there is evidence of gene flow

TABLE 3. Genic recombination indicated by polymorphisms among clones recovered from *P. foliosus* × *P. pusillus* hybrids from Mudge Pond, Connecticut. Positions in the nrITS alignment that were polymorphic for nucleotide identity (letters) or indels (0 or 1) are indicated by parentheses and bold type. For phylogenetic analysis, consensus sequences were used, which represented the majority nucleotide/indel configuration observed for a given taxon. Data for other taxa studied are provided for comparison. Only sites that differed between *P. foliosus* and *P. pusillus* are shown.

	Alignment position				
Taxon	72	441	456	460	505
P. foliosus	G	T	T	1	T
clone 1.2	G	T	T	1	T
clone 1.4	G	T	T	1	T
clone 1.6	G	T	T	1	T
clone 1.7	**(A)**	T	T	1	**(C)**
clone 1.8	G	T	T	1	T
clone 1.9	G	T	T	1	T
clone 1.11	G	T	T	1	T
P. pusillus	A	C	C	0	C
clone 1.1	**(G)**	C	C	0	C
clone 1.3	A	C	C	0	C
clone 1.5	A	C	C	**(1)**	C
clone 1.10	**(G)**	C	C	0	C
P. berchtoldii	A	C	T	1	C
P. clystocarpus	A	C	T	1	C
P. compressus	A	T	T	1	C
P. cristatus	A	C	T	1	T
P. gemmiparus	A	C	T	1	C
P. octandrus	A	C	T	1	T
P. vaseyi	A	C	T	1	C
P. zosteriformis	A	C	T	1	C

between these taxa, an observation that would weaken a case to retain them as distinct species. A similar argument could be made with respect to *P. clystocarpus*, which Haynes (1974) insisted could not be differentiated from *P. pusillus* (including *P. berchtoldii*) in the absence of fruits. In our molecular analysis *P. clystocarpus* differed from *P. berchtoldii* only by a single indel. Although Whittall et al. (2004) concluded that there was no evidence of gene flow between *P. clystocarpus* and other taxa they surveyed, their own data (see their Table 3) indicated several accessions of *P. berchtoldii* (apparently mistaken as *P. pusillus*) to be polymorphic for that indel, thereby clearly indicating gene flow between the taxa. Taken together, the minor degree of morphological and genetic divergence separating *P. berchtoldii*, *P. clystocarpus,* and *P. gemmiparus*, coupled with the restricted geographical distributions of the latter two taxa, along with evidence of gene flow of both taxa with *P. berchtoldii*, is a compelling example of subspecific differentiation. Consequently, we have proposed two new combinations below to accommodate this revised taxonomic interpretation.

Because most of the taxa we surveyed were characterized by only minor genetic variation, our data have limited use for evaluating geographical patterns of differentiation. Accessions of *P. vaseyi* were invariant across its range (Connecticut, Maine, Minnesota, and Vermont), as was the less extensively sampled *P. gemmiparus* (Connecticut, New Hampshire). *Potamogeton berchtoldii* surveyed from a wide geographical range of accessions (Connecticut, Maine, Minnesota, New Hampshire, and Texas) exhibited only one nucleotide difference in nrITS, which occurred in two Connecticut plants, and one nucleotide difference in *trnK*, which occurred in plants from Long Lake, Minnesota. North American accessions of *P. pusillus* differed only slightly from Chinese material. Similarly, only minor variation was detected between accessions of *P. foliosus* from Connecticut and Texas.

The low level of genetic variation may be a consequence of clonal, vegetative reproduction, which occurs in all the species we surveyed. Plants of *P. vaseyi* often do not develop floating leaves or flowers, especially when growing in deeper water, and it is likely that vegetative reproduction by turions would be their dominant reproductive mode. There also is the possibility that postglacial establishment of *P. vaseyi* populations involved a genetic bottleneck as a consequence of founder effect, which could account for low levels of detectable genetic variability. Turions are also common in *P. berchtoldii*, *P. gemmiparus,* and *P. pusillus*. In contrast, turions are uncommon in *P. foliosus* (Haynes and Hellquist 2000), which exhibited somewhat higher genetic variability than the other species we studied.

Before the implementation of genetic data, evidence of hybridization in *Potamogeton* was weak at best (Les and Philbrick 1993). Fortunately, a number of studies have since appeared, providing firm genetic evidence of hybridization in the genus (e.g. Hollingsworth et al. 1995; Fant et al. 2001, 2005; King et al. 2001; Fant and Preston 2004; Kaplan and Fehrer 2004; Du et al. 2009). Our observations confirm that hybridization in *Potamogeton* is a significant process of fairly broad occurrence. We encountered three instances of interspecific hybridization (*P. berchtoldii* × *P. vaseyi*, *P. foliosus* × *P. pusillus*, and *P. gemmiparus* × *P. vaseyi*) during the course of our investigations, which were not even directed at hybrid detection. There also was evidence of hybridization involving several other taxa (*P. berchtoldii* × *P. gemmiparus* and *P. berchtoldii* × *P. clystocarpus*), which we would categorize as intraspecific. *Potamogeton* hybrids create difficulty in identification, and those we encountered did not exhibit obvious characteristics that would readily betray their hybrid nature. Clearly, there is a need to conduct large-scale surveys for hybrids in *Potamogeton* throughout the range of most species, even where no morphological indication of hybridization might occur.

The evaluation of nrITS sequence data is somewhat problematic when dealing with *Potamogeton* hybrids and requires molecular cloning for clarification. Because the nrITS region undergoes concerted evolution, repeated reproductive events are expected to eventually convert the two distinct copies present in a hybrid genome into a single copy of one parental or recombinant sequence, a process primarily attributed to unequal crossing-over (Álvarez and Wendel 2003; Eickbush and Eickbush 2007). However, prolonged vegetative reproduction can slow the process, making it possible to distinguish both parental alleles in relatively recent hybrid plants (Moody and Les 2002). In *Potamogeton* hybrids we not only recovered parental alleles but also encountered several instances where single cloned alleles represented a mosaic of parts from both parental genomes (Tables 2–3). Such "chimeric" sequences have been attributed to genic recombination, which occurs commonly in plant hybrids (Álvarez and Wendel 2003), and has been observed in other *Potamogeton* hybrids (Du et al. 2009). These observations provide some insight into the concerted evolutionary process in *Potamogeton*; moreover, they indicate the necessity to clone accessions when there are indications of hybridity.

The ability of molecular data to resolve species limits for problematic *Potamogeton* taxa greatly facilitates their evaluation by conservation organizations. Previously, the high level of identification error made it difficult to assess conclusively the status of *P. gemmiparus* and *P. vaseyi* in New England where they are both listed as imperiled. By adopting a more precise means of identification using molecular markers, and coupling that work with more extensive field surveys, the status of both taxa in the region has been clarified. For instance, *P. gemmiparus* was regarded as possibly extirpated in New Hampshire. However, this study confidently documents the presence of the species from Pemigewasset Lake. Previously, *P. vaseyi* was regarded in Connecticut as the rarer of the two taxa, but so far this study has documented even fewer sites in the state for *P. gemmiparus*. Also, some sites previously attributed to *P. gemmiparus* (e.g. Coventry Lake, Crystal Lake, Connecticut) have now been clarified as misidentifications of *P. vaseyi* specimens that lacked floating leaves.

We have presented a reliable method that easily distinguishes *P. gemmiparus* and *P. vaseyi* and recommend a thorough genetic survey of all reported occurrences of these taxa using this approach. For this purpose it would be necessary only to sequence the nrITS region, which provided the better resolution of the loci investigated, and has the additional advantage of being able to detect natural hybrids. The nrITS region also is among the most easily sequenced plant loci due to its high copy number, relatively short length, and conserved primer regions. The presence of diagnostic indels also would make it possible to design specific primers selected for each taxon to facilitate the genetic screening process using a simple, PCR-based methodology.

Phylogenetic investigations of *Potamogeton* using molecular data are promising and should be continued in the genus.

With respect to insight gained by the present study, it seems necessary to re-evaluate the status of *P. berchtoldii* and *P. pusillus* throughout North America and other potential relationships of these species in particular. Notably, *Potamogeton fibrillosus* Fernald could represent another case of mistaken identity within this group. It most recently has been recognized as either a variety (Haynes and Reveal 1973) or subspecies (Haynes and Hellquist 2000) of *P. foliosus*, despite some specimens possessing anomalous characteristics typically attributed to *P. pusillus* such as nodal glands, completely fused stipular sheaths, and interrupted spikes. Given the phylogenetic proximity of *P. pusillus* and *P. foliosus* indicated by the present study, a closer relationship to the former species would seem plausible, and this possibility should be investigated further.

Taxonomic Treatment

1. **Potamogeton berchtoldii** Fieber subsp. **clystocarpus** (Fernald) D. Les & N. P. Tippery, comb. et stat. nov. *Potamogeton clystocarpus* Fernald, Mem. Amer. Acad. Arts 17(1): 79, pls. 15, 30, Fig. 5. 1932.—TYPE: U.S.A. Texas: "deep sluggish pool in rock, Little Aguja Canyon, alt. 1,575 m., Davis Mts., Jeff Davis Co.," June 15, 1931, *J. A. Moore & J. A. Steyermark 3088* (holotype: GH).

2. **Potamogeton berchtoldii** Fieber subsp. **gemmiparus** (J. W. Robbins) D. Les & N. P. Tippery, comb. nov. *Potamogeton pusillus* L. var. *gemmiparus* J. W. Robbins, in A. Gray, Manual ed. 5, 489. 1867.—TYPE: U.S.A.: Massachusetts, "Valley of the Blackstone, Uxbridge," *J. W. Robbins s.n.* (lectotype: NY, selected by Haynes [1974]).

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Appendix 1. Accessions of *Potamogeton* used in molecular analyses. (O) = outgroup sequence; (D) = sequences downloaded from the GenBank database and contain the locality followed only by the accession number(s). Accession numbers separated by a comma indicate nrITS data and *trnK* intron data respectively; n/a = no sequence available. All newly reported sequences include collector information (italicized) and are deposited at the University of Connecticut herbarium (CONN). Hybrid accessions are listed last.

Potamogeton berchtoldii Fieber (Slender Pondweed). **USA. Connecticut**. Beseck Lake, *Les 715a*, GQ247388, GQ247471; *Les 715b*, GQ247389, GQ247472; *Les 715c*, GQ247390, GQ247473; EF526403 (D), n/a. Bigelow Hollow, *Murray 07-066*, GQ247391, GQ247474. Bolton Lake, *Murray 07-045*, GQ247392, n/a. Clear Lake, EF526402 (D), n/a. Columbia Lake, *Mayne, s.n.*, GQ247393, GQ247475. Coventry Lake, *Murray 06-115*, GQ247394, GQ247476; *Murray 06-117*, GQ247395, GQ247477. Mansfield Hollow, *Les, Benoit & Sheldon, s.n.*, GQ247396, GQ247478. Pachaug Pond, *Murray 07-085*, GQ247397, GQ247479. Quaddick Reservoir, *Murray 06-106*, GQ247398, GQ247480. **Maine**. Wilson Pond, *Cameron s.n.*, GQ247399, GQ247481. **Minnesota**. Captive Lake, *Kuchinsky s.n.*, GQ247400, GQ247482. Donna's Pond, *Perleberg s.n.*, GQ247401, GQ247483. Greer Lake, *Loss s.n.*, GQ247402, GQ247484. Long Lake, *Wandrie s.n.*, GQ247403, GQ247485. Pt. Pleasant, *Perleberg s.n.*, GQ247404, GQ247486. "**New England**", AY714289 (D), n/a. **New Hampshire**. Bearcamp Pond, *Les 713/Murray 06-060*, GQ247405, GQ247487; *Les 714/Murray 06-061*, GQ247406, GQ247488. Kanasatka Lake, *Les 710/Murray 06-067*, GQ247407, GQ247489. Lees Pond, *Les 712/Murray 06-064*, GQ247408, GQ247490. Pemigewasset Lake, *Les 707/Murray 06-062*, GQ247409, GQ247491. **Texas.** Madera Creek, AY714288 (D), n/a. ***P. clystocarpus*** Fernald (Little Aguja Pondweed). **USA. Texas.** Aguja Creek, AY714283 (D), n/a; AY714284 (D), n/a. ***P. compressus*** L. (Flatstem Pondweed). **China.** DQ840310 (D, O), n/a. ***P. cristatus*** Regel & Maack (Crested Pondweed). **China**. DQ840323 (D), n/a. ***P. foliosus*** Rafinesque (Leafy Pondweed). **USA. Connecticut**. Beseck Lake, *Les, Murray & Hunter s.n.*, GQ247410, GQ247492. Mt. Higby Reservoir, *Murray 07-004*, GQ247411, GQ247493. Orange, *Murray s.n.*, GQ247412, GQ247494. **Texas.** Aguja Creek, AY714292 (D), n/a; AY714293 (D), n/a. ***P. gemmiparus*** (J. W. Robbins) J. W. Robbins ex Morong (Budding Pondweed). **USA. Connecticut**. Bigelow Hollow, *Murray 06-045*, GQ247413, GQ247495; *Murray 06-048*, GQ247414, GQ247496; *Knocklein s.n.*, GQ247415, GQ247497. Pickerel Lake, *Murray 06-014.5*, GQ247416, GQ247498; *Murray 06-014.8*, GQ247417, GQ247499. Powers Lake, *Murray 06-013*, GQ247418, GQ247500. **New Hampshire**. Pemigewasset Lake, *Les 708/Murray 06-063*, GQ247419, GQ247501. ***P. octandrus*** Poir. (Pondweed). **China**. DQ840316 (D), n/a. ***P. pusillus*** Linnaeus (Slender Pondweed). **China.** DQ840294 (D), n/a; DQ840295 (D), n/a; DQ840296 (D), n/a. **USA**. **Minnesota**. Pine Mountain, *Perleberg & Vacinek s.n.*, GQ247420, GQ247502. Ten Mile Lake, *Perleberg, Bulman & Loso s.n.*, GQ247421, GQ247503. ***P. vaseyi*** J. W. Robbins (Vasey's Pondweed). **USA. Connecticut**. Beseck Lake, *Murray 07-011*, GQ247422, GQ247504. Coventry Lake, *Murray 06-116*, GQ247423, GQ247505. Crystal Lake, *Murray 06-040*, GQ247424, GQ247506; *Murray 06-042*, GQ247425, GQ247507; *Murray 06-044*, GQ247426, GQ247508. Gardner Lake, *Murray 06-101*, GQ247427, GQ247509. Lake Marie, *Murray 07-027*, GQ247428, GQ247510. Mt. Higby Reservoir, *Murray 07-005*, GQ247429, GQ247511. Pachaug Pond, *Murray 07-084*, GQ247430, GQ247512. Wintergreen Lake, *Les 716*, GQ247431, GQ247513. **Minnesota**. Long Lake, *Loso & Bulman s.n.*, GQ247432, GQ247514. Ozawindib Lake, *Myhre 09864*, GQ247433, n/a. Sand Lake, *Perleberg s.n.*1, GQ247434, GQ247515; *Perleberg s.n.*2, GQ247435, GQ247516. **Vermont**. Missisquoi, *Les 493*, GQ247436, GQ247517. Lake Memphremagog, *Hellquist s.n.*, GQ247437, GQ247518. ***P. zosteriformis*** Fernald (Flatstem Pondweed). **USA. Vermont.** Missisquoi, *Les 494*, GQ247438, n/a (O). ***P. berchtoldii*** × ***P. vaseyi*** (hybrid pondweed). **USA. Minnesota.** Stark Pond, *Perleberg & Bulman s.n.*, GQ247439-GQ247440, GQ247519. ***P. foliosus*** × ***P. pusillus*** (hybrid pondweed). **USA. Connecticut**. Mudge Pond, *Murray 07-039*, GQ247441-GQ247451, GQ247520. ***P. gemmiparus*** × ***P. vaseyi*** (hybrid pondweed). **USA. Maine**. Wilson Pond, *Cameron s.n.*, GQ247452-GQ247470, GQ247521.