

## Molecular phylogenetic analysis of *Ceratophyllum* L. taxa: a new perspective

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*Ceratophyllum* (Ceratophyllaceae) is among the most enigmatic of angiosperm taxa, with its phylogenetic placement changing almost continuously over the last few decades. *Ceratophyllum* spp. are difficult to identify using vegetative morphology alone, which is commonly all that is available for these highly clonal plants. A molecular phylogenetic approach was used to examine relationships in the genus and provide molecular markers to facilitate the identification of *Ceratophyllum* spp. This study included all known *Ceratophyllum* spp. that have been distinguished in the last two taxonomic treatments of the genus. Sequence data for ITS and *matK* were used to examine phylogenetic relationships among species. The molecular analyses readily distinguish five clades which correspond taxonomically to *C. echinatum*, *C. demersum*, *C. australe* (including *C. tanaiticum*), *C. submersum* and *C. muricatum* (including *C. muricatum* subsp. *muricatum* and *C. m.* subsp. *kossinskyi*). In these analyses, accessions of the morphologically distinct *C. platyacanthum* were not clearly differentiated from some accessions of *C. demersum*, perhaps as a consequence of the probable polyploid origin of this taxon. Overall, the molecular data disagree with some previous studies based on morphology by indicating (1) the presence of more than two species in the genus, (2) that *C. echinatum* is not the closest relative of *C. submersum*, (3) that *C. muricatum* is related more closely to *C. submersum* than to *C. australe* and (4) that *C. tanaiticum* is resolved in a well-supported clade with *C. australe*, which is distinct from accessions of *C. muricatum*. Although *C. tanaiticum* and *C. australe* fall in the same clade in the current analysis, their high level of genetic divergence, extreme geographical isolation and significantly different morphologies support their recognition as distinct species. We also discuss the chorological and palaeobotanical aspects of the new results.

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## INTRODUCTION

In the first half of the 19<sup>th</sup> century, Schleiden referred to *Ceratophyllum* L. as a ‘vegetable vagabond’ the taxonomic placement of which was renowned for its interfamilial wanderings (see Les, 1986a). The systematic position of *Ceratophyllum* has been reconsidered several times, especially after more recent analyses, as evidenced by the molecular-based classifications recommended by the Angiosperm Phylogeny Group (APG, 1998; APG II, 2003; APG III, 2009; APG IV, 2016).

*Ceratophyllum* is a genus of perennial, freshwater herbs with an essentially cosmopolitan distribution. Extensive morphological variability has caused considerable taxonomic confusion in the genus, and its systematic relationships are poorly understood. Adaptation to an aquatic existence has been accompanied by conspicuous morphological reduction, especially in floral structures (Les, 1986b; Iwamoto, Shimizu & Ohba, 2003).

According to the most recent reviews of the genus, there are either six extant species (Les, 1989, 1993) or two species each composed of four varieties (Wilmot-Dear, 1985). In her proposed classification, Wilmot-Dear (1985) considered the range of fruit and leaf variation with regard to geographical distribution, although that treatment was not supported by quantitative evaluations or statistical analyses of the morphological characters. The acceptance of the latter taxonomic concept by European botanists has negatively affected the conservation status by de-emphasizing the significance of several rare taxa that were relegated to varietal status in that treatment (see Jalas & Suominen, 1989; Lansdown, 2018).

Subsequently, the genus was subdivided into three sections (each having two species) by Les (1989): section *Ceratophyllum* (*C. demersum* L., *C. platyacanthum* Cham.), section *Submersum* (*C. submersum* L., *C. echinatum* A.Gray) and section *Muricatum* (*C. muricatum* Cham., *C. tanaiticum* Sapjegin). This scheme was well supported by numerical studies, chemosystematic data and phytogeographical relationships, and Les’s concept has been accepted by some (e.g. Velasquez, 1994; Cook, 1996, 2004).

It also has been widely accepted that the fruit of *Ceratophyllum* provides the most important morphological characters for distinguishing different taxa in the genus (e.g. Sapjegin, 1902; Les, 1986a, b, 1988a, b, 1989; Scribailo & Alix, 2002), but some species rarely produce fruit. Although some statistically significant differences in vegetative characters can be

useful for species identification (Csiky *et al.*, 2010), we believed that a study utilizing molecular data would shed additional light on taxonomic and phylogenetic relationships in the genus.

The purpose of this study is to present a new perspective on phylogenetic relationships in *Ceratophyllaceae* using ITS and *matK* nucleotide sequences of those extant *Ceratophyllum* spp. recognized in the most recent taxonomic treatments. To the best of our knowledge, no phylogenetic classification of *Ceratophyllum* based on an analysis of DNA sequence data has been previously attempted.

## MATERIAL AND METHODS

### PLANT MATERIAL

Taxa sampled in the current study are listed according to Les (1989), with one exception in the ranking of taxa [*C. australe* Griseb. was treated as *C. muricatum* subsp. *australe* (Griseb.) Les by Les (1989)].

Family Ceratophyllaceae Gray, Nat. arr. Brit. pl. 554. 1821 (Gray, 1821).

Genus *Ceratophyllum* L., Sp. pl. ed. 1: 992. 1753 (Linnaeus, 1753).

1. *Ceratophyllum demersum* L., Sp. pl. ed 1: 992. 1753 (Linnaeus, 1753).
- 2.a *Ceratophyllum platyacanthum* Cham., Linnaea 4: 504. 1829 (Chamisso, 1829).
- 2.b *Ceratophyllum platyacanthum* Cham. subsp. *oryzeterum* (Kom.) Les, Syst. Bot. 1988b (Les, 1988b).
3. *Ceratophyllum echinatum* A.Gray, Fl. N. Amer. 1: 56. 1838 (Torrey & Gray, 1838).
4. *Ceratophyllum submersum* L., Sp. pl. ed. 2: 1409. 1763 (Linnaeus, 1763).
- 5.a *Ceratophyllum muricatum* Cham., Linnaea 4: 504. 1829 (Chamisso, 1829).
- 5.b *Ceratophyllum muricatum* Cham. subsp. *kossinskyi* (Kuzen.) Les, Syst. Bot. 1988a (Les, 1988a).
6. *Ceratophyllum australe* Griseb., Symb. Fl. argent. 14. 1879 (Grisebach, 1879).
7. *Ceratophyllum tanaiticum* Sapjegin, Trudy Obsc. Isp. Prir. Imp. Harkovsk. Univ. 37: 315. 1902 (Sapjegin, 1902).

### DNA PROCESSING AND SEQUENCING

Twenty-eight *Ceratophyllum* accessions belonging to nine different taxa were analysed (Table 1). Polymerase chain reactions (PCRs) were performed

**Table 1.** Voucher information for nuclear ribosomal ITS (nrITS) and *matK* sequences of studied taxa used in the cladistic analyses of *Ceratophyllum* taxa

ID	Name	Voucher	Collector	Origin	<i>matK</i>	nrITS
A1	<i>C. muricatum</i> Cham. subsp. <i>aus-trale</i> (Griseb.) Les	<i>C. C. Jaco</i> CA1	C. C. Jaco	St. Vincent Island, FL, USA	MG837727	MG831576
A2	<i>C. muricatum</i> Cham. subsp. <i>aus-trale</i> (Griseb.) Les	<i>E. Terneus</i> CA2	E. Terneus	La Tembladera, Oro Province, Ecuador	MG837728	MG831577
D1	<i>C. demersum</i> L.	<i>J. Csiky</i> CD1	J. Csiky 2009	Bogádmindszent, Dráva Plain, Hungary	KM582618	KM582603
D2	<i>C. demersum</i> L.	<i>A. Mesterházy</i> CD2	D. Ganzha, B.A. Baranovszkij, A. Mesterházy 2010	Ivano-Mykhailovka, Dnepropetrovsk region, Ukraine	KM582619	KM582604
cD3	<i>C. demersum</i> L.	<i>A. Mesterházy</i> CD3	A. Mesterházy 2011	Athiémé-Houeyagbé, Mono, Benin	KM582620	KM582605
D4	<i>C. demersum</i> L.	<i>Budnik</i> s.n. (CONN)	Budnik	Volgograd, Russia	–	MG831578
D5	<i>C. demersum</i> L.	<i>Les 596</i> (CONN)	D. Les	Reid River, Queensland, Australia	MG837729	MG831579
D6	<i>C. demersum</i> L.	<i>Les 802</i> (CONN)	D. Les	Otter Creek Reservoir, Piute County, UT, USA	MG837730	–
D7	<i>C. demersum</i> L.	<i>Les 1016</i> (CONN)	D. Les	Arivaca Lake, Pima County, AZ, USA	MG837731	MG831580
D8	<i>C. demersum</i> L.	<i>Les 751</i> (CONN)	D. Les	Lake Moultrie, Berkeley County, SC, USA	MG837732	–
D9	<i>C. demersum</i> L.	<i>Les 849</i> (CONN)	D. Les	Big Lake, San Patricio County, TX, USA	MG837733	MG831581
E	<i>C. echinatum</i> A. Gray	<i>R. W. Scribailo</i> CE1	R. W. Scribailo 2008	Valparaiso, IN, USA	KM582621	KM582606
K1	<i>C. muricatum</i> Cham. subsp. <i>kossinskii</i> (Kuzen.) Les	<i>A. P. Laktionov</i> CK1	A. P. Laktionov, V.G. Papchenkov, N.O. Vostrikova 2012	Sergievka, Astrakhan region, Russia	KM582622	KM582607
K2	<i>C. muricatum</i> Cham. subsp. <i>kossinskii</i> (Kuzen.) Les	<i>A. Mesterházy</i> CK2	A. P. Laktionov, A. A. Bobrov, D. Pifko, A. Mesterházy 2012	Kamüzjak, Astrakhan region, Russia	KM582623	KM582608
M1	<i>C. muricatum</i> Cham.	<i>Leach 2856</i> (CONN)	Leach	Tipperary Station, Northern Territory, Australia	MG837734	MG831582
M2	<i>C. muricatum</i> Cham.	<i>K. Kaushik</i> CM2	K. Kaushik	Bhawal, Sonipat district, Haryana state, India	MG837735	MG831583
M3	<i>C. muricatum</i> Cham.	<i>A. Mesterházy</i> CM3	A. Mesterházy 2014	Rufisque-Dakar, Region Niayes, Senegal	KP842567	KP842566
O1	<i>C. platyacanthum</i> Cham. subsp. <i>oryzatorum</i> (Kom.) Les	<i>K. Evzhenko</i> CO1	K. Evzhenko 2008	Krapivka, Omsk region, Russia	KM582624	KM582609
O2	<i>C. platyacanthum</i> Cham. subsp. <i>oryzatorum</i> (Kom.) Les	<i>L. M. Kipriyanova</i> CO2	L. M. Kipriyanova 2011	Verkh-Irmen, Novosibirsk region, Russia	KM582629	KM582614
P	<i>C. platyacanthum</i> Cham.	<i>A. Mesterházy</i> CP1	D. Ganzha, B.A. Baranovszkij, A. Mesterházy 2010	Ivano-Mykhailovka, Dnepropetrovsk region, Ukraine	KM582628	KM582613

Table 1. Continued

ID	Name	Voucher	Collector	Origin	<i>matK</i>	nrITS
S1	<i>C. submersum</i> L.	<i>A. Mesterházy CS1</i>	A.P. Laktionov, A. A Bobrov, D. Pifko, A. Mesterházy 2012	Terkin, Volgograd region, Russia	KM582626	KM582611
S2	<i>C. submersum</i> L.	<i>J. Csiky CS2</i>	J. Csiky 2009	Vajszló, Dráva Plain, Hungary	KM582627	KM582612
S3	<i>C. submersum</i> L.	<i>J. Csiky CS3</i>	J. Csiky 2009	Sellye, Dráva Plain, Hungary	KM582615	KM582600
S4	<i>C. submersum</i> L.	<i>Budnik s.n. (CONN)</i>	Budnik	Volgograd, Russia	MG837736	MG831584
S5	<i>C. submersum</i> L.	<i>Les 388 (CONN)</i>	D. Les	Grossee, Andelfingen, Switzerland	MG837737	MG831585
T1	<i>C. tanaiticum</i> Sapiegin	<i>J. Csiky CT1</i>	J. Csiky 2008	Drávafook, Dráva Plain, Hungary	KM582625	KM582610
T2	<i>C. tanaiticum</i> Sapiegin	<i>A. Mesterházy CT2</i>	D. Pifko-A. Mesterházy 2012	Uldyuchiny, Kalmykia Republic, Russia	KM582616	KM582601
T3	<i>C. tanaiticum</i> Sapiegin	<i>A. Mesterházy CT3</i>	A. Mesterházy 2010	Decs, Gemenc, Hungary	KM582617	KM582602

Herbarium specimens of A1–2, D1–3, E, K1–2, M2–3, O1–2, P, S1–3 and T1–3 are deposited in the Herbarium of the University of Pécs (JPU), whereas D4–9, M1 and S4–5 can be found in the Herbarium of the University of Connecticut (CONN).

directly from leaf pieces 2–3 mm in length, with a Phire Plant Direct PCR Kit (Finnzymes), using the ‘Dilution protocol’ of the manufacturer. ITS4 and ITS5 primers (White *et al.*, 1990) were used for the amplification of nuclear ITS regions. Primers CDmatKFW (TTATGTGACCTATGCGAAGCC) and CDmatKRv: GTGGTACACAAGAATCGTTCGG) amplifying the plastid *matK* region were newly designed based on the *C. demersum* plastid genome (GenBank accession: EF614270.1; Moore *et al.*, 2007). PCRs were run in a PTC-200 Thermal Cycler (MJ Research) in 20- $\mu$ L reaction volumes with the following programme: 98 °C for 5 s; 59 °C (in the case of the ITS primers) or 65 °C (in the case of the CDmatK primers) for 5 s; and 72 °C for 20 s, with an initial denaturation step at 98 °C for 5 min.

Agarose gel electrophoresis (1% agarose gel 1 $\times$  TBE buffer) was used to test if primers amplified targeted loci in all samples. A GeneJET Gel Extraction Kit (Thermo Scientific) was used to recover DNA fragments according to the supplier’s instructions. DNA quantities and qualities of the samples were checked with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Samples were sequenced using the Big Dye Direct Cycle Sequencing Kit (Life Technologies). Cleaned cycle sequencing products were analysed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Each sequence was derived from at least two independent sequencing reactions.

#### PHYLOGENETIC ANALYSES

Nucleotide sequences were aligned using the ClustalW method with default settings (as implemented in the JalView computer program), edited manually and concatenated using Geneious v.6.1.5. created by Biomatters (available from: <http://www.geneious.com/>). The phylogenetic placement of *Ceratophyllum* is controversial, having been placed as sister to monocots (Zanis *et al.*, 2003), sister to eudicots (APG III, 2009) and sister to all angiosperms (APG, 1998); thus, it is difficult to determine a suitable outgroup for the rooting of phylogenetic trees, and the phylogenetic tree showing evolutionary relationships among the studied species is represented as an unrooted polytomy. Maximum parsimony (MP) analyses of the separate and combined ITS and *matK* sequence data were performed using PAUP\* 4.0 (Swofford, 2001), with the following options: keeping best trees only; when multiple starting trees exist swap on best only; 10000 random sequence addition replicates; hold one tree at each step; TBR branch swapping algorithm; multrees option off; swap on best only in effect. The number of bootstrap replicates was 1000. Tree statistics included the consistency index (CI), homoplasy index (HI) and retention index (RI).



For Bayesian analyses, a nucleotide substitution model was selected using ModelTest 3.7 (Posada & Crandall, 1998). The AIC and BIC model selection criteria proposed the same substitution model (GTR+I) for ITS and *matK* markers and the combined data sets. Separate and combined Bayesian likelihood analyses were performed with MrBayes (Ronquist & Huelsenbeck, 2003) using a Markov chain Monte Carlo (MCMC) method. The analyses comprised two runs of six independent MCMC chains each, monitored for one million generations. Every 100<sup>th</sup> generation was sampled, and the temperature coefficient was set to 0.15. Twenty-five per cent of the first generations were excluded from the consensus as a burn-in phase. Nodes were considered to be supported when Bayesian posterior probabilities (PP) were  $\geq 0.95$  and bootstrap support (BS) values were  $> 60$ .

## RESULTS

Initially, separate Bayesian and parsimony analyses were performed on the ITS and *matK* datasets to assess the possibility of combined data analysis (data not shown). Phylogenetic trees resulting from these separate analyses had identical topologies. In addition, the combined analysis topologies were also examined for conflict with the 0.95 posterior intervals of the single gene analyses. As no conflict was observed, the two datasets were assumed to be congruent and appropriate for combined analysis. A summary of the tree data and information on variable and potentially parsimony-informative characters from the separate and combined MP analyses are given in Table 2. A phylogenetic tree inferring evolutionary relationships among the *Ceratophyllum* taxa studied is presented as an unrooted polytomy in Figure 1. Two major clades including different taxa are the well-supported ‘Clade SMAT’ and ‘Clade D’ (in both cases PP: 1; BS: 100); *C. echinatum* appears as the third branch of the basal polytomy.

The first clade (‘Clade SMAT’) comprised a ‘Clade AT’ (PP: 1; BS: 98), including a strongly supported *C. australe* (A1-2) group (PP: 1; BS: 95) with the

*C. tanaiticum* (T1–3) accessions, and the ‘Clade SM’ (PP/BS: 1/100), including accessions of *C. submersum* (S1–5), *C. muricatum* subsp. *muricatum* (M1–3) and *C. muricatum* subsp. *kossinskyi* (K1–2). The *C. submersum* accessions (S1–5) resolved as a strongly supported clade (PP: 1; BS: 99) that was sister to a somewhat less strongly supported clade (PP: 0.98; BS: 77) containing accessions of *C. muricatum* subsp. *kossinskyi* (K1–2) and *C. muricatum* subsp. *muricatum* (M1–3). Indian material of *C. muricatum* (M2) resolved in the ‘Clade SM’, despite the presence of several unique single nucleotide polymorphisms (SNPs) in ITS2, which distinguished it from all other accessions of *C. muricatum*.

The second major group (‘Clade D’) comprised accessions of *C. demersum* (D1–9), *C. platyacanthum* subsp. *platyacanthum* (P) and *C. platyacanthum* subsp. *oryzetorum* (O1–2). Two statistically unsupported groups can be distinguished in this clade. In the first (PP: 0.88; BS: 63), the single accession of *C. platyacanthum* subsp. *platyacanthum* and the Ukrainian material identified as *C. demersum* clustered in a group, distinct from the accessions of *C. platyacanthum* subsp. *oryzetorum* and *C. demersum*. The second (PP: 0.87; BS: 69) includes three (but not all) accessions of *C. demersum* from the USA.

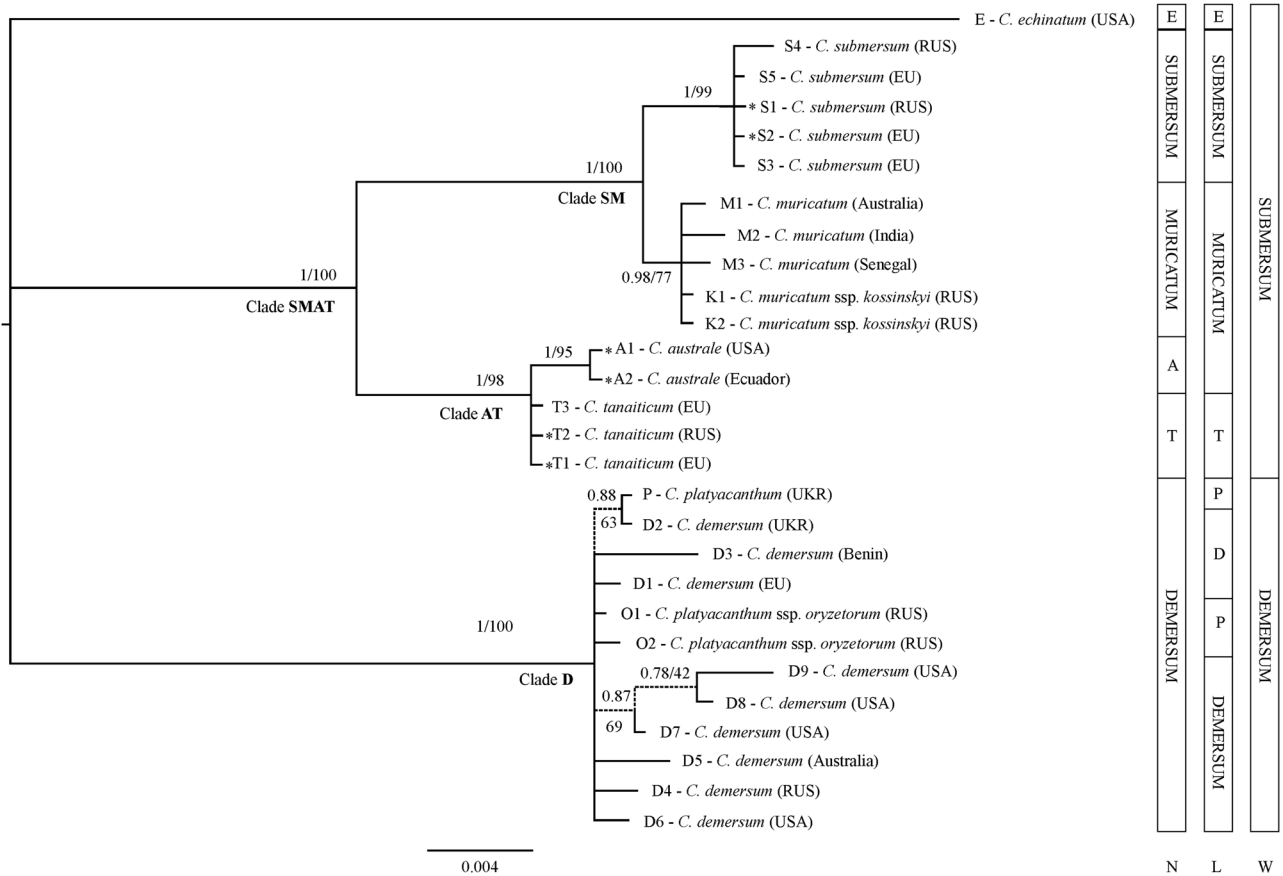
## DISCUSSION

### CERATOPHYLLUM ECHINATUM

*Ceratophyllum echinatum* is both morphologically (Les, 1985, 1986a, 1989) and phylogenetically (Fig. 1) the most distinct taxon of *Ceratophyllum*. A 10-bp insertion occurs in the ITS2 region of *C. echinatum*, which was absent in all other samples sequenced. Evidence of this unique insertion and the other distinctive polymorphisms in the locus are supported by an independently sequenced accession of *C. echinatum* (GenBank: AY335971), which is 100% congruent with the present ITS sequence in the overlapping region. Wilmot-Dear (1985) subsumed this species as a variety of *C. submersum* (Table 3), but our data strongly support its retention as a separate species.

**Table 2.** Descriptive tree statistics for parsimony analyses

Datasets	ITS	<i>matK</i>	Combined ITS/ <i>matK</i>
Length of aligned matrices (bp)	559	940	1499
Variable characters	113	62	149
Potentially parsimony-informative characters	52 (9.3%)	42 (4.5%)	93 (6.2%)
Consistency index (CI)	0.9028	0.9020	0.9483
Homoplasy index (HI)	0.0972	0.0980	0.0517
Retention index (RI)	0.9216	0.9752	0.9800



**Figure 1.** Phylogenetic relationships among the studied *Ceratophyllum* taxa based on Bayesian analysis of ITS and *matK* gene sequences. The scale bar indicates the number of substitutions per site. Significant posterior probabilities/bootstrap support are indicated by numbers; unsupported branches are indicated by broken lines. A: *C. australe*; D: *C. demersum*; E: *C. echinatum*; M: *C. muricatum*; P: *C. platyacanthum*; S: *C. submersum*; T: *C. tanaiticum*. Vertical stripes indicate the new treatment (N) and the most recent classifications of the genus: L (Les, 1989), W (Wilmot-Dear, 1985). An asterisk (\*) indicates identical DNA sequences of operational taxonomic units in a clade.

The most distinctive phenotypic characters of this North American species, which separate it from the other taxa, are its plumule morphology (Muenscher, 1940) unique in the genus, the length of lateral spines and a specific flavonoid chemistry (Les, 1985, 1986a).

#### CLADE D

This group includes those taxa characterized by leaves having a maximum leaf order of two (Les, 1993). Although this distinction holds well for the majority of material examined, a few exceptions have been noted (Wilmot-Dear, 1985; Les, 1986a). Some specimens identified as *C. demersum* have at least some leaves that are third-order dichotomously divided. In some cases (e.g. fertile population in West Africa; A. Mesterházy pers. obs.) the distal portions of the leaf are finely textured and soft (collapsing upon removal from the water) and bear denticles that are more or

less inconspicuously exerted, which are features more characteristic of *C. submersum* or *C. muricatum*. Because sterile populations of *Ceratophyllum* are frequent, it is possible to confuse them in the field. However, in other regions (Australia and India; D. H. Les pers. obs.), some third-order leaves have been observed with second-order leaves on fertile plants that resemble *C. demersum* in every other respect. Although an inverse correlation exists between the mean number of ultimate leaf segments of a taxon (which is related to the leaf order) and latitude, it is extremely rare for a second-order species to produce third-order leaves at any latitude (Les, 1986a). Regardless, the ability of authentic *C. demersum* to produce third-order leaves appears to be more of a geographical (i.e. Africa, southern Asia and Australia) rather than latitudinal phenomenon.

*Ceratophyllum demersum* and *C. platyacanthum* s.l. (including *C. platyacanthum* subsp. *platyacanthum* and

**Table 3.** Reconciliation between three systems of *Ceratophyllum* species according to the phylogenetic results and the most recent classifications of the genus; shading indicates the same taxonomic treatment

New, phylogenetic results	Les, 1986a, 1989	Wilmot-Dear, 1985
<i>Ceratophyllum demersum</i> L.	<i>C. demersum</i> L. <i>C. platyacanthum</i> Cham. <i>C. platyacanthum</i> subsp. <i>oryzeturum</i> (Kom.) Les	<i>C. demersum</i> L. <i>C. demersum</i> L. var. <i>platyacanthum</i> (Cham.) Wimm.
<i>Ceratophyllum echinatum</i> A.Gray	<i>C. echinatum</i> A.Gray	<i>C. submersum</i> L. subsp. <i>muricatum</i> (Cham.) Wilmot-Dear var. <i>echinatum</i> (A.Gray) Wilmot-Dear
<i>Ceratophyllum submersum</i> L.	<i>C. submersum</i> L.	<i>C. submersum</i> L. subsp. <i>submersum</i>
<i>Ceratophyllum muricatum</i> Cham.	<i>C. muricatum</i> Cham. <i>C. muricatum</i> Cham. subsp. <i>kossinskyi</i> (Kuzen.) Les	<i>C. submersum</i> L. subsp. <i>muricatum</i> (Cham.) Wilmot-Dear var. <i>echinatum</i> (A.Gray) Wilmot-Dear <i>C. submersum</i> L. subsp. <i>muricatum</i> (Cham.) Wilmot-Dear var. <i>manschuricum</i> Miki
<i>Ceratophyllum australe</i> Griseb.	<i>C. muricatum</i> Cham. subsp. <i>australe</i> (Griseb.) Les	<i>C. submersum</i> L. subsp. <i>muricatum</i> (Cham.) Wilmot-Dear var. <i>echinatum</i> (A.Gray) Wilmot-Dear
<i>Ceratophyllum tanaiticum</i> Sapjegin	<i>C. tanaiticum</i> Sapjegin	<i>C. submersum</i> L. subsp. <i>muricatum</i> (Cham.) Wilmot-Dear var. <i>echinatum</i> (A.Gray) Wilmot-Dear

*C. platyacanthum* subsp. *oryzeturum*) have been treated as distinct species in some reviews (e.g. Les, 1989). Our analysis indicates an unsupported branch (PP: 0.88; BS: 63) in ‘Clade D’, which visually separates all of the other accessions from this study, and includes the one *C. platyacanthum* subsp. *platyacanthum* accession. However, a *C. demersum* accession also resolved in this group, which could be interpreted as evidence to merge the taxa as proposed by Wilmot-Dear (1985). Although the molecular analysis does not distinguish the morphologically distinct *C. platyacanthum* s.l. from *C. demersum* as distinct clades, this result could be attributed to several factors. Some field observations have noted the co-occurrence of fertile *C. platyacanthum* subsp. *platyacanthum* and *C. demersum* phenotypes in the same stand (e.g. in Ukraine; A. Mesterházy pers. obs.), which could reflect some variability in the expression of the phenotypic traits (i.e. facial spines and a spiny marginal wing) in a single taxon. The distributions of the allopatric *C. platyacanthum* subsp. *platyacanthum* and *C. platyacanthum* subsp. *oryzeturum* do occur in the range of the more cosmopolitan *C. demersum* (Wilmot-Dear, 1985; Les, 1986a, 1988b), but they persist as conspicuous phenotypes. Moreover, *C. platyacanthum* s.l. is reported to be a hexaploid taxon as evidenced by chromosome counts for *C. platyacanthum* subsp. *oryzeturum* ( $2n = 72$ ; Okada & Tamura, 1981). If recently derived from a specific subset of diploid *C. demersum* ( $2n = 24$ ) populations, which is likely, it would not be unusual for those *C. demersum* sequences to be similar to those of *C. platyacanthum* s.l. Similarly, *C. platyacanthum* subsp. *platyacanthum* shares a virtually identical flavonoid profile with European populations of *C. demersum*,

which is distinct from *C. demersum* populations in other regions (Les, 1986a). This hypothesis needs further testing, and implications of polyploidy with respect to the status of *C. platyacanthum* s.l. require additional investigation. It should also be noted that *C. platyacanthum* subsp. *oryzeturum* and *C. demersum* have different ecological niches and life-history strategies according to some evidence from West Siberia and Kazakhstan (Sviridenko, 2000; Kipriyanova, 2009).

The morphological distinctness of *C. platyacanthum* s.l., its unique genetic association with European populations of *C. demersum* showing the same flavonoid profile and the disjunct distribution of *C. platyacanthum* s.l. nested in the range of *C. demersum* add credence to the possibility that the taxon is a recently diverged polyploid derivative of *C. demersum*.

#### CLADE SMAT

This group closely parallels Wilmot-Dear’s (1985) *sensu lato* taxonomic concept of *C. submersum* and includes those species with leaves dichotomously divided into three or four orders, with the exception of *C. echinatum*. ‘Clade SMAT’ contains two well-supported groups, ‘Clade AT’ and ‘Clade SM’. This particular association of species contrasts with earlier classifications that were based primarily on morphological studies (e.g. Wilmot-Dear, 1985; Les, 1986a).

#### CLADE SM

In the current taxonomic treatment of *Ceratophyllum*, the most intriguing result is the topology of ‘Clade SM’. Because the *C. submersum* accessions form a strongly

supported group (PP: 1; BS: 99), and its supported sister branch (PP: 0.98; BS: 77) comprises all the *C. muricatum* subsp. *muricatum* and *C. muricatum* subsp. *kossinskyi* samples, we suggest treating them as two different species: *C. submersum* and *C. muricatum*. Both monophyletic groups can be distinguished by several synapomorphic morphological characters (Les, 1986a, 1988a).

‘Clade SM’ incorporates the spiny-margined *C. muricatum* subsp. *muricatum* and *C. muricatum* subsp. *kossinskyi* and the spineless *C. submersum* accessions in accordance with Wilmot-Dear’s classification (Fig. 1, Table 3). All of these taxa are more or less allopatric. According to Les (1986a, 1989) the global distribution of *C. submersum* is restricted to Europe and adjacent temperate Asia (Komarov, 1937; Malyshev, Peschkova & Baikov, 2003), with some enigmatic, disjunct occurrences in Subsaharan Africa. Although *C. submersum* is absent in the larger part of Asia, *C. muricatum* subsp. *kossinskyi* occupies similar habitats in temperate zones of the Far East and Europe (Komarov, 1937; Markova, 1970; Fu & Les, 2001).

In many previous studies it has been widely accepted that the fruit of *Ceratophyllum* provides the most important morphological characters that make it possible to distinguish different species in the genus (e.g. Sapjegin, 1902; Les, 1986a, b, 1988a, b, 1989; Scribailo & Alix, 2002), and this is certainly the case with some taxa. However, total or partial spine reduction occurs sporadically in all spiny-margined species distinguished by Les (1989) and Csiky *et al.* (2010). If we accept that the spineless fruit of *C. submersum* is a result of reduction in the clade, the new phylogenetic results are in congruence with the distribution ranges of the extant Old World species. *Ceratophyllum muricatum* subsp. *muricatum* would represent ‘Clade SM’ in the tropical zone (Africa, India, Indonesia and Melanesia), whereas *C. muricatum* subsp. *kossinskyi* and *C. submersum* would represent the same group in the temperate zone (Far East and Europe). This treatment is partly in accordance with Wilmot-Dear’s (1985) concept, which merges these species under *C. submersum* (Fig. 1, Table 3). However, in Wilmot-Dear’s system other species are also subsumed in *C. submersum*; these form the distinct, strongly supported ‘Clade AT’ (e.g. *C. australe* and *C. tanaiticum*) and *C. echinatum* in the current phylogenetic analysis (Fig. 1, Table 3).

#### CLADE AT

Although Les (1989) classified *C. tanaiticum* and *C. muricatum* in one section, according to the present analysis, samples of *C. tanaiticum* and *C. muricatum* (except *C. m.* subsp. *australe*) are separated into two fully supported distinct clades (‘Clades SM’ and ‘Clade

AT’). Moreover, two geographically separated taxa, i.e. *C. m.* subsp. *australe* and *C. tanaiticum*, are the closest relatives in the *C. submersum* clade. According to Les (1986a), *C. muricatum* subsp. *muricatum* and *C. m.* subsp. *kossinskyi* are similar morphologically, whereas *C. m.* subsp. *australe* is fairly distinct from *C. m.* subsp. *muricatum*. The most important characters that distinguish both species of ‘Clade AT’ from the other spiny-margined taxa are (1) the length of the peduncle, which on average is longer than 1 mm, (2) the number of marginal spines (more than three on each side) and (3) the length to width ratio of the fruit body, which is greater than or equal to 1.6 (Les, 1986a). Although the phylogenetic results do not resolve accessions of *C. tanaiticum* as a distinct clade, they are distinct from the accessions of *C. m.* subsp. *australe*, which are separated in a strongly supported group in ‘Clade AT’ (Fig. 1). Because of their disjunct distribution (separated by the Atlantic Ocean) and their specific morphological characters, we confidently recognize them as two distinct species: *C. australe* and *C. tanaiticum*. Despite the substantial distance between the two *C. australe* samples of > 3000 km (Florida–Ecuador), we did not detect any sequence differences between them. These accessions of *C. australe* were so similar that they lacked any unique SNPs. The accessions of *C. australe* clustered in a strongly supported group, whereas those of *C. tanaiticum* resolved as a polytomy in ‘Clade AT’.

The results presented here particularly affect the taxonomic and conservation status of *C. tanaiticum*, which has been noted as a species of dubious validity in some publications (see Jalas & Suominen, 1989; Lansdown, 2018). The phylogenetic tree also supports the separation of this species from *C. submersum* and from the regionally closest representative of *C. muricatum* (*C. muricatum* subsp. *kossinskyi*), which were formerly thought to be the closest relatives of *C. tanaiticum*. This rather rare, but characteristic element of the East European steppe and forest–steppe zones certainly deserves conservation and recognition as an important endemic species of the region. Elevating *C. australe* from subspecies rank allows proposal of conservation status for this rare entity in the southeastern United States where habitat loss from coastal development severely limits its distribution.

#### PALAEOBOTANICAL ASPECTS

Findings from the current study, which provide new insights into the phylogenetics of *Ceratophyllum*, have implications for the palaeobotanical history of the genus on a world-wide scale. Several fossils of *Ceratophyllum* have been reported previously in the palaeobotanical literature; however, their assignment to a particular species has sometimes been questionable (Dorofeev, 1988; Herendeen, Les & Dilcher, 1990). A potential example



of this problem can be found with the fossils of *C. cf. muricatum* subsp. *muricatum* discovered in a Miocene (16 Myr old) stratum in China (Wang, Ferguson & Li, 2005). According to Wang *et al.* (2005: figs 8 and 10; cf. Haynald, 1881: figs I-IIIa and B), the morphology of the basal and stylar spines (e.g. length, angle of contingence), the winged marginal spines and the edge on the facial side of the fruit refer to an ancient type of *C. platyacanthum* subsp. *platyacanthum* (cf. Chamisso, 1829: fig. 6a, Wilmot-Dear, 1985: fig. 3M). The leaf divisions of these fossils (i.e. Wang *et al.*, 2005: fig. 9) also refer to section *Ceratophyllum* (Les, 1989), as the leaves dichotomously divide into two to four ultimate segments with larger leaves forking twice (three times). Specimens with marginal wings and facial spines of section *Ceratophyllum* (i.e. *C. platyacanthum* subsp. *platyacanthum*) were reported exclusively from Europe (Les, 1986a). Dorofeev (1988) suggested that *C. demersum* is the youngest species among the taxa of Ceratophyllaceae and probably appeared in the upper Miocene, but became frequent in the Pliocene and Pleistocene. Findings from this paper (Wang *et al.*, 2005) would be the first to indicate the presence of *C. demersum* s.l. (including *C. platyacanthum*) in the Miocene.

Certain fossils from the Tertiary of North America (Herendeen *et al.*, 1990) and Asia (Avakov, 1962, Dorofeev, 1988) resemble the taxa of 'Clade AT'; however, the fruit characters on the fossils are intermediate between the values observed for *C. australe* and *C. tanaiticum*. According to achene morphology, *C. muricatum* subsp. *incertum* (Berry) Herendeen, Les & Dilcher and *C. zaisanicum* Avakov are somewhat more similar to *C. australe* (e.g. peduncle length, spines, achene length to width ratio), whereas fossils of *C. furcatispinum* Herendeen, Les & Dilcher are closer to *C. tanaiticum* (e.g. wings, achene length to width ratio) (cf. Les, 1986a). The presence of such fossils in the Palaeocene–Eocene strata of North America and in the Oligocene–Miocene deposits in Asia suggests a common ancestor of these species evolved in North America in the early Palaeogene. Like several angiosperms at that time, descendents of this taxon supposedly migrated (1) to Asia via the Bering land bridge (Wen, Nie & Ickert-Bond, 2016), which was continuous throughout the Palaeogene and intermittent since (ancestors of *C. tanaiticum*), and (2) to South America via the Panamic bridges (Cione *et al.*, 2015), which was continuous in the Palaeogene and in the Pliocene–Pleistocene (the ancestors of *C. australe*).

If the dating of these fossils is correct it would suggest that the morphological features of the aforementioned *Ceratophyllum* spp. are representative of ancient (at least Palaeocene) character states in the genus (e.g. on the fruit cf. Avakov, 1962; Dorofeev, 1988). As an example, the structure of the (*Ceratophyllum* leaf-like)

cylindrical bracts with lateral teeth on Ukrainian and Hungarian *C. tanaiticum* specimens and the long peduncles on both extant species of 'Clade AT' perhaps preserve a primitive state of the involucre reduction (cf. Les, 1986a; Csiky *et al.*, 2010).

Les (1989) hypothesized that the present distribution of *Ceratophyllum* spp. and the three subspecies of *C. muricatum* was due to tectonic events that occurred during the breakup of Gondwanaland. Taking the present and former (Les *et al.*, 2003) results into consideration, it may be possible to explain some of the recent distributions (and not the origin of the taxa) by climatic events that occurred during the Pleistocene and/or in the Holocene. It is likely that the temperate European *C. submersum* (Les, 1989) survived the glacial periods in lake refugia in close proximity to the Mediterranean Sea and expanded its distribution to its current range in the Holocene (see *Fagus sylvatica* L. in Europe, Magri, 2008; cf. Katz, Katz & Kipiani, 1965). Outside of the contiguous range of *C. submersum* the enigmatic occurrences of this species in Subsaharan Africa can possibly be explained in a biogeographical context. According to Les (1986a) the primary mechanism of long-distance dispersal in the genus is endozoochory by waterfowl. Although it has been suggested in former studies (Les, 1986a) that the African stands are relict populations of a once wider distribution, we hypothesize that *C. submersum* is an 'occasional species' in Africa and that the Subsaharan populations are recent introductions. In this regard it is noteworthy that (1) with the exception of Africa, *C. submersum* has never been found outside of the temperate zone, (2) the actual routes of European migratory birds notably cross the sporadic localities of *C. submersum* in Africa (Flack *et al.*, 2016) and (3) the barrier is a 1800-km desert between the European (core) and African (sink) populations (cf. Hultén & Fries, 1986; Les, 1986a, 1989; Alerstam, 1990; Strandberg *et al.*, 2010). The probability that a fruit of a temperate *Ceratophyllum* taxon would survive the migration via birds from Europe to Africa, then germinate and form populations in a suitable habitat decreases from the north to south in Africa (cf. Strandberg *et al.*, 2010). The unlikelihood of the latter might explain why this taxon is absent from the southern part of tropical Africa and there are not any reconfirmed *C. submersum* (Les, 1989) populations from Subsaharan Africa (except one in Tanzania; Les, 1986a).

In the post-glacial period it appears likely that *C. muricatum* subsp. *muricatum* was widely distributed from 10° to 30° latitudes in Africa and the Middle East, as the climate of this area was much wetter during the Holocene (Drake *et al.*, 2011). After the Holocene climatic optimum an increase in aridity divided the formerly continuous savanna region into two isolated parts, the larger in Africa and the smaller in India. This climatic

change might ultimately have led to the isolation of two *Ceratophyllum muricatum* subsp. *muricatum* populations, one in Africa and one in India. A number of wetland taxa, including *Utricularia* L., *Blyxa* Noronha ex Thouars and *Wiesneria* Micheli (Cook, 1996, 2004), exhibit a disjunct distribution similar to that seen in *Ceratophyllum muricatum* subsp. *muricatum*. It is also relevant that the migratory routes of waterfowl, which are largely north–south, reduce the likelihood of long-distance endozoochoric dispersal between Africa and India, thus reinforcing the isolation of these relict populations (cf. Palm *et al.*, 2015; Flack *et al.*, 2016).

Today, *C. tanaiticum* has a relatively small range in the steppe and forest–steppe regions of the Pontic and Turanian regions, with some isolated populations in the Carpathian Basin (Csiky *et al.*, 2010). During and after the last glacial period, *C. tanaiticum* could have survived in the contracted and then partially fused Black Sea and Caspian Sea region (Dolukhanov, 2007) and subsequently expanded its ranges with climatic

warming at the end of the Holocene (cf. Katz *et al.*, 1965; Dubyna, Chorna & Borimska, 1985).

## CONCLUSIONS

From the perspective of data presented in the current study, neither Wilmot-Dear's (1985) nor Les' (1986a, 1989) overall concept of species delimitation in the genus *Ceratophyllum* is entirely supported. In summary, results from this study indicate (1) the presence of more than two species in the genus, (2) that *C. echinatum* is not the closest relative of *C. submersum*, (3) that *C. muricatum* is more closely related to *C. submersum* than to *C. tanaiticum* and (4) that *C. tanaiticum* resolves in a well-supported clade with *C. australe*, distinct from accessions of *C. muricatum*. The results also emphasize that morphological diversity of *C. demersum* s.l. is not entirely congruent with genetic diversity of the selected markers (ITS, *matK*).

### NEW KEY

Using the phylogenetic analysis presented here as a framework for species delimitation the following key for *Ceratophyllum* is presented based on the morphological and distribution data of Les (1986a) and Csiky *et al.* (2010). This key uses distinctive characters based on statistical analyses, but some of the fruit and leaf characters are variable and overlapping in their lower or upper domains in the case of certain species (Les, 1986a).

- 1a** Leaves forked on the first node of the plumule. Leaves dichotomously divided to three or four orders, fine-textured, soft, collapsing upon removal from the water, with inconspicuously exerted denticles. Achenes with elongate stylar spine and margins typically with numerous lateral spines ..... *C. echinatum* A.Gray  
Distribution: temperate North America.
- 1b** Leaves simple on the first node of the plumule..... **2**
- 2a** Leaves dichotomously divided to two (one to three) orders, coarse-textured, rigid, retaining their form when removed from the water, with strongly exerted denticles. Achenes with an elongate stylar and two basal spines; facial spines present or absent. Spines occasionally winged, rarely totally absent... *C. demersum* L.  
Distribution: cosmopolitan.
- 2b** Leaves dichotomously divided to three or four orders, fine-textured, soft, collapsing upon removal from the water, with inconspicuously exerted denticles..... **3**
- 3a** Peduncle short ( $\leq 1$  mm), fruit body length/width ratio  $> 1.6$ . Achene margins spineless or with several spines..... **4**
- 3b** Peduncle  $> 1$  mm, fruit body length/width ratio  $< 1.6$ . Achene margins with several spines..... **5**
- 4a** Achene spineless. Fruit body length  $> 4.5$  mm, stylar spine  $\leq 2$  mm..... *C. submersum* L.  
Distribution: Europe and the adjacent regions of Asia and Africa.
- 4b** Achene margin with at least two basal spines. Fruit body length  $\leq 4.5$  mm, stylar spine usually  $> 2$  mm ..... *C. muricatum* Cham.  
Distribution: Africa, Asia and Melanesia, sporadic in Europe and Australia.
- 5a** Bract flattened, peduncle  $< 2$  mm. Leaves  $> 2$  cm. Achene margin with several spines and occasionally slightly winged..... *C. australe* Griseb.  
Distribution: Tropical and warm temperate Americas near or North from the Equator.
- 5b** Bract cylindrical, peduncle  $> 2$  mm. Leaves  $\leq 2$  cm. Achene margin with a well-developed wing typically with several spines..... *C. tanaiticum* Sapjegin  
Distribution: endemic to the steppes of East Europe.

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