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REGULAR PAPER

Molecular phylogenetic species delimitation in the aquatic genus *Ottelia* (Hydrocharitaceae) reveals cryptic diversity within a widespread species

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Abstract

Ottelia, a pantropical genus of aquatic plants belonging to the family Hydrocharitaceae, includes several narrowly distributed taxa in Asia. Although the Asian species have received comparatively more research attention than congeners in other areas, various key taxonomic questions remain unaddressed, especially with regards to apparent cryptic diversity within *O. alismoides*, a widespread species complex native to Asia, northern Australia and tropical Africa. Here we test taxonomic concepts and evaluate species boundaries using a phylogenetic framework. We sampled five of the seven species of *Ottelia* in Asia as well as each species endemic to Africa and Australia; multiple samples of *O. alismoides* were obtained from across Asia. Phylogenetic trees based on five plastid DNA markers and the nuclear ITS region shared almost identical topologies. A Bayesian coalescent method of species delimitation using the multi-locus data set discerned one species in Africa, one in Australia and four in Asia with the highest probability. The results lead us to infer that a population sampled in Thailand represents a hitherto unrecognised cryptic taxon within the widespread species complex, although the apparent lack of unambiguous diagnostic characters currently precludes formal description. Conversely, no molecular evidence for distinguishing *O. cordata* and *O. emersa* was obtained, and so the latter is synonymised under the former. Two accessions that exhibit inconsistent positions among our phylogenetic trees may represent cases of chloroplast capture, however incomplete lineage sorting or polyploidy are alternative hypotheses that ought to be tested using other molecular markers.

Keywords Alismatales · Indo-Burma Biodiversity Hotspot · Monocotyledons · New species · Species delimitation · STACEY

Introduction

Ottelia is a genus of aquatic plants belonging to the family Hydrocharitaceae (Cook and Urmi-König 1984; Cook et al. 1983). The genus has two main centres of diversity, one in tropical Africa (13 species) and the other in Southeast Asia (six species) (Cook and Urmi-König 1984; Cook et al.

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1983). In their interfamilial phylogenetic analysis of Alismatidae (Alismatales), Les et al. (1997) included samples of the Asian *Ottelia alismoides* (L.) Pers. and the African *O. ulvifolia* (Planch.) Walp. However, significant phylogenetic insights into the subgeneric classification of *Ottelia* were not gained until Chen et al. (2012) published their molecular phylogenetic analysis of Hydrocharitaceae, in which relationships among five *Ottelia* species (one accession each of *O. acuminata* (Gagnep.) Dandy, *O. alismoides*, *O. emersa* Z.C. Zhao & R.L. Luo, *O. ovalifolia* (R.Br.) Rich. and *O. sinensis* (H. Lév. & Vaniot) H. Lév. ex Dandy) were examined. This study revealed the Australian *O. ovalifolia* to be sister to the four Asian species. Nevertheless, *Ottelia* still represents one of the three "hydrocharit genera most in need of more comprehensive study" (Les and Tippery 2013). Of particular interest is the widely distributed *O. alismoides*, which exhibits extensive morphological variation throughout its range. Molecular systematic studies of a diverse range of other groups containing similarly widespread and morphologically variable taxa, including frogs (Angulo and Icochea 2010; Funket al. 2012), lizards (Oliver et al. 2009), birds (Lohman et al. 2010; Manthey et al. 2011) and crabs (Phiri and Daniels 2016), have uncovered evidence of considerable cryptic speciation.

Cook and Urmi-König (1984) revised the number of Asian species to six, namely, *O. acuminata* (South China), *O. alismoides* (widespread in tropical and subtropical Asia and northern Australia), *O. balansae* (Gagnep.) Dandy (South China and Northern Vietnam), *O. cordata* (Wall.) Dandy (South China (Hainan), Bangladesh, Cambodia, Myanmar and Thailand), *O. mesenterium* (Hallier f.) Hartog (Sulawesi) and *O. sinensis* (South China) (Table 1). A seventh Asian species, *O. emersa*, was described more recently from Southwest China (Luo and Wang 1987), but He and Sun (1990) rejected the distinctiveness of this entity based on morphological and isozyme evidence, and so synonymised it under *O. cordata*. Further, He et al. (1990) studied the morphology of *O. balansae* and *O. sinensis* and concluded that the two are impossible to distinguish and thus the latter was subsumed under the synonymy of the former. In their treatment of Hydrocharitaceae for the Flora of China, Wang et al. (2010) followed He et al.'s (1990) conclusion in treating *O. sinensis* as a synonym of *O. balansae*, but disagreed with He and Sun (1990) and instead accepted both *O. cordata* and *O. emersa* as distinct species because "This species [*O. emersa*] differs from *Ottelia cordata* by its leaves emersed and not dimorphic, male flowers in each spathe with up to 47–60 flowers, and seeds densely hairy". Accepted nomenclature according to Govaerts et al. (2018) mostly follows Wang et al. (2010). *Ottelia acuminata* and *O. balansae* are morphologically distinct, although identification is sometimes confused if it is ambiguous whether specimens are functionally bisexual or not (Cook and Urmi-König 1984).

The aim of the present study was to assess two taxonomic questions central to an improved understanding of the phylogenetic history of *Ottelia*: (i) whether undetected cryptic diversity exists within the widespread *O. alismoides*, and (ii) whether *Ottelia cordata* and *O. emersa* are conspecific. To do so, we undertook expanded taxon sampling as compared with the earlier studies, with a particular focus on the species of Asia. Here we apply a phylogenetic species concept to resolve the boundaries within a multi-locus data set comprising both plastid markers (hereinafter ptDNA) and the nuclear ribosomal ITS region (hereinafter nrITS). We utilise our findings to reappraise the taxonomy of the Asian members of the genus.

Materials and methods

Taxon sampling

Samples of *Ottelia* were collected in the field or obtained from herbarium specimens (Fig. 1; Table S1). For specimen identification, we used the taxonomic treatments of Cook et al. (1983) and Cook and Urmi-König (1984), with cross-referencing to the following local floras: den Hartog (1957), Cook (1996), Haynes (2001), Wang et al. (2010) and Tanaka (2015) for Asian species, and Jacobs and McColl (2011) for the Australian species. Hutchinson and Dalziel (1958) was consulted to confirm if our collections from Burkina Faso and Senegal corresponded to *O. ulvifolia*, the only species recorded in that flora. Identification to infraspecific level

Table 1 Summery of taxonomic studies on *Ottelia* in Asia

	O. acuminata	*O. alismoides*	*O. balansae*	*O. cordata*	*O. emersa*	*O. mesenterium*	*O. sinensis*
Cook (1984)	*O. acuminata*	*O. alismoides*	*O. balansae*	*O. cordata*	–	*O. mesenterium*	*O. sinensis*
Luo and Wang (1987)	–	–	–	–	*O. emersa*	–	–
He et al. (1990)	–	–	*O. balansae*	–	–	–	*O. balansae*
He and Sun (1990)	–	–	–	*O. cordata*	*O. cordata*	–	–
Wang et al. (2010)	*O. acuminata*	*O. alismoides*	*O. balansae*	*O. cordata*	*O. emersa*	–	*O. balansae*
WCSP (2018)	*O. acuminata*	*O. alismoides*	*O. balansae*	*O. cordata*	*O. emersa*	–	*O. balansae*

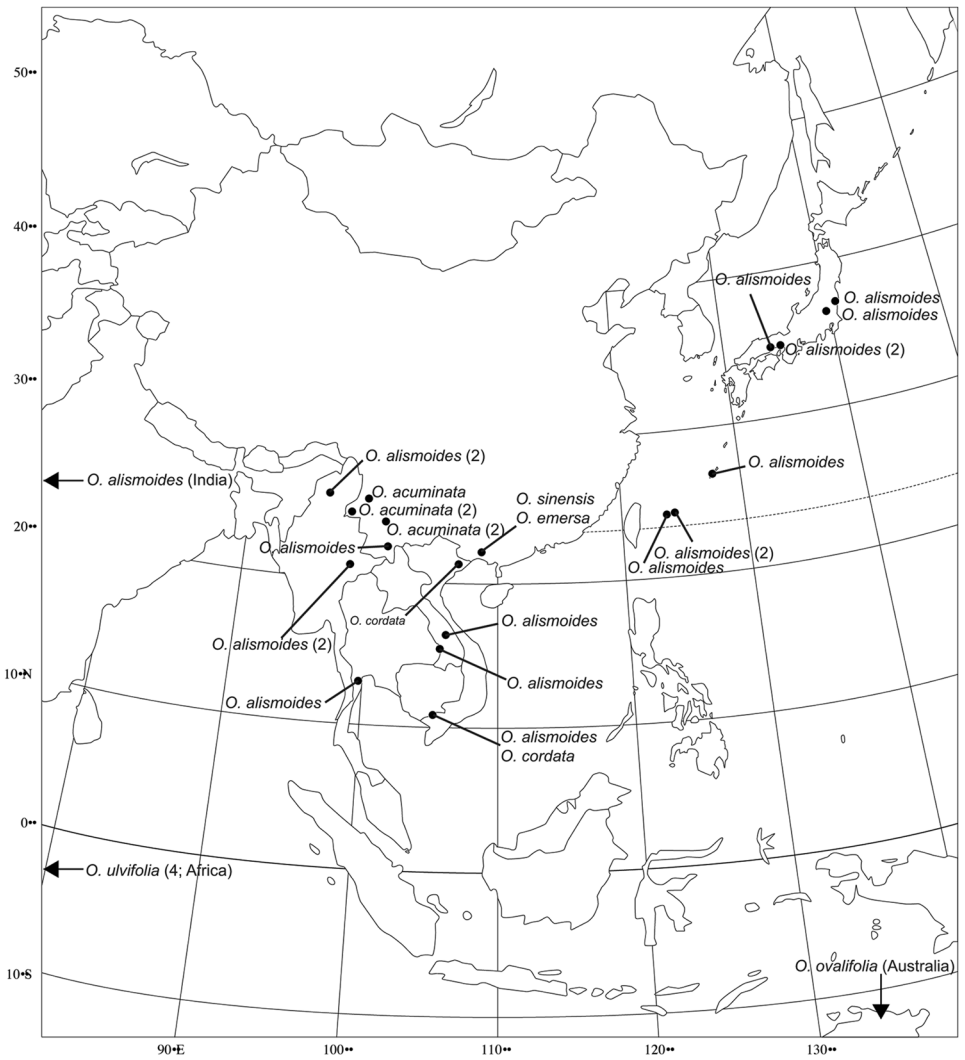

Fig. 1 Map of sampling localities of *Ottelia* species included in the present study. The number of specimens per species per area is shown in parentheses. Only the native accessions are shown here; the samples of garden origin (one each for *O. acuminata*, *O. alismoides* and *O. ulvifolia*) are not shown

within *O. acuminata*, as defined by Cook and Urmi-König (1984) and Wang et al. (2010), was not conducted because our focus was on inter-specific relationships. Taxa sampled for our study were *O. acuminata* (three samples, including one from Chen et al. 2012), *O. alismoides* (20 samples), *O. cordata* (four samples), *O. emersa* (a single sample from Chen et al. 2012), *O. ovalifolia* (one sample), *O. sinensis* (a single sample from Chen et al. 2012) and *O. ulvifolia* (four samples) (Fig. 1). Multiple samples of *O. alismoides* were obtained from across Asia yet no samples were available from northern Australia and tropical Africa. The sample of *O. emersa* originated from the type locality, which is given as "Guixian (Guiguang), Guangxi, China" (Chen et al. 2012). *Blyxa* Noronha ex Thouars and *Elodea* Michx., representing the most and one of the second most closely related genera in the phylogeny of Hydrocharitaceae, respectively, were chosen as outgroup taxa following Tanaka et al. (1997), Les et al. (1997, 2006), Chen et al. (2012), and Les and Tippery (2013), even though *Ottelia* was found to be non-monophyletic, with some species instead grouping with *Blyxa* species in the *rbcL* analyses of Les et al. (1997) and Les and Tippery (2013).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica gel-dried leaf tissue using the CTAB method described in Ito et al. (2010). Five regions of ptDNA (*matK*, *ndhF*, *rbcL*, *rpoB* and *rpoC1*) and nrITS were PCR amplified with the following primers: trnK-Hy2F (5′-ACACAGTTTCATACCCAATC) and trnK-Bly3R (5′-CCTTGTTCTGACCATATCGC) for *matK*; ndhF-F2 (Oxelman et al. 1999) and ndhF-1955R.re (Ito et al. 2017) modified from the primer ndhF-1955R published by Olmstead and Sweere (1994) for *ndhF*; rbcL-F1F (Wolf et al. 1994) and rbcL-1379R (Little and Barrington 2003) for *rbcL*; '2f' (5′-ATGCAACGTCAAGCAGTTCC) and '4r' (5′-GATCCCAGCATCACAATTCC) for *rpoB* (published by Chase et al. 2007); and '1f' (5′-GTGGATACACTTCTT

GATAATGG) and '3r' (5′- TGAGAAAACATAAGTAAA CGGGC) for *rpoC1* (published by Chase et al. 2007); ITS-4 and ITS-5 for nrITS (Baldwin 1992). PCR amplification was conducted using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan). PCR cycling conditions were 94 °C for 60 s; then 30 cycles of 94 °C for 45 s, 52 °C for 30 s, 72 °C for 60 s, with a final extension of 72 °C for 5 min. PCR products were cleaned using ExoSAP-IT purification (GE Healthcare, Piscataway, New Jersey), and then amplified using Big Dye Terminator ver. 3.1 (Applied Biosystems, Foster City, California) using the same primers as those used for the PCR amplifications. DNA sequencing was performed with a 3130xl Genetic Analyzer (Applied Biosystems). Automatic base-calling was checked by eye in Genetyx-Win ver. 3 (Software Development Co., Tokyo, Japan). All sequences generated in the present study have been submitted to the DNA Data Bank of Japan (DDBJ), which is linked to GenBank, and their accession numbers and voucher specimen information are presented in Table S1.

Molecular phylogenetic analysis

Sequences were aligned using Mafft ver. 7.058 (Katoh and Standley 2013) and then inspected manually. Indels were not coded because length variations were either ambiguous (nrITS) or observed only between outgroup and ingroup taxa (*matK* and *ndhF*). Analyses were independently performed for ptDNA (*matK*, *ndhF*, *rbcL*, *rpoB*, and *rpoC1*) and nrITS data sets respectively to identify possible incongruences between different genomic regions. All 34 ingroup and the two outgroup accessions were included in the ptDNA data set, while 34 ingroup and one outgroup (*Blyxa*) accessions were included in the nrITS data set to allow accurate alignment of the fast-evolving nrITS region.

Phylogenies were reconstructed using maximum parsimony (MP) in PAUP* ver. 4.0b10 (Swofford 2002), maximum likelihood (ML), and Bayesian inference (BI; Yang and Rannala 1997). In the MP analysis, a heuristic search was performed with 100 random addition replicates and tree-bisection-reconnection (TBR) branch swapping, with the MulTrees option in effect. The MaxTrees option was set at 100,000. Bootstrap analyses (Felsenstein 1985) were performed using 1,000 replicates with TBR branch swapping and simple addition sequences. The MaxTrees option was set at 1,000 to avoid entrapment in local optima.

For the ML analysis, we used the RAxML BlackBox online server (http://phylobench.vital-it.ch/raxml-bb/), which supports GTR-based models of nucleotide substitution (Stamatakis 2008). The maximum likelihood search option was used to find the best-scoring tree after bootstrapping. The gamma model of rate heterogeneity was selected. Statistical support for branches was calculated by rapid bootstrap analyses of 100 replicates (Stamatakis et al. 2008).

BI analyses were conducted with MrBayes ver. 3.2.2 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) run on the CIPRES portal (Miller et al. 2010) after the best models had been determined in MrModeltest ver. 3.7 (Nylander 2002); these models were GTR + I+G and HKY + G for ptDNA and nrITS data sets, respectively. Analyses were run for 3,000,000 generations for ptDNA and nrITS data sets, respectively, sampling every 1,000 generations and discarding the first 25% as burn-in. The convergence and effective sampling sizes (ESS) of all parameters were checked in Tracer ver. 1.6 (Rambaut et al. 2014). All trees were visualised using FigTree ver. 1.3.1 (Rambaut 2009). Nodes were recognised as strongly (≥ 90% MP bootstrap support (BS), ≥ 90% ML BS or ≥ 0.95 posterior probability (PP)), moderately (≥ 70% MP BS, ≥ 70% ML BS or ≥ 0.9 PP), or weakly (< 70% MP BS, < 70% ML BS or < 0.9 PP) supported. The data matrices and the MP, RAxML and BI trees were deposited in Treebase (http://purl.org/phylo/treebase/phylows/study/TB2:S20673?x-access-code=ac4d65e4ca13b2a554a1d9522f8d47ee&format=html).

Test of topological incongruence

The Shimodaira Approximately Unbiased (AU) test (Shimodaira 2002) was implemented in PAUP* to test for topological conflict. We used the RAxML alignment in NEXUS format and a file composed of the best trees from each constrained RAxML analysis using the default likelihood settings, with 10,000 bootstrap replicates. The following conflicting nodes discerned upon visual inspection were used as constraints with the other data set: Constraint pt-1, a monophyletic clade of clade VI plus *O. alismoides* TD5603 and *O. alismoides* YI1157 in Fig. 2b, and Constraint nr-1, a monophyletic clade of singleton V plus *O. alismoides* TD5603 and *O. alismoides* YI1157 in Fig. 2a.

Species delimitation using STACEY

A Bayesian coalescent method of species delimitation was performed using STACEY (species tree estimation using DNA sequences from multiple loci; Jones 2017), which is an extension of DISSECT (Jones et al. 2015). STACEY was implemented in BEAST ver. 2.4.4 (Bouckaert et al. 2014; Drummond and Rambaut 2007; Drummond et al. 2006).

We ran STACEY using a multilocus data set (ptDNA and nrITS) with all ingroup species; outgroup species were excluded to avoid rate differences and hidden substitutions between ingroup and outgroup species (B. Oxelman, personal communication, November 22, 2016). We performed two independent runs of ten million generations of the MCMC chains, sampling every 1 000 generations. Convergence of the stationary distribution was checked by visual inspection of plotted posterior estimates using Tracer ver.

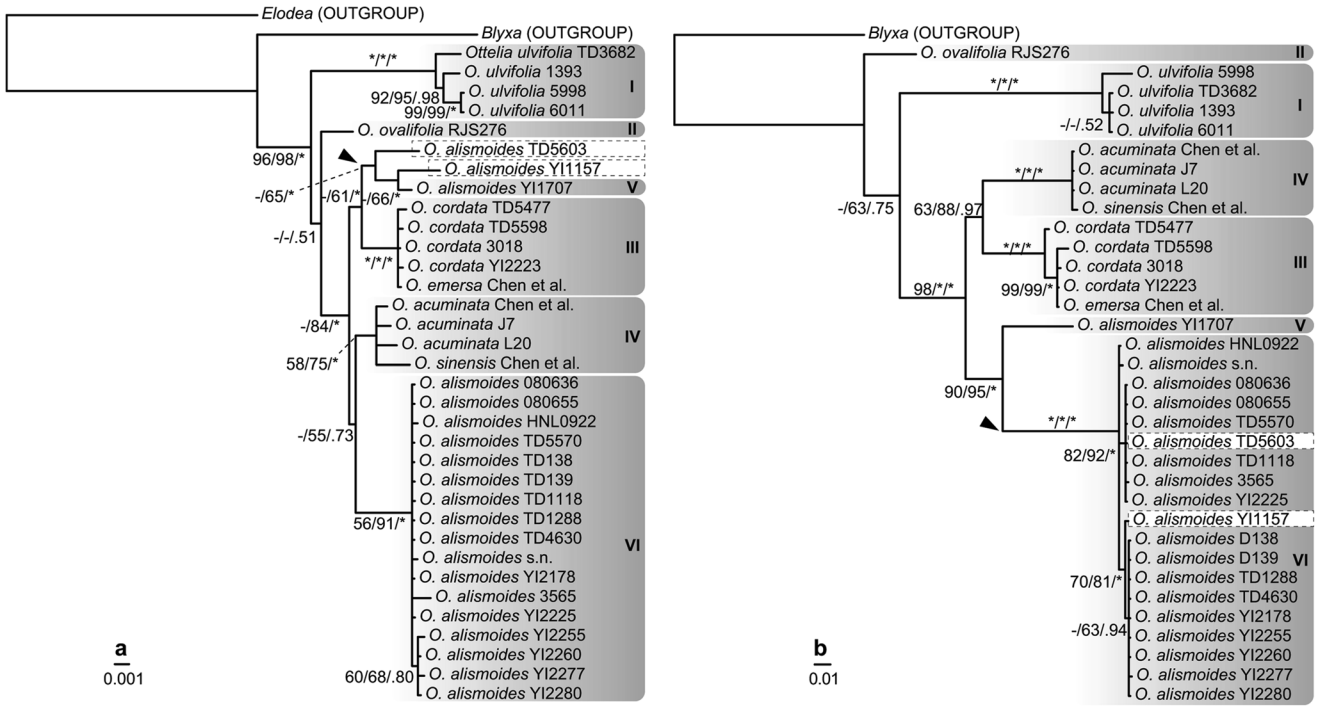

Fig. 2 MrBayes trees of *Ottelia* based on **a** ptDNA (*matK*, *ndhF*, *rbcL*, *rpoB*, and *rpoC1*) and **b** nrITS data sets. Numbers above or below the branches indicate bootstrap support (BP) calculated in maximum parsimony (MP BS) and maximum likelihood (RAxML BS) analyses and Bayesian prior probabilities (PP). BP < 70% and PP < 0.9 are indicated by hyphens while those of ≥ 95% and ≥ 0.99 are indicated by asterisks. Well-supported clades and singletons are highlighted by grey rectangles with Roman numerals. Two accessions whose position differed between the ptDNA and nrITS trees are enclosed in dashed boxes. Arrows indicate nodes in conflict between the ptDNA and nrITS trees

1.6 (Rambaut et al. 2014). After discarding the first 1 000 trees as burn-in, the samples were summarised in the maximum clade credibility tree using TreeAnnotator ver. 1.6.1 (Drummond and Rambaut 2007) with a posterior probability limit of 0.5 and summarising of mean node heights. The results were visualised using FigTree ver. 1.3.1 (Rambaut 2009).

Results

Molecular phylogeny

The ptDNA data set comprising five genes included 4,611 aligned characters, of which 111 were parsimony-informative. The percentage of missing characters was 14.27% for *matK*, 50.20% for *ndhF*, 4.11% for *rbcL*, 2.17% for *rpoB* and 3.83% for *rpoC1*. Analysis of this data set yielded the imposed limit of 100 000 MP trees (tree length = 395 steps; consistency index = 0.90; retention index = 0.92). The strict-consensus MP tree, the RAxML tree, and the MrBayes BI 50% consensus tree showed no incongruent phylogenetic relationships; thus, only the MrBayes tree is presented here (Fig. 2a).

It is ambiguous which species of *Ottelia* diverged first. In the RAxML analysis, *O. ovalifolia* (singleton II) is sister to all remaining species (58% RAxML BS), whereas in the MrBayes tree, *O. ulvifolia* (clade I) is sister to the rest (0.51 PP); neither relationship receives statistical support in the MP analysis. The core group excluding these two species is supported in both the RAxML (84% RAxML BS) and MrBayes (1.0 PP) analyses, and is resolved as comprising two distinct lineages: one containing clade III (*O. cordata*-*O. emersa*), singleton V (*O. alismoides* YI1707), *O. alismoides* TD5603 and *O. alismoides* YI1157 (61% RAxML BS; 1.0 PP), and the other containing clade IV (*O. acuminata*–*O. sinensis*) and clade VI (*O. alismoides*) (55% RAxML BS; 0.73 PP). However, neither of these lineages is supported in the MP tree.

The nrITS data set included 845 aligned characters, of which 216 were parsimony-informative. The percentage of missing characters was 12.97%. Analysis of this data set yielded the imposed limit of 100,000 MP trees (tree length = 538 steps; consistency index = 0.83; retention index = 0.93). The strict-consensus MP tree, the RAxML tree and the MrBayes BI 50% consensus tree showed no incongruent phylogenetic relationships; thus, only the MrBayes tree is presented here (Fig. 2b).

It is again ambiguous which species of *Ottelia* diverged first. In the RAxML and MrBayes analyses, *O. ovalifolia* (singleton II) is sister to all other taxa (63% RAxML BS; 0.75 PP), whereas in the MP tree *O. ulvifolia* (clade I) is sister to them (69% MP BS). The core group excluding these two species is strongly supported (98% MP BS; 100% RAxML BS; 1.0 PP) and clearly divided into two lineages: one comprising clade III (*O. cordata–O. emersa*) and clade IV (*O. acuminata–O. sinensis*) (63% MP BS; 88% RAxML BS; 0.97 PP), and the other comprising singleton V (*O. alismoides* YI1707), clade VI (*O. alismoides*), *O. alismoides* TD5603 and *O. alismoides* YI1157 (90% MP BS; 95% RAxML BS; 1.0 PP).

Topological incongruence

We observed two cases of hard incongruence (≥ 70% BS; Johnson and Soltis 1998) between ptDNA and nrITS trees, both of which were attributed to two accessions, *O. alismoides* TD5603 and *O. alismoides* YI1157: the two were positioned next to singleton V in the ptDNA data set, but were grouped in clade VI in the nrITS data set (arrows in Fig. 2). Constraining these conflicting nodes onto the other data set retrieved trees with maximum likelihood scores that were significantly worse than those derived from an unconstrained search, according to the AU test ($P < 0.05$ in both analyses; Tables S2, S3).

Species delimitation using STACEY

SpeciesDelimitationAnalyser generated 265 clusterings from the MCMC runs. The highest posterior probability was 0.33 PP for a classification with six species or minimal clusters, i.e. comprising four clades and two singletons (Fig. 3). The similarity matrix revealed that the individuals within the four clades had less than 0.07 PP belonging to a different cluster or a singleton (data not shown). While the similarity score among the 17 *O. alismoides* samples was 0. 9 858 739 on average, the score between these 17 samples and the single *Ottelia* sp. sample averaged 0. 0997812 (Table S4). Similarly, the average similarity score among the three samples of *O. acuminata* was 0. 9790857, whereas the score between the three *O. acuminata* samples and one *O. sinensis* sample was 0. 9255969 (Table S4). Further, the average similarity score among the four *O. cordata* samples was 0. 9544697, but the score between these four samples and the single *O. emersa* sample was 0. 9669628 (Table S4).

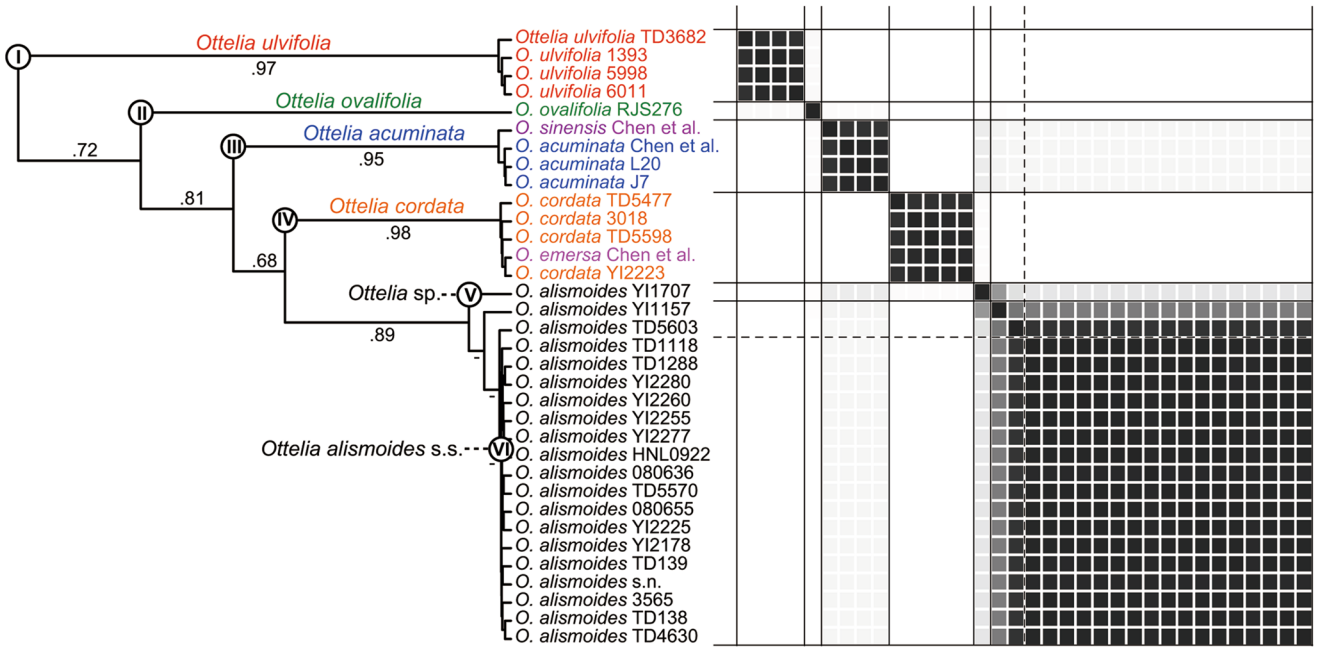

Fig. 3 Maximum sum of clade credibility SMC-tree based on multilocus (plastid DNA, nuclear ITS) data set of *Ottelia* from BEAST 2 analysis and similarity matrix from STACEY analysis. Posterior probabilities from BEAST 2 are given for major clades on the branches. The squares in the matrix represent posterior probabilities (white = 0, black = 1) for pairs of individuals belonged to the same cluster. Roman numerals on the clades of the SMC-tree, correspond to those in Fig. 2, representing the six minimal clusters or species of *Ottelia* delimited in STACEY analysis. The similarity scores of the two accessions that exhibited conflicting placement in Fig. 2 are indicated with dotted lines

Discussion

The present study examined intergeneric relationships within *Ottelia* using a phylogenetic approach. A Bayesian coalescent method of species delimitation using the multilocus data set discerned six species in our sample set. Here we discuss the implications of these findings in terms of the taxonomy of *Ottelia* species drawing on the phylogenetic species concept and with a particular focus on traditional morphological characters to distinguish species.

Cryptic diversity within *Ottelia alismoides*

Multiple molecular systematic studies have uncovered cryptic diversity within widespread taxa belonging to various life forms. In the present study, we sought to re-evaluate the status of the geographically widespread and morphologically variable *Ottelia alismoides* (Cook and Urmi-König 1984), which is conventionally diagnosed by its "spathe with 3 or more wings" (Cook and Urmi-König 1984; Cook et al. 1983), through phylogenetic analysis of multiple samples collected throughout its range in Asia and encompassing much of the morphological variation reviewed by Cook and Urmi-König (1984). *Ottelia alismoides* is, in all of our molecular phylogenetic analyses, divided into two lineages (Fig. 2), both of which are also recovered in species delimitation analysis (with an average similarity score of < 0.1) as belonging to the same species or minimal clusters (Fig. 3; Table S4). We therefore infer that *O. alismoides* sensu Cook and Urmi-König (1984) contains at least one cryptic species, in addition to *O. alismoides* sensu stricto (hereinafter referred to as *O. alismoides* s.s.). This cryptic species is represented in our study by the single accession YI1707 from Thailand.

Cook and Urmi-König (1984) summarised the extensive morphological variation in *O. alismoides* in terms of discrepancies in leaf outline (narrowly elliptic to very widely ovate), spathe morphology (urceolate, ellipsoidal, or cylindrical), flower colour (white, pink, bluish, violet, or light purple), and the number of stamens (3–12) and carpels (3–10). We confirmed much of this morphological variation among the 20 accessions of *O. alismoides* sensu Cook and Urmi-König (1984) included in our phylogenetic analyses, but nevertheless found no clear-cut difference between *O. alismoides* s.s. and *O. alismoides* YI1707. Given the wide karyotype variation that has been reported within *O. alismoides*, with diploid numbers ranging from $2n = 22$ to $2n = 132$ (Cook and Urmi-König 1984), it is possible that the cryptic taxon detected here may be distinguishable on the basis of chromosome number, but this character was not assessed in our samples. For the time being, we refrain from making any firm conclusion on the taxonomic status of *O. alismoides* until further material belonging to the same operational taxonomic unit as *O. alismoides* YI1707 becomes available.

Conspecific nature of *Ottelia cordata* and *O. emersa*

The present study aimed to test two alternative hypotheses pertaining to the status of *Ottelia cordata* and *O. emersa* in tropical Asia: He and Sun (1990) rejected the distinctiveness of the two species based on morphological and isozyme evidence, whereas Wang et al. (2010) and WCSP (2018) accepted both. The present study included four accessions of *O. cordata* and a single sample of *O. emersa* from the type locality in Guangxi, South China (Chen et al. 2012). The results of our phylogenetic analyses reveal *O. emersa* to be nested within *O. cordata* (with an average similarity score of 0. 9669628; Table S4), suggesting that the two are conspecific (Figs. 2, 3). Given that *O. cordata* is the earlier name, it takes nomenclatural priority over *O. emersa* (McNeil et al. 2012: Art. 11.3). The morphological differences between the two entities documented by Wang et al. (2010) (i.e. leaves emersed, submerged or floating, 10–30 or 47–60 male flowers per spathe and seeds densely hairy or smooth), should accordingly be considered infraspecific variation, and thus the description of *O. cordata* is here revised (see Taxonomic treatment).

Are *Ottelia acuminata* and *O. sinensis* distinct?

Ottelia acuminata is a dioecious species described from southwest China (Cook and Urmi-König 1984). It has since been recorded also from southeast China (Wang et al. 2010). In contrast, *O. sinensis* is a monoecious species known to occur in southwest China (Cook and Urmi-König 1984) and in northern Vietnam under the synonym *O. balansae*. The present study indicates that the single accession from Guangxi, southeast China, identified as *O. sinensis* by Chen et al. (2012), is phylogenetically indistinguishable from the three accessions of *O. acuminata* (with an average similarity score of 0.93; Table S4), possibly suggesting that the two are conspecific. However, because the locality in Guangxi has been reported to harbour both *O. acuminata* and *O. sinensis* (Cook and Urmi-König 1984; Wang et al. 2010), we cannot rule out the possibility that Chen et al. (2012) misidentified *O. acuminata* as *O. sinensis*. For the time being, we refrain from making any firm conclusion on the taxonomic status of either species until further material of *O. sinensis* becomes available, ideally from northern Vietnam where *O. sinensis* has been reported (under the synonym *O. balansae*), but where *O. acuminata* is not known to be distributed.

Evidence of reticulate evolution in *Ottelia*?

Two accessions of *Ottelia alismoides* from Myanmar and India (TD5603, YI1757) occupy inconsistent positions in the ptDNA and nrITS trees (Fig. 2; Tables S2, S3). Cases of such topological incongruence are often reported in phylogenetic studies (reviewed by Degnan and Rosenberg 2009; Wendel and Doyle 1998). Among the known causes, which include the particular genetic marker selected for phylogenetic reconstruction and incomplete lineage sorting (Wendel and Doyle 1998), chloroplast capture may best explain the phenomenon in *Ottelia* because the two accessions have unique ptDNA haplotypes but share nrITS ribotypes with *O. alismoides*, and also lack unique, heterogeneous nrITS (Fig. 2). It is noteworthy that the two accessions may represent independent taxa of hybrid origin, as in the recently reported case involving *Nymphoides montana* Aston (Menyanthaceae; Tippery and Les 2011), but this scenario is less likely because the specimens exhibited no distinct morphological differences. Additional material representing these entities from the two countries will be subjected to further phylogenetic and taxonomic analysis, in which alternative, more discriminatory approaches, such as low-copy nuclear DNA markers and AFLP, will be applied in order to provide greater clarity on the cause of this ambiguous finding.

Taxonomic treatment

Ottelia cordata (Wall.) Dandy, J. Bot. (London), 72: 137, 1934.

≡ *Boottia cordata* Wall., Pl. Asiat. Rariores, 1: 52, t. 65, 15 July 1830.—TYPE: BURMA. "ripae Irawaddi prope Avam" (Ava). Lectotype (designated by Cook and Urmi-König in Aquat. Bot. 20: 146. 1984): t. 65 in Wall., Pl. Asiat. Rariores, 1. 15 July 1830 (*non vidi* photo!).

= *Ottelia emersa* Z.C. Zhao et R.L. Luo, J. Wuhan Bot. Res., 5: 339, f. 1, November 1987. —TYPE: CHINA. Guangxi, Guixian county, in ponds and canals. Lectotype (designated by He and Sun in Aquat. Bot. 36: 397. 1990): 8 Oct. 1980, Z.C. Zhao 0595 (WH *non vidi*); paratype (designated herein): 22 Sep. 1986, R.L. Luo 030 (WH *non vidi*).

Diagnostic Features—Specimens having only emmersed leaves, densely hairy seeds, and 47–60 male flowers in the male spathe that were identified by Wang et al. (2010) as *O. emersa* are here treated as morphological variation within *O. cordata*.

Distribution and Habitat—Cambodia, China (Guangxi, Hainan), Myanmar, Thailand, Vietnam.

Notes—Two specimens were cited in the protologue of *Ottelia emersa* by Luo and Wang (1987). He and Sun (1990) apparently chose the older one (Z.C. Zhao 0595) as a lectotype; thus, the other specimen (R.L. Luo 030) is herein selected as a paratype, which He and Sun (1990) incorrectly treated as an "isotype".

IUCN conservation assessment—More than ten populations are known to occur in China (Guangxi, Hainan), Myanmar, Thailand, and Vietnam, including those confirmed in the course of the present study, with a total population size in excess of 1,000 individuals. Plants are known to set fruit and recruit freely at most of these sites. Although wetlands are in general threatened by land conversion and other modifications to natural drainage patterns throughout this region, there is at present no evidence that this species has undergone decline as a result. Therefore, we follow the current conservation assessment of Least Concern (LC) prepared by Juffe-Bignoli (2011).

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