Phylogeny and a revised classification of the Chinese species of *Nyssa* (Nyssaceae) based on morphological and molecular data

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Abstract The taxonomy of *Nyssa* (Nyssaceae) in China is confused and uncertain due to differing views about how many species should be recognized, and a scarcity of good morphological differential characters. In the present study, we examined 52 morphological characters from up to fifty accessions each of six of the seven species recognized in the *Flora of China*: *N. yunnanensis*, *N. javanica*, *N. sinensis*, *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis.* Based on both principal coordinate analysis and UPGMA cluster analysis, *N. yunnanensis* and *N. javanica* were both morphologically distinct. However, accessions of *N. sinensis*, *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis* were intermixed in both analyses and could not be discriminated from each other. Sequence data from nuclear ITS and the chloroplast regions *trnH-psbA*, *rps16r-f*, *trnL-rps32F*, *trnS-G* and *trnL-F* were used to further analyze the *Nyssa* species listed above, together with *N. aquatica* and *N. sylvatica* from North America. All analyses of the DNA data using maximum parsimony, maximum likelihood and Bayesian methods strongly supported the monophyly of *Nyssa*. Within *Nyssa*, *N. yunnanensis* and *N. javanica* were resolved as sister species, which were genetically distinct. The other four Chinese taxa were barely differentiated by molecular data, with only *N. shangszeensis* and *N. wenshanensis* weakly supported as monophyletic. Hence, based on morphological and molecular data, we recommend that *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis* be reduced to synonyms of *N. sinensis*, and only *N. sinensis*, *N. yunnanensis* and *N. javanica* be recognized for *Flora of China*. The American *N. aquatica* and *N. sylvatica* were sister species in the cpDNA but not in the ITS analyses, indicating possible reticulate evolution.

Keywords China; molecular data; *Nyssa*; PCO; phylogeny; taxonomic revision; UPGMA

Supplementary Material The alignment files are available in the Supplementary Data section of the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

Nyssa L. is one of three genera comprising Nyssaceae, together with *Camptotheca* Decne. and *Davidia* Baill. (Qin & Phengklai, 2007). The family has a Tertiary relict distribution, with *Camptotheca* and *Davidia* currently restricted to eastern Asia, whereas *Nyssa* is disjunct between eastern Asia and eastern North America (Eyde, 1966; Fang & al., 1983; Wen & Stuessy, 1993; Wu & al., 2003). In the Americas, *Nyssa* comprises three species in North America (*Nyssa aquatica* L., *N. ogeche* Marshall, *N. sylvatica* Marshall), and one in Costa Rica (*N. talamancana* Hammel & Zamora). However, the number of species in Asia is disputed: of the seven species recognized in *Flora of China* (Qin & Phengklai, 2007), only four were recognized by Wen & Stuessy (1993). Initially, only two species were proposed for eastern Asia: *N. sinensis* Oliv. and *N. javanica* (Blume) Wangerin (Eyde, 1963). Based on detailed morphological studies including fossils, Eyde (1963) considered *N. sinensis* to be most closely related to *N. sylvatica,* and *N. javanica* to be most closely related to *N. aquatica*. Subsequently, *Alangium shweliense* W.W. Sm. (Smith, 1921) was transferred to *Nyssa* as *N. shweliensis* (W.W. Sm.) Airy Shaw (Airy Shaw, 1969). Later, another four species were described from China, i.e. *N. shangszeensis* W.P. Fang & Soong, *N. leptophylla* W.P. Fang & T.P. Chen, *N. wenshanensis* W.P. Fang & Soong (Fang & Soong, 1975), and *N. yunnanensis* W.C. Yin (Wu & Fan, 1977).

Nyssa is defined by the following synapomorphies: flowers polygamodioecious, axillary inflorescence, single flower per pistillate inflorescence, and imbricate aestivation

(Wen & Stuessy, 1993). Based on a survey of 18 characters selected from herbarium specimens, Wen & Stuessy (1993) recognized only four species in eastern Asia, i.e., *N. javanica*, *N. shangszeensis, N. shweliensis* and *N. sinensis*; the other three (*N. leptophylla*, *N. wenshanensis*, *N. yunnanensis*) were treated as dubiously distinct from *N. javanica*. Wen & Stuessy (1993) hence regarded these last four species as a species complex in need of critical taxonomic re-evaluation. Differences between these four species appeared very small in the characters used, namely leaf shape, size and texture, number of leaf veins, petiole length, leaf and inflorescence pubescence, pedicel length, and fruit size. However, DNA barcode data indicated that *N. yunnanensis* is not closely related to *N. javanica* (N. Wang & al., 2012). However, for distinguishing species in *Nyssa* 18 morphological characters may be insufficent, and certain important characters such as fruit color can be lost in herbarium material. Therefore, a systematic analysis including a larger number of characters combined with detailed field observations appeared necessary to evaluate the status of these species.

To properly elucidate relationships and determine the number of *Nyssa* species in China, a detailed analysis combining morphometric and molecular data is necessary. The objectives of the present study are to: (1) reconstruct the phylogeny of the extant Chinese *Nyssa* species plus two North American species (*N. aquatica*, *N. sylvatica*) via phylogenetic analyses; and (2) evaluate the species status of Chinese *Nyssa* species using morphological data and molecular data. To address these objectives, nuclear ITS and five cpDNA markers (*trnH-psbA*, *rps16r-f*, *trnL-rps32F*, *trnS-G*, *trnL-F*) were used to investigate the phylogeny of *Nyssa*, and 52 morphological characters from 300 accessions of six Chinese species, from localities covering most of their distribution ranges, were examined and analyzed using principal coordinate analysis (PCO) and unweighted pair-group method of averages (UPGMA).

■ MATERIALS AND METHODS

Taxon sampling. — Of the seven *Nyssa* species listed in *Flora of China*, six were included in the current study; *N. leptophylla* could not be included because detailed locality information was unavailable and no samples of this species could be obtained. Type specimens of each Chinese species were examined; however, these provided relatively few measurable characters. Hence they were used to confirm that material from each type locality matched the species as originally described, but not incorporated into the analysis.

Material of *N. yunnanensis* and *N. javanica* was collected from one locality each (Appendix), and could be assigned to these species based on morphology. However, past taxonomic confusion combined with morphological similarity made identification of the other four species problematic. *Nyssa shangszeensis* and *N. wenshanensis* have only been collected from their type localities (Appendix), and in each case no other *Nyssa* species has ever been recorded from the vicinity of the type localities. Our own field observations confirmed the presence of only one species matching the type description (and type specimen) at each type locality. Therefore, material from the type localities was considered to represent these species. Similarly, material from localities where *N. sinensis* (and no other species) and *N. shweliensis* (and no other species) had previously been recorded was examined, confirmed to match descriptions of each species, and therefore assigned to *N. sinensis and N. shweliensis*, respectively. In addition, some cultivated accessions of *N. sinensis* from Kunming were examined. Localities and vouchers of specimens are listed in the Appendix, and voucher specimens were deposited in KUN.

For five species fifty living individuals were sampled for morphological analysis. However, only 37 individuals of *N. yunnanensis* are known in the wild, and therefore thirteen individual plants were sampled twice, from different parts of the same individual, to raise the number of accessions to 50 per species in order to avoid possible skewness in the analysis.

For each accession, some characters were recorded on the living plant, and at least one herbarium specimen was made, on which further characters were measured. Multiple specimens were made in cases where the first specimen did not contain all necessary characters. These specimens were deposited in KUN.

For molecular analysis, 10 species of Nyssaceae, represented by 18 accessions, were examined (Appendix). Multiple accessions were included for all Chinese species: *N. yunnanensis* (2 accessions), *N. javanica* (2), *N. sinensis* (4), *N. shangszeensis* (2), *N. shweliensis* (2) and *N. wenshanensis* (2). From each locality, one pistillate and one staminate individual were sampled (Appendix) which avoided any possibility of sampling the same genet twice. One accession each of the American *N. sylvatica* and *N. aquatica* obtained from the Arnold Aboretum of Harvard University were included, and one each of *Davidia involucrata* Baill. and *Camptotheca acuminata* Decne. as outgroups. The status of these genera as sister groups to *Nyssa* was confirmed by Xiang & al. (2002).

Morphological analysis. — A total of 52 characters were measured from each accession, comprising 33 qualitative (Table 1) and 19 quantitative (Table 2) characters. Morphological variation was assessed using univariate statistics. Measurements were made by hand with ruler, protractor and a binocular microscope. Coding of discrete character states was not designed to reflect evolutionary progressions (Möller & al., 2007). To visualize variation among individuals, the morphometric data were subjected to a PCO clustering analysis implemented in the software PAST v.1.75 (Hammer & al., 2006). The normal distribution of the continuous was tested prior to analysis and no character showed skewness. Plots were generated for the first two principal components (PCs). Because of the different nature of the characters (Casgrain & Legendre, 2001: 108), SPSS v.17.0 was used to generate a correlation matrix (by dividing each value by the variable's standard deviation). This eliminates the effect of the different measurement scales used and produces variables without physical dimensions (Casgrain & al., 2005). Principal components were used as input variables for a cluster analysis using UPGMA to generate a dendrogram in PAST.

Table 1. Qualitative morphological characters of *Nyssa* species examined for use in PCO and UPGMA cluster analysis.

Character[a]	*N. yunnanensis*	*N. javanica*	*N. sinensis*	*N. shweliensis*	*N. shangszeensis*	*N. wenshanensis*
1. Foliage	Evergreen (1)	Deciduous (0)	Deciduous (0)	Deciduous (0)	Deciduous (0)	Deciduous (0)
2. Bark color	Gray (2)	Taupe (1)	Brown (0)	Brown (0)	Brown (0)	Brown (0)
3. Lenticel density	Sparse (0)	Sparse-dense (1)	Dense (2)	Dense (2)	Dense (2)	Dense (2)
4. Bark surface	Smooth (0)	Smooth (0)	Rough (1)	Rough (1)	Rough (1)	Rough (1)
5. Calyx indumentum density	Dense (1)	Dense (1)	Sparse (0)	Sparse (0)	Sparse (0)	Sparse (0)
6. Calyx lobe shape	Semicircle (1)	Semicircle (1)	Triangle (0)	Triangle (0)	Triangle (0)	Triangle (0)
7. Bracteole shape	Triangular (0)	Triangular (0)	Filamentous (1)	Filamentous (1)	Filamentous (1)	Filamentous (1)
8. Breeding system	Androdioecy (0)	Androdioecy (0)	Tridioecy (1)	Tridioecy (1)	Tridioecy (1)	Tridioecy (1)
9. Staminate inflorescence form	Capitulum (0)	Capitulum (0)	Raceme (1)	Raceme (1)	Raceme (1)	Raceme (1)
10. Staminate peduncle indumentum	Dense (2)	Glabrous (0)	Sparse (1)	Sparse (1)	Sparse (1)	Sparse (1)
11. Staminate pedicel	Absent (1)	Absent (1)	Present (0)	Present (0)	Present (0)	Present (0)
12. Staminate disc color	Kelly green (0)	Kelly green (0)	Cyan (1)	Cyan (1)	Cyan (1)	Cyan (1)
13. Bisexual inflorescence form	Capitulum (0)	Capitulum (0)	Raceme (1)	Raceme (1)	Raceme (1)	Raceme (1)
14. Bisexual peduncle indumentum	Dense (2)	Glabrous (0)	Sparse (1)	Sparse (1)	Sparse (1)	Sparse (1)
15. Bisexual pedicel	Absent (0)	Absent (0)	Present (1)	Present (1)	Present (1)	Present (1)
16. Style	Bifid (0)	Bifid (0)	Entire or bifid (1)	Entire or bifid (1)	Entire or bifid (1)	Entire or bifid (1)
17. Disc color of bisexual flower[b]	Kelly (0)	Deep green (1)	Cyan (2)	Cyan (2)	Cyan (2)	Cyan (2)
18. Lamina shape	Ovate or obovate (0)	Ovate or obovate (0)	Ovate or obovate (0)	Ovate or obovate (0)	Ovate or obovate (0)	Ovate or obovate (0)
19. Leaf apex shape	Acute or acuminate (0)	Acute or acuminate (0)	Acute or acuminate (0)	Acute or acuminate (0)	Acute or acuminate (0)	Acute or acuminate (0)
20. Leaf base shape	Broadly cuneate (1)	Broadly cuneate (1)	Cuneate (0)	Cuneate (0)	Cuneate (0)	Cuneate (0)
21. Petiole indumentum	Dense (2)	Glabrous (0)	Sparse (1)	Sparse (1)	Sparse (1)	Sparse (1)
22. Leaf texture	Leathery (1)	Papery (0)	Papery (0)	Papery (0)	Papery (0)	Papery (0)
23. Leaf indumentum above	Dense (2)	Glabrous (0)	Sparse (1)	Sparse (1)	Sparse (1)	Sparse (1)
24. Leaf indumentum below	Dense (2)	Glabrous (0)	Sparse (1)	Sparse (1)	Sparse (1)	Sparse (1)
25. Young branch indumentum	Dense (1)	Sparse (0)	Sparse (0)	Sparse (0)	Sparse (0)	Sparse (0)
26. Mature branch indumentum	Sparse (1)	Glabrous (0)	Sparse (1)	Sparse (1)	Sparse (1)	Sparse (1)
27. Leaf color in autumn	Green (1)	Red (0)	Red (0)	Red (0)	Red (0)	Red (0)
28. Ovary loculus number	One (0)	One (0)	One or two (1)	One or two (1)	One or two (1)	One or two (1)
29. Ovary ovule number	One (0)	One (0)	One or two (1)	One or two (1)	One or two (1)	One or two (1)
30. Fruit shape	Broadly oblong (1)	Ellipsoid (0)	Ellipsoid (0)	Ellipsoid (0)	Ellipsoid (0)	Ellipsoid (0)
31. Fruit color	Red (0)	Red (0)	Purple (1)	Purple (1)	Purple (1)	Purple (1)
32. Seed shape	Flatly ellipsoid (0)	Flatly triangular (2)	Flatly cone (1)	Flatly cone (1)	Flatly cone (1)	Flatly cone (1)
33. Seed episperm rib	Lengthwise (0)	Almost absent (1)	Lengthwise (0)	Lengthwise (0)	Lengthwise (0)	Lengthwise (0)

[a] Numbers in parentheses following states indicate how each character state was coded for the analyses.
[b] Since two breeding systems exist for *Nyssa* species (androdioecy and tridioecy), bisexual flower is used to represent hermaphrodite flowers and pistillate flowers for easy comparison.

DNA isolation, amplification and sequencing. — Genomic DNA was extracted from 30–50 mg of silica-gel dried leaves of each accession using a modified CTAB (cetyl trimethyl ammonium bromide) method (Doyle & Doyle 1987). The extracted total DNA was diluted to a final concentration of 50–80 ng/µl for subsequent use. Amplifications of the target gene regions were performed via the polymerase chain reaction (PCR) in a GeneAmp PCR System 9700 DNA Thermal Cycler (PerkinElmer, city, state, U.S.A.) or Eppendorf (Hamburg, Germany).

Six DNA regions were included in this study: the nuclear ribosomal internal transcribed spacer (nrITS) region and the chloroplast regions *trnH-psbA*, *rps16r-f*, *trnL-F*, *trnL-rps32F* and *trnS-G*. The nrDNA ITS region was amplified using primers ITS4 and ITS5 (White & al., 1990). For the chloroplast regions, primers psbA and trnH^{GUG} (Tate & Simpson, 2003), rps16F and rps16R (Shaw & al., 2005), trnL^{UAG} and rps32-F (Wakasugi & al., 1998), c and f (Taberlet & al., 1991) and trnS-F and trnR-R (Li & al., 2007) were used to PCR amplify *trnH-psbA*, *rps16r-f*, *trn*L-*rpl*32F, *trnL-F* intron-spacer region and *trnS-G*, respectively. All PCR reactions were carried out in 25 µL volumes. The reaction mix for both ITS and chloroplast regions amplification contained 0.3 U AmpliTaq polymerase, 10× buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.5 µmol/L primer, 1 µL DMSO (dimethyl sulfoxide) and 30–50 ng genomic DNA. The PCR consisted of an initial denaturation at 95°C for 4 min, followed by 32–34 cycles of 1 min at 94°C, 50 s at 52°C, 1.5 min at 72°C, and ended with an extension step of 7 min at 72°C. The PCR products were purified using the GELase Agarose (Epicentre Technologies, Madison, Wisconsin, U.S.A.) according to the manufacturer's protocol for sequencing PCR reactions. Sequencing reactions were performed using the PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, California, U.S.A.). The sequencing products were run on an ABI 3700 automated sequencer (Perkin Elmer).

All sequences were obtained by direct sequencing of PCR products. All sequences were deposited in GenBank (see Appendix for accession numbers).

Phylogenetic analyses. — A multiple alignment (see Supplementary Data) of DNA sequences was conducted using CLUSTAL X with default parameters (Thompson & al., 1997) and was adjusted manually where necessary. A maximum parsimony (MP) analysis was carried out using the software PAUP* v.4.0b10 (Swofford, 2002). All characters were treated as unordered and were equally weighted. Each analysis consisted of a heuristic search with 1000 random sequence addition replicates (saving 100 trees per replicate), stepwise addition, Multrees, and tree-bisection-reconnection (TBR) branch swapping. Clade support was estimated using the bootstrap method (Felsenstein, 1985) with 1000 replicates and the same settings as above.

Table 2. Quantitative morphological characters of *Nyssa* species examined for use in PCO and UPGMA cluster analysis.

Character	*N. yunnanensis*	*N. javanica*	*N. sinensis*	*N. shweliensis*	*N. shangszeensis*	*N. wenshanensis*
34. Staminate peduncle, length [mm]	16.26 ± 0.96[a]	16.77 ± 1.37[a]	21.88 ± 2.78[b]	21.70 ± 2.72[b]	21.22 ± 2.98[b]	22.31 ± 2.73[b]
35. Staminate pedicel, length [mm]	0 ± 0[a]	0 ± 0[a]	2.80 ± 0.31[b]	2.83 ± 0.25[b]	2.78 ± 0.25[b]	2.72 ± 0.23[b]
36. Staminate flower, no. of petals	5.38 ± 0.64[a]	5.10 ± 0.36[b]	5.00 ± 0[c]	5.00 ± 0[c]	5.00 ± 0[c]	5.00 ± 0[c]
37. Staminate flowers, no. per inflorescence	17.12 ± 3.89[a]	15.38 ± 3.24[b]	16.12 ± 3.25[b]	16.62 ± 2.86[b]	15.26 ± 2.45[b]	16.02 ± 2.52[b]
38. Staminate flower, no. of stamens	10.92 ± 1.35[a]	10.36 ± 0.88[a]	9.68 ± 0.26[b]	9.64 ± 0.78[b]	9.36 ± 0.94[b]	9.47 ± 0.49[b]
39. Bisexual[d] peduncle, length [mm]	16.00 ± 0.96[a]	16.70 ± 1.04[a]	21.94 ± 2.53[b]	21.63 ± 2.89[b]	21.66 ± 2.74[b]	21.05 ± 2.45[b]
40. Bisexual pedicel, length [mm]	0 ± 0[a]	0 ± 0[a]	2.70 ± 0.24[b]	2.82 ± 0.16[b]	2.79 ± 0.18[b]	2.79 ± 0.14[b]
41. Bisexual flower, no. of petals	5.04 ± 0.40[a]	5.00 ± 0.20[a]	5.00 ± 0[a]	5.00 ± 0[a]	5.00 ± 0[a]	5.00 ± 0[a]
42. Bisexual flowers, no. per inflorescence	8.96 ± 1.41[a]	8.08 ± 0.99[a]	3.68 ± 0.94[b]	3.80 ± 0.69[b]	3.50 ± 1.09[b]	3.80 ± 1.05[b]
43. Bisexual flower, no. of stamens	5.72 ± 0.78[a]	4.74 ± 0.72[a]	0.88 ± 0.90[b]	0.76 ± 0.72[b]	0.82 ± 0.75[b]	0.36 ± 0.66[b]
44. Lamina, length-to-width ratio	1.99 ± 4.89[a]	2.06 ± 0.46[a]	2.08 ± 0.51[a]	2.09 ± 0.52[a]	2.11 ± 0.55[a]	2.10 ± 0.55[a]
45. Petiole, length [mm]	19.38 ± 4.89[a]	27.95 ± 1.79[a]	20.35 ± 2.62[a]	21.15 ± 1.96[a]	19.37 ± 2.03[a]	21.90 ± 1.14[a]
46. Lamina, no. of lateral veins	12.98 ± 1.91[a]	8.82 ± 0.92[b]	7.66 ± 0.98[b]	7.36 ± 0.72[b]	7.92 ± 0.72[b]	6.60 ± 0.61[b]
47. Fruit length [mm]	19.50 ± 0.71[a]	17.20 ± 0.63[b]	17.12 ± 0.54[b]	17.05 ± 0.73[b]	17.25 ± 0.77[b]	16.49 ± 0.80[b]
48. Fruit width [mm]	15.80 ± 0.57[a]	15.27 ± 0.59[a]	14.78 ± 0.44[a]	14.65 ± 0.47[a]	14.51 ± 0.20[a]	14.52 ± 0.21[a]
49. Seed length including episperm [mm]	16.18 ± 1.02[a]	14.67 ± 0.21[b]	9.87 ± 0.60[c]	9.00 ± 0.61[c]	9.23 ± 0.70[c]	7.59 ± 0.17[c]
50. Seed length excluding episperm [mm]	9.71 ± 0.56[a]	7.50 ± 0.19[b]	5.56 ± 0.27[c]	3.96 ± 0.44[c]	4.64 ± 0.37[c]	4.69 ± 0.21[c]
51. Seed thickness including episperm [mm]	4.20 ± 0.11[a]	3.64 ± 0.23[a]	5.52 ± 0.25[a]	3.93 ± 0.44[a]	4.60 ± 0.37[a]	4.66 ± 0.21[a]
52. Seed, no. of ribs on the episperm	4.20 ± 0.94[a]	1.44 ± 0.50[b]	7.74 ± 0.99[c]	6.80 ± 1.54[c]	6.70 ± 1.82[c]	8.20 ± 0.83[c]

[a,b,c] For each character, mean and 95% confidence interval are given. Letters following ranges indicate statistical differences, e.g., a range followed by "a" is statistically different from any followed by "b" or "c" at the 5% level.

[d] Since two breeding systems exist for *Nyssa* species (androdioecy and tridioecy), bisexual flower is used to represent hermaphrodite flowers and pistillate flowers for easy comparison.

A maximum likelihood (ML) analysis was carried out using an online version of the program RaxML (http://phylobench.vital-it.ch/raxml-bb/; Stamatakis & al., 2008). Additionally, a Bayesian inference (BI) analysis was conducted using the program MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003), allowing different models for each region. The combined chloroplast dataset was partitioned into three parts (nst = 6, rates = equal; nst = 2, rates = equal; nst = 6, rates = gamma), and each partition was allowed to have its own parameters as suggested by Nylander (2004). Three independent runs of 5,000,000 generations were completed with four chains each (three heated, one cold). Trees were sampled every 500 generations and the first 25% of runs were discarded as burn-in. A majority-rule consensus of the remaining trees from the three runs was produced and used as the Bayesian inference tree with posterior probabilities (PP).

The incongruence length difference (ILD) test (Farris & al., 1994) implemented in PAUP* v.4.0b10 (Swofford, 2002) was conducted to asses the congruence of chloroplast datasets. We used 1000 homogeneity replicates. Heuristic search with initial trees obtained by random addition, NNI (nearest-neighbor interchange) branch swapping, and 10 random addition sequences following Sjolin & al. (2005). The ILD value for the combined chloroplast dataset was 1.00, indicating congruence among the datasets. Hence the datasets were combined.

■ RESULTS

Morphological analysis

Principal coordinate analysis. — Based on 50 accessions of each species, the first two axes in the PCO analysis accounted for 57.44% and 25.41% of the total variance, respectively, while the third axis explained only 2.40% of the variance. Because 82.85% of all variance was represented by the first two axes, the data was displayed in 2-dimensional plots and other axes were not considered. In the PCO plot, all material fell into one of three discrete clusters. All material of *N. javanica* formed the first cluster, all material of *N. yunnanensis* formed the second cluster, while the third cluster contained all accessions of the remaining four species: *N. sinensis*, *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis* (Fig. 1). The third axis also failed to separate *N. sinensis*, *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis* (data not shown). These four species, therefore, could not be discriminated based on this analysis. There was no overlap between the three clusters.

UPGMA cluster analysis of morphological data. — Each of the three clusters resolved by PCO analysis was also present in the UPGMA analysis (Fig. 2). *Nyssa javanica* and *N. yunnanensis* were clearly differentiated, each forming a discrete cluster; however, accessions of *N. sinensis*, *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis* were intermixed in the third cluster (Fig. 2). The UPGMA analysis thus also could not differentiate these four species morphologically.

Diagnostic characters for each species. — No qualitative or quantitative characters were found to distinguish between *N. sinensis*, *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis*. The following paragraph therefore discusses only three identifiable entities: *N. sinensis*, *N. yunnanensis* and *N. javanica*.

Regarding qualitative characters, *N. yunnanensis* differs from all other species in that it is evergreen (character 1) and has broadly oblong fruits (character 30), whereas other species are deciduous with ellipsoid fruits. *Nyssa yunnanensis* tends to be densely pubescent, whereas *N. javanica* is glabrous and *N. sinensis* sparsely pubescent; this was evident on the calyx (character 5), peduncles (both staminate and bisexual; characters 10/14), petiole (character 21), and upper and lower leaf surface (characters 23, 24; Fig. 3). The three species can also be distinguished by lenticel density (character 3), bark

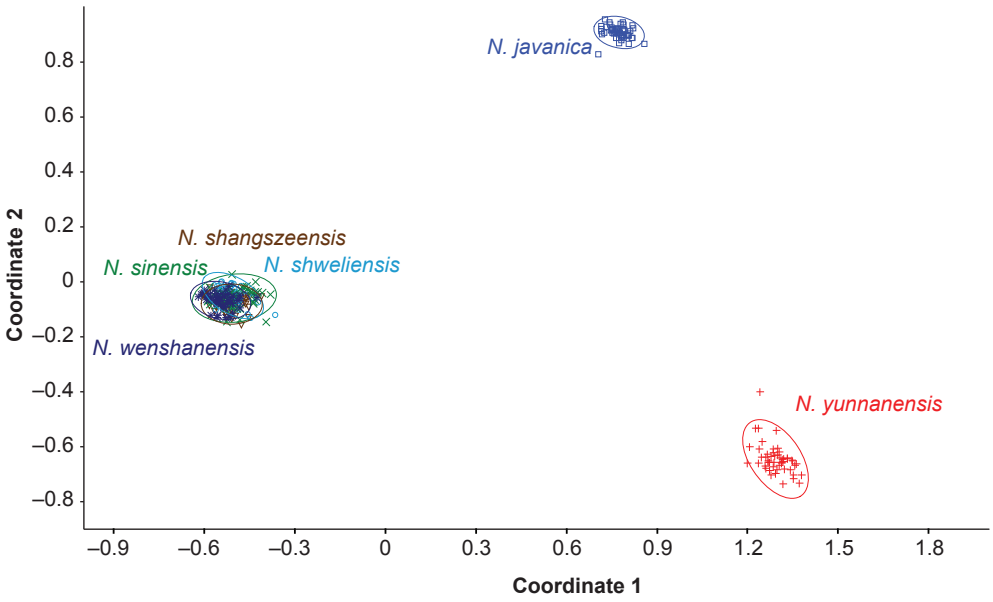

Fig. 1. PCO scatter plot based on 52 vegetative characters of 50 accessions of each of six *Nyssa* species.

color (character 2) and disc color of bisexual flowers (character 17); various other characters distinguish one species from the other two in various combinations (Table 1).

Regarding quantitative characters, the three species show clear and statistically significant differences for characters 49 (seed length including episperm), 50 (seed length excluding episperm), and 52 (number of ribs on the episperm). Various other quantitative characters separate one species from the other two in various combinations (Table 2).

Molecular analysis

ITS phylogenetic analysis. — Among the 18 accessions analyzed, the length of the complete ITS region varied from 590 to 609 bp. The aligned ITS matrix comprised 677 nucleotide sites, of which 207 were variable and 76 were phylogenetically informative (Table 3). MP analysis yielded two most parsimonious trees with a consistency index (CI) of 0.914 and a retention index (RI) of 0.888. The strict consensus of these two trees

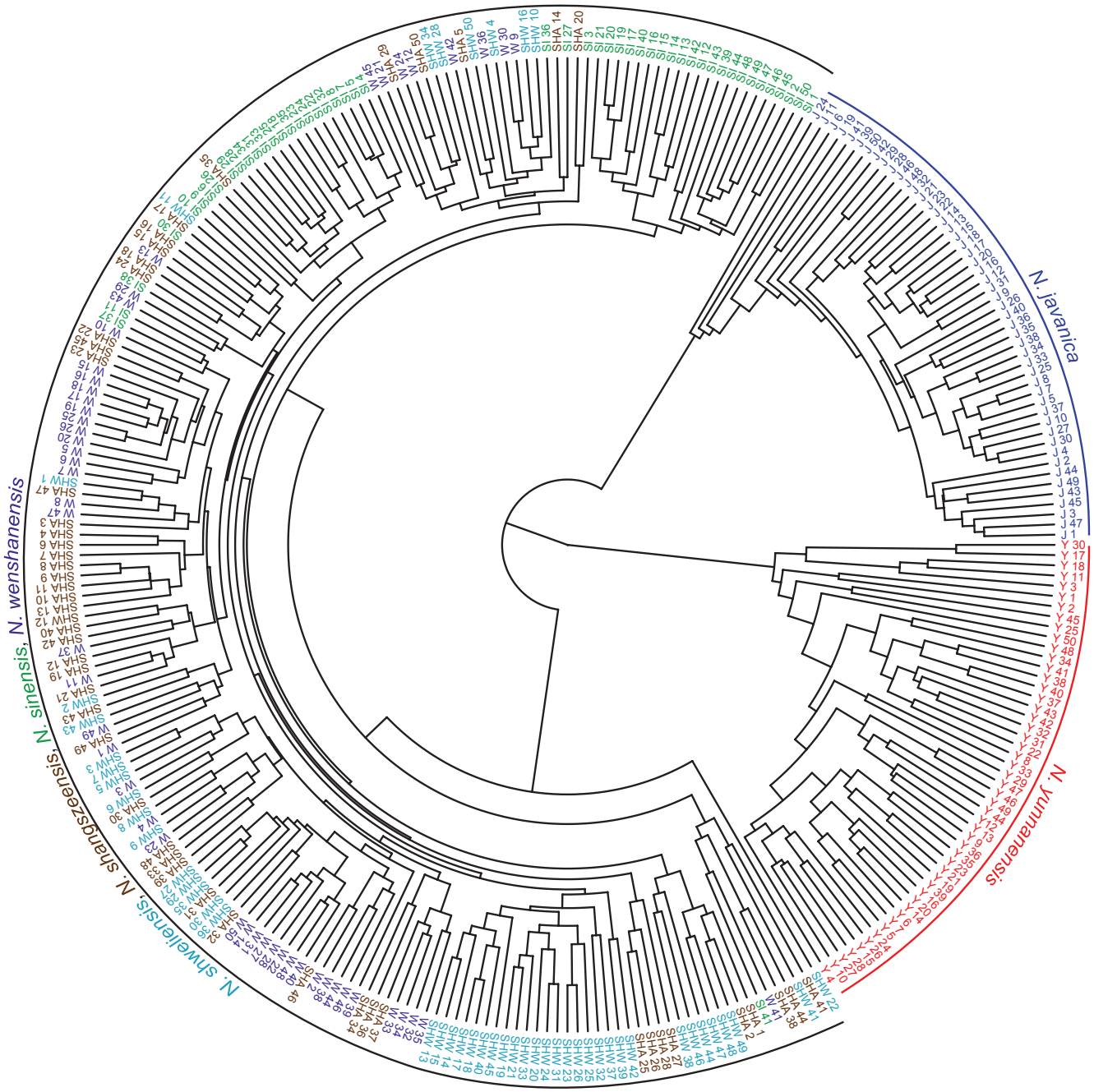

Fig. 2. Cluster analysis using UPGMA of morphological measurements of 52 characters of 50 accessions each of six *Nyssa* species. Color of accessions corresponds to color of species names.

was congruent with the topologies recovered by both ML and Bayesian analysis of the ITS sequence data. Therefore the MP strict consensus tree is presented with MP bootstrap, ML and Bayesian support for each clade (Fig. 4).

Nyssa was strongly supported (Bootstrap = 100%, ML = 89%, Bayesian PP = 100%) as monophyletic. It comprised a fairly well supported (92%, 88%, 100%) clade containing *N. yunnanensis* and *N. javanica,* plus a weakly supported (75%, 53%, 86%) clade containing all other species. Within the latter clade, *N. sylvatica* is sister to a very well-supported (99%, 99%, 100%) clade of *N. sinensis*, *N. shangszeensis*, *N. wenshanensis* and *N. shweliensis*. Within this clade, *N. shangszeensis* and *N. wenshanensis* are moderately supported as monophyletic, but *N. shweliensis* is not monophyletic, and *N. sinensis* is polyphyletic (Fig. 4).

Phylogenetic analysis of the combined chloroplast dataset. — After alignment, the combined chloroplast dataset comprised 4035 nucleotide sites, of which 227 were variable but uninformative, and 111 were phylogenetically informative (Table 3). MP analysis yielded one most parsimonious tree with a CI of 0.926 and a RI of 0.843. The topology of this tree was congruent with the topologies recovered by both ML and Bayesian analysis of the combined chloroplast data (Fig. 5).

The monophyly of *Nyssa* receives 100% support in all three analyses. However, there is one difference to the ITS phylogeny: *N. aquatica* is sister to *N. sylvatica* with 97% bootstrap,

Table 3. Gene region, sequence length, number of variable characters and number of potentially parsimony-informative characters.

Gene region	Sequence length [bp]	Variable characters (%)	Informative characters (%)
ITS	677	207 (30.6%)	76 (11.2%)
trnH-psbA	464	45 (9.7%)	17 (3.7%)
rps16r-f	857	53 (6.2%)	18 (2.1%)
trnL-F	899	58 (6.5%)	15 (1.7%)
trnL-rps32F	999	80 (8.0%)	18 (1.8%)
trnS-G	816	102 (12.5%)	43 (5.3%)
cpDNA combined	4035	338 (8.4%)	111 (2.8%)

Fig. 3. Some morphological characters that separate *N. yunnanensis*, *N. javanica* and *N. sinensis*. **1–4:** *N. yunnanensis*. **1,** mature branch with leaves; **2,** staminate flower; **3,** staminate inflorescence; **4,** fruiting branch. **5–6:** *N. javanica*. **5,** mature branch with leaves and fruits; **6,** staminate inflorescence. **7–10:** *N. sinensis*. **7,** mature branch with leaves and fruits; **8,** staminate inflorescence; **9,** young fruit; **10,** fruiting branch. — Scale bars 1, 3–10: 1 cm; 2: 1 mm.

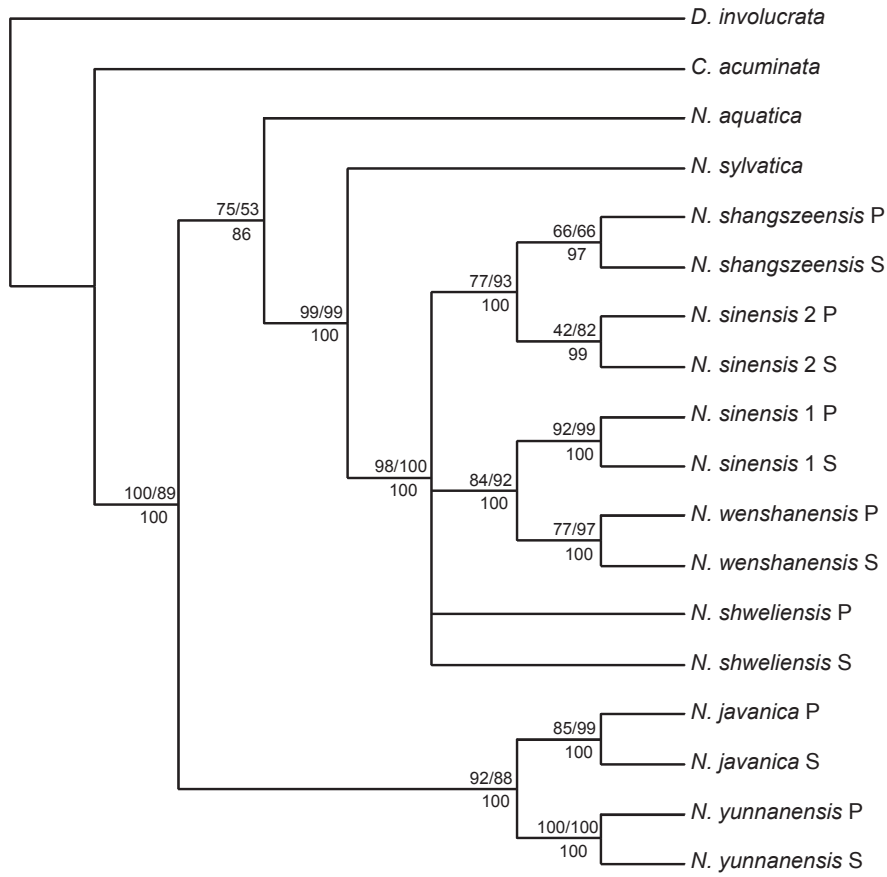

Fig. 4. Strict consensus tree based on ITS sequences. Figures above branches show maximum parsimony bootstrap/ maximum likelihood bootstrap support; figures below branches show Bayesian posterior probability, all as percentages.

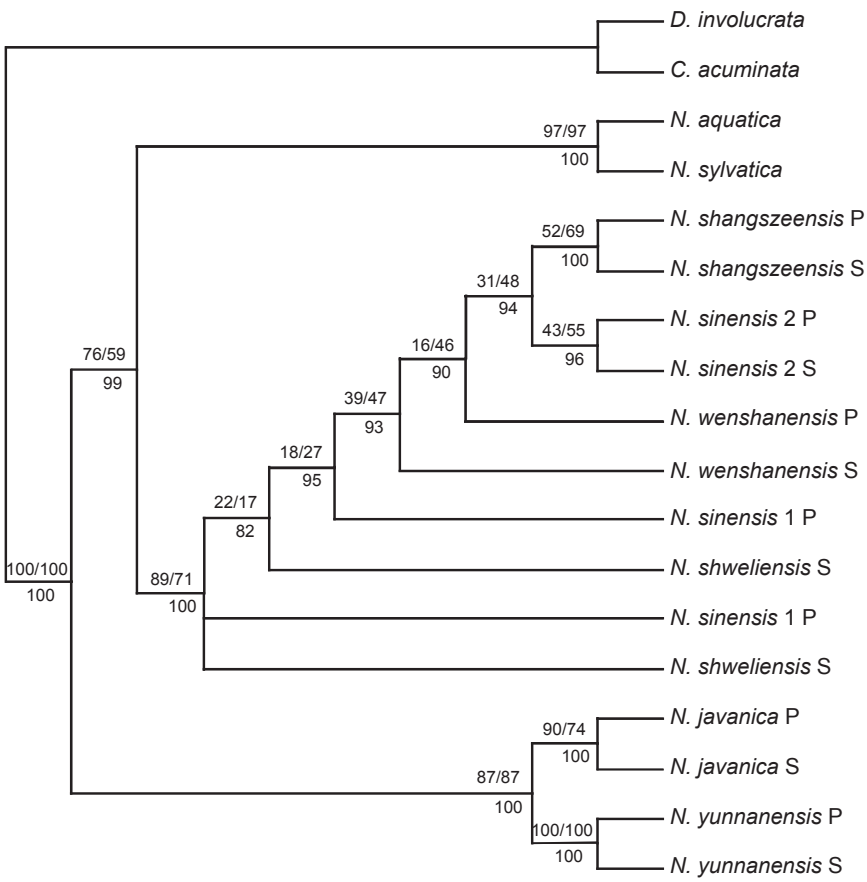

Fig. 5. Strict consensus tree based on the combined sequences of five cpDNA regions (*trnH-psbA*, *rps16r-f*, *trnL-rps32F*, *trnS-G*, *trnL-F*). Figures above branches show maximum parsimony bootstrap/ maximum likelihood bootstrap support; figures below branches show Bayesian posterior probability, all as percentages.

97% ML and 100% Bayesian support. The genus therefore comprises *N. aquatica*/*N. sylvatica* plus two other clades, also with 100% Bayesian support but less support in the other analyses. The *N. javanica*/*N. yunnanensis* clade is sister to all other taxa, and the *N. aquatica*/*N. sylvatica* clade is sister to a clade of *N. sinensis*, *N. shangszeensis*, *N. wenshanensis* and *N. shweliensis*. Relationships between these last four species are barely resolved, and only *N. shangszeensis* is supported as monophyletic with strong support only in the Bayesian analysis.

■ DISCUSSION

How many *Nyssa* species? — Based on our analysis, *Nyssa* contains only three species in eastern Asia: *N. javanica*, *N. yunnanensis*, and *N. sinensis*. Three other described species, *N. shangszeensis*, *N. wenshanensis* and *N. shweliensis*, cannot be differentiated from *N. sinensis* by morphology (Figs. 1–2), and neither are they clearly distinct based on molecular data (Figs. 4–5; N. Wang & al., in press). Although *N. wenshanensis* and *N. shangszeensis* were each weakly supported as monophyletic, recognizing them as distinct would make *N. sinensis* polyphyletic. Isolated populations within a species can become monophyletic for molecular markers (Edwards & al., 2008), so monophyly alone is no reason to recognize these as species. Our data therefore supports the synonymising of *N. shangszeensis*, *N. wenshanensis* and *N. shweliensis* under *N. sinensis*. The alternative to sinking these species completely would be to refer to them as the *N. sinensis* species complex. From the original descriptions, *N. shweliensis* differs from *N. sinensis* in that branchlets, petioles and pedicels are persistently pilose in *N. shweliensis,* but become subglabrous when old in *N. sinensis* (Fang & Soong, 1975); however, we found this character to be variable within *N. shweliensis*.

Both *N. wenshanensis* and *N. shangszeensis* were separated from *N. sinensis* and *N. shweliensis* because the staminate inflorescences in the first two species form heads of shortly pedicellate flowers, whereas those of the latter two form umbels or racemes (Fang & Soong, 1975). However, at the time of description, pistillate flowers of *N. wenshanensis*, *N. shangszeensis* and *N. shweliensis* were unknown, and fruits of *N. wenshanensis* and staminate flowers of *N. shangszeensis* were similarly unknown (Fang & Soong, 1975). Hence descriptions were based on staminate inflorescences only, and the current analysis found no consistent differences in these between the four species. The supposed difference between *N. wenshanensis* and *N. shangszeensis* is that their leaves are membranaceous and coriaceous, respectively, but our field examination showed that leaves of *N. shangszeensis* vary in thickness due to factors such as age, so that this character again does not hold.

In contrast, *N. yunnanensis* is very clearly distinct from both *N. sinensis* and *N. javanica* based on both morphology and molecular data. Although *N. yunnanensis* was previously treated as part of the *N. javanica* species complex (Wen & Stuessy, 1993), our analysis detected 17 qualitative characters and 7 quantitative characters that differed between the two species (Tables 1–2). Indeed, *N. javanica* does not appear closely related to any of the species previously placed in the *N. javanica* species complex.

Uncertainty remains regarding the status of *N. leptophylla*, material of which could not be located for this study. Based on herbarium specimens we have seen, it might also be synonymous with *N. sinensis*, but at present we suggest that it be listed as a species incertae sedis.

Phylogenetic relationships among *Nyssa* species revealed by molecular data. — Leaving aside *N. aquatica*, which will be discussed below, our data clearly indicate that *N. yunnanensis* and *N. javanica* are sister to each other, whereas *N. sinensis* is more closely related to the American *N. sylvatica* than to any Asian species. This latter finding matches the conclusion by Eyde (1963) and Chen & Qian (1991), based on fossil morphology and embryology, respectively. Therefore, East Asian *Nyssa* does not form a monophyletic group.

Different relationships are indicated for *N. aquatica* by ITS data, which places this as sister to the rest of the genus, and cpDNA data, which resolves it as sister to *N. sylvatica*. Both relationships have reasonable support. Hence the ITS tree indicates the existence of two separate lineages in North America, whereas cpDNA indicates only one. A possible explanation of the incongruency of ITS and cpDNA data is transfer of genetic material via introgression, and a possible scenario is as follows. *Nyssa aquatica* arrived first in North America and acquired cpDNA of *N. sylvatica* via chloroplast capture after that species had arrived (e.g., Rieseberg & Soltis, 1991; Tsitrone & al., 2003: Milne & al., 2010). Alternatively, hybridization between *N. sylvatica* and a now extinct lineage, which donated its ITS, could have resulted in the phylogenetic position of *N. aquatica*. To better understand what has happened here, the other two New World species (*N. ogeche*, *N. talamancana*) need to be examined for ITS and chloroplast markers.

Implications for conservation. — The most widespread Asian *Nyssa* species is *N. javanica*, which occurs in SE and SW China, and whose range extents west into India and south to Indonesia. Within China, *Nyssa sinensis* is more widespread than *N. javanica*; if taken to include *N. shangszeensis*, *N. shweliensis*, and *N. wenshanensis*, it ranges from central China to SE and SW China. It usually grows in wet forests by streams, and in valleys. Conversely, *N. yunnanensis* is known only from one site in Yunnan. It grows in mountainous bogs and marshes, mostly in riparian vegetation along rivers, in association with species of *Quercus* L., *Juglans* L., *Persea* Mill., and *Laurus* L. Our field observations detected three small populations distributed along a stream, around a well and adjacent to an old dam, indicating a habitat severely disturbed by human activity. This restricted range could indicate a far narrower ecological tolerance range for *N. yunnanensis* than for the other species.

Habitat destruction is a severe threat to all localities for *Nyssa* in China, and therefore it may be necessary to prioritize certain sites for conservation. In this context, having a clear knowledge of the taxonomy of species is a key step towards an effective conservation strategy. Previously, *N. shangszeensis*, *N. shweliensis, N. wenshanensis* and *N. yunnanensis* all had the same taxonomic status, with *N. yunnanensis* listed as a critically endangered species (S. Wang & Xie, 2004)

and *N. shangszeensis* and *N. shweliensis* included in the Red List of Chinese species (S. Wang & Xie, 2004). Our analysis, however, indicates that of these, only *N. yunnanensis* should be recognized as a species; therefore its one remaining known wild site should receive a much higher conservation priority than any other *Nyssa* sites in China. Populations of *N. shangszeensis*, *N. shweliensis* and *N. wenshanensis* are of lesser importance, but remain valuable as reservoirs of genetic diversity within *N. sinensis*. Nonetheless, the practical outcome of our work should be that conservation resources are focused as much as possible on *N. yunnanensis*.

■ CONCLUSION

The taxonomy of *Nyssa* within China has been confused in the past. Our analyses provided clear evidence of the number of species present in China, which contradicts both the recognition of seven distinct species in *Flora of China* (Qin & Phengklai, 2007) and the grouping of *N. javanica*, *N. yunnanensis*, *N. wenshanensis* and *N. leptophylla* as the *N. javanica* complex (Wen & Stuessy, 1993). Instead, we found that *N. wenshanensis* and two other species should be included in *N. sinensis*. We propose the recognition of the following species for the *Flora of China*: *N. javanica*, *N. sinensis* and *N. yunnanensis*. This demonstrates the value of using a large number of characters and accessions in a morphological analysis of a group of superficially similar species, and also shows the importance of field observations (notably the evergreen habit in *N. yunnanensis*). Moreover, tackling the problem with both molecular and morphological data allowed conclusions to be made with some confidence. It is hoped that this work will lead to an increased conservation effort for *N. yunnanensis*, of which only 37 specimens remain at its only known wild site (C.-Q. Zhang, pers. obs.) and which appears to be the only narrow endemic *Nyssa* species that exists in China.

■ ACKNOWLEDGMENTS

We thank Yu-Xiao Zhang and Li-Na Dong at Kunming Institute of Botany, Chinese Academy of Sciences (CAS), for their help with laboratory work and data analysis. We also sincerely thank the two reviewers: M. Weigend and T. Stuessy, for their valuable comments and advices on this manuscript. The research was funded by the Natural Science Foundation of China (No. 30770139), the Bureau of Science and Technology of Yunnan, China (No. 2009BB001) and the Large-Scale Scientific Facilities of the Chinese Academy of Sciences (No. 2009-LSF-GBOWS-01).

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Appendix. GenBank accession numbers for each sequence region used in this study.

Species: locality, latitude, longitude; inflorescence type, voucher (*S* = *Sun*; *Z* = *Zhang*; ITS; *trnH-psbA*, *rps16r-f*, *trnL-F*, *trnL-rps32F*, *trnS-G*

C. acuminata Decne.: Yunnan, Puwen, 22°24′N, 101°05′E; *S2007032102*; EU734436, EU734454, EU734472, EU734490, EU734508, EU734526. — ***D. involucrata*** Baill.: Yunnan, Puwen, 22°24′N, 101°05′E *S2007032106*, EU734435, EU734453, EU734471, EU734489, EU734507, EU734525. — ***N. aquatica*** L.: Arnold Arboretum, cultivated; unknown inflorescence type: *ZJW966-79*, EU734437, EU734455, EU734473, EU734491, EU734509, EU734527. — ***N. javanica*** (Blume) Wangerin: Yunnan, Mengsong, 21°29′N, 100°30′E; staminate: *S2005032702*, EU734450, EU734468, EU734486, EU734504, EU734522, EU734540; pistillate: *S2005032704*, EU734449, EU734467, EU734485, EU734503, EU734521, EU734539. — ***N. shangszeensis*** W.P. Fang & Soong: Guangxi, Shangsi, 21°53′N, 107°54′E; staminate: *S2007040504*, EU734440, EU734458, EU734476, EU734494, EU734512, EU734530; pistillate: *S2007040901*, EU734439, EU734457, EU734475, EU734493, EU734511, EU734529. — ***N. shweliensis*** (W.W. Sm.) Airy-Shaw: Yunnan, Honghe, 23°15′N, 103°30′E; staminate: *S2007040204*; EU734448, EU734466, EU734484, EU734502, EU734520, EU734538; pistillate: *S2007040201*, EU734447, EU734465, EU734483, EU734501, EU734519, EU734537. — ***N. sinensis*** Oliv. 1: Yunnan, Pingbian, 22°54′N, 103°42′E; staminate: *S2007040502*, EU734444, EU734462, EU734480, EU734498, EU734516, EU734534; pistillate: *S2007040501*, EU734443, EU734461, EU734479, EU734497, EU734515, EU734533. ***N. sinensis*** 2: Kunming Botanic Garden, cultivated; staminate: *S2004041503*, EU734442, EU734460, EU734478, EU734496, EU734514, EU734532; pistillate: *S2004041501*, EU734441, EU734459, EU734477, EU734495, EU734513, EU734531. — ***N. sylvatica*** Marshall: Arnold Arboretum, cultivated; unknown inflorescence type: *ZJW374-94*, EU734438, EU734456, EU734474, EU734492, EU734510, EU734528. — ***N. wenshanensis*** W.P. Fang & Soong: Yunnan, Wenshan, 23°22′N, 103°14′E; staminate: *S2007041301*, EU734446, EU734464, EU734482, EU734500, EU734518, EU734536; pistillate: *S2006032801*, EU734445, EU734463, EU734481, EU734499, EU734517, EU734535. — ***N. yunnanensis*** W.C. Yin: Yunnan, Puwen, 22°24′N, 101°05′E; staminate: *S2005031104*, EU734452, EU734470, EU734488, EU734506, EU734524, EU734542; pistillate: *S2005032101*, EU734451, EU734469, EU734487, EU734505, EU734523, EU734541.