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Research Article

Phylogenetic position of *Sonneratia griffithii* based on sequences of the nuclear ribosomal internal transcribed spacer and 13 nuclear genes

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Abstract Compared with single gene analysis, multi-locus sequence data usually perform better in resolving phylogenetic relationships of closely related species. *Sonneratia* (Lythraceae s.l.), a genus of mangroves, is widely distributed in the Indo-West Pacific region. *Sonneratia griffithii* Kurz is one of the rarest species of this genus and morphological analyses have produced conflicting results regarding its affinity to other species of *Sonneratia*. In this study, we aimed to resolve the phylogenetic position of *S. griffithii* by using sequence data of the nuclear ribosomal internal transcribed spacer (nrITS) and 13 nuclear genes. We first reconstructed the phylogeny of five *Sonneratia* species using nrITS and the nuclear gene *rpl9*, which showed that *S. caseolaris* (L.) Engl. diverged first from the other species and that *S. griffithii* was closer to *S. apetala* Buch.-Ham. and *S. ovata* Backer; however, the relationships among *S. griffithii*, *S. apetala*, and *S. ovata* could not be resolved. Further phylogenetic analysis based on sequences of nrITS and 13 nuclear genes and using *S. caseolaris* as an outgroup showed that *S. griffithii* and *S. apetala* were sister species with high bootstrap support. We conclude that *S. griffithii* and *S. apetala* have the closest relationships. Furthermore, our phylogenetic analyses do not support previous intrageneric classifications of *Sonneratia*.

Key words: mangroves, nuclear genes, phylogeny, *Sonneratia*.

Sonneratia (Lythraceae s.l.), a genus of mangroves in the Indo-West Pacific region, ranges from East Africa through the Indo-Malay Peninsula to China, Japan, and tropical Australia (Tomlinson, 1986; Duke & Jackes, 1987; Duke, 1994). This genus is composed of six species (*S. alba* Griff., *S. caseolaris* (L.) Engl., *S. ovata* Backer, *S. apetala* Bach.-Ham., *S. lanceolata* Blume, and *S. griffithii* Kurz) and three interspecific hybrids (Wang & Chen, 2002; Zhou et al., 2005; Qiu et al., 2008). Among these six species, *S. alba* has the widest distribution, followed by *S. caseolaris*, *S. ovata*, and *S. apetala*; *S. lanceolata* and *S. griffithii* are narrowly distributed species.

Sonneratia griffithii, a rare *Sonneratia* species (Blasco et al., 2001), is restricted to the coasts of the Andaman Sea, from Bengal to the upper Malay Peninsula (Tomlinson, 1986). Although described as locally common, it is rarely collected. One of our previous studies identified the occasional occurrence of natural hybrids between *S. alba* and *S. griffithii* along the western coasts of Thailand (Qiu et al., 2008). *Sonneratia griffithii* was first described as "allied to, but distantly different from *S. caseolaris*" (Backer & van Steenis, 1951). Tomlinson (1986) and Aksornkoae et al. (1992) considered *S. griffithii* to be close to, but distinct from *S. caseolaris* due to the prominent leaf veins, absence of petals, white filament, and hard fruit of *S. griffithii*. During our field survey, we found the leaves of *S. griffithii* to be obovate to suborbicular, which is highly similar to those of *S. ovata*. In addition, the absence of petals, a conspicuous trait in *Sonneratia*, is only observed in *S. griffithii*, *S. ovata*, and *S. apetala*. Hence, the phylogenetic position of *S. griffithii* is elusive based solely on the available morphological data.

DNA sequence-based phylogenetic analyses have been implemented as a means of resolving the taxonomic status and phylogenetic relationships among various plant and animal groups. However, single molecular markers do not always permit strong and unambiguous resolution and leave uncertainties and unanswered questions, especially for closely related species, due to the lack of sufficiently informative sites. In recent years, multi-locus sequences have been successfully used to resolve phylogenetic relationships of recently diverged species, which could not previously be resolved based on the sequences of single or even several genes. For example, the phylogenetic relationships among the species in the genera *Oryza* and *Pinus* were successfully resolved using 142 and 191 genes, respectively (Zou et al., 2008; Niu et al., 2013). In the present study, we aimed to infer the phylogenetic position of *S. griffithii* using sequences data from multiple DNA fragments. We reconstructed the phylogeny of *Sonneratia* by first combining the sequences of the internal transcribed spacers (ITS) of nuclear ribosomal (nr) DNA with the sequence of a nuclear gene *rpl9* from five of the six *Sonneratia* taxa. *Sonneratia lanceolata* was not included in this study because it was incorporated into *S. caseolaris* based on our population genetics study (Yang Y-C et al., 2014, unpublished data). Because the phylogenetic relationships of *S. ovata*, *S. apetala*,

and *S. griffithii* were not resolved, we then used sequences from 12 additional nuclear genes to resolve the phylogenetic position of *S. griffithii*.

Material and Methods

Plant materials

Sampling information of five non-hybrid taxa of *Sonneratia* is listed in Table 1. Because the two widespread species, *S. alba* and *S. caseolaris*, show a high level of among-population differentiation (Zhou et al., 2007, 2011; Triest, 2008), we collected samples of the two species from three different countries: China, Thailand, and Australia (Table 1). *Duabanga grandiflora* Walp. was used as an outgroup based on the results of Shi et al. (2000). For DNA extraction, leaves from these individuals were collected in plastic bags containing silica gels. Voucher specimens were deposited in the Herbaria of Sun Yat-sen University (SYS).

Polymerase chain reaction and sequencing

Total genomic DNA was extracted from silica-dried leaf tissues using the CTAB method according to Doyle & Doyle (1987). The complete ITS region (including ITS-1, the 5.8S rRNA gene, and ITS-2) was amplified using the universal primers ITS-4 and ITS-5 (White et al., 1990). Based on the sequences of over 200 clones from a leaf cDNA library of *S. caseolaris*, we previously designed 70 pairs of primers to amplify the targeted nuclear genes (*rpl9*, *cpi*, *ppi*, *phi*, *cci*, *idr*, *map*, *ndmd*, *lia*, *sbd*, *rpl33*, *tcl*, and *drp*) from the genomic DNA of four *Sonneratia* species (Zhou et al., 2007, 2011). Genes were also identified as orthologous using primers anchoring different locations (Zhou et al., 2007). In this study, we amplified 13 of the 70 nuclear genes for the five *Sonneratia* species and the outgroup *D. grandiflora*. Double-stranded fragments were amplified using 35 cycles of the polymerase chain reaction (PCR; 94 °C for 4 min; 94 °C for 40 s, 51–57 °C for 30 s, 72 °C for 1.25 min; and 72 °C for 8 min) carried out in a 30-μL reaction. Annealing temperatures were changed to 45 °C as needed to improve the quality of the PCR products for the outgroup. The gene IDs, sequence lengths, gene descriptions, and primer sequences for the 13 nuclear genes are listed in Table 2. The PCR products were purified by using the Pearl gel extraction kit (Pearl Bio-tech, Guangzhou, China) following 1.2% agarose gel electrophoresis. Sequencing was carried out in an ABI 3730 automated DNA sequencer with the BigDye Terminator Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the same primer sets used for PCR amplifications. All sequences were deposited in GenBank with accession numbers KJ511903–KJ512059 and KJ535307–KJ535319 (Table 1).

Phylogenetic analyses

For the outgroup species *D. grandiflora*, only the nrITS and the nuclear gene *rpl9* have been successfully amplified and sequenced. Therefore, we first reconstructed the phylogeny of *Sonneratia* using the combined sequence data from these two fragments. Prior to combining the sequences, the congruence was examined using the partition–homogeneity test (Farris et al., 1995). These sequences were aligned in CLUSTALX (version 1.7; Thompson et al., 1997) and manually

Table 1 GenBank accessions of *Sonneratia* and an outgroup used in this study

Taxon	Voucher	Source	ITS	*rpl9*	*cpi*	*ppi*	*phi*	*cci*	*idr*	*map*	*ndmd*	*lia*	*sbd*	*rpl33*	*tcl*	*drp*
S. alba Griff.	C. Zhong 200908-QH02	Qionghai, Hainan, China	KJ511906	KJ511932	KJ511951	KJ511980	KJ511987	KJ511965	KJ511941	KJ512016	KJ512021	KJ512035	KJ511926	KJ512046	KJ512053	KJ512002
	S. Shi 200908-RT01	Ranong, Thailand	KJ511903	KJ511931	KJ511959	KJ511977	KJ511988	KJ511970	KJ511945	KJ512012	KJ512024	KJ512030	KJ511917	KJ512048	KJ512057	KJ512005
	S. Shi 200902-QA01	Queensland, Australia	KJ511914	KJ511935	KJ511956	KJ511979	KJ511986	KJ511967	KJ511942	KJ512009	KJ512019	KJ512031	KJ511923	KJ512045	KJ512054	KJ512006
S. caseolaris (L.) Engl.	C. Zhong 200908-QH03	Qionghai, Hainan, China	KJ511904	KJ511933	KJ511958	KJ511976	KJ511995	KJ511971	KJ511940	KJ512011	KJ512027	KJ512032	KJ511924	KJ512040	KJ512052	KJ512007
	S. Shi 200908-RT01	Ranong, Thailand	KJ511915	KJ511930	KJ511960	KJ511984	KJ511991	KJ511968	KJ511950	KJ512013	KJ512026	KJ512038	KJ511916	KJ512042	KJ512058	KJ512004
	S. Shi 200902-QA04	Queensland, Australia	KJ511905	KJ511929	KJ511954	KJ511982	KJ511992	KJ511969	KJ511949	KJ512015	KJ512025	KJ512037	KJ511922	KJ512049	KJ512059	KJ511998
S. ovata Backer (1)	S. Shi 200209-WC04	Wenchang, Hainan, China	KJ511911	KJ511934	KJ511957	KJ511983	KJ511994	KJ511972	KJ511943	KJ512010	KJ512023	KJ512034	KJ511921	KJ512047	KJ512055	KJ512000
S. ovata Backer (2)	S. Shi 200209-WC05	Wenchang, Hainan, China	KJ511910	KJ511937	KJ511952	KJ511975	KJ511990	KJ511966	KJ511939	KJ535312	KJ535314	KJ512036	KJ511918	KJ535317	KJ535318	KJ535310
S. apetala Bach.-Ham. (1)	S. Shi 200605-SZ01	Cultivated in Futian Mangroves Reserve, Shenzhen, Guangdong, China	KJ511913	KJ511938	KJ511953	KJ511981	KJ511996	KJ511973	KJ511948	KJ512014	KJ512020	KJ512039	KJ511925	KJ512044	KJ512056	KJ512003
S. apetala Bach.-Ham. (2)	S. Shi 200605-SZ03	Cultivated in Futian Mangroves Reserve, Shenzhen, Guangdong, China	KJ511908	KJ535308	KJ511955	KJ511978	KJ511993	KJ535309	KJ511946	KJ535313	KJ535315	KJ512033	KJ511919	KJ535316	KJ535319	KJ535311
S. griffithii Kurz (1)	S. Shi 200709-RT02	Ranong, Thailand	KJ511912	KJ511928	KJ511961	KJ511985	KJ511997	KJ511963	KJ511947	KJ512008	KJ512018	KJ512028	KJ511920	KJ512041	KJ512050	KJ511999
S. griffithii Kurz (2)	S. Shi 200709-RT03	Ranong, Thailand	KJ511907	KJ511936	KJ511962	KJ511974	KJ511989	KJ511964	KJ511944	KJ512017	KJ512022	KJ512029	KJ511927	KJ512043	KJ512051	KJ512001
Duabanga grandiflora Walp.	R. Zhou 200801-Dg04	Cultivated in South China Agricultural University Campus, Guangzhou, Guangdong, China	KJ511909	KJ535307	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Voucher specimens are at the Herbarium of Sun Yat-sen University (SYS). Numbers in parentheses after species names represent sample accession numbers. ITS, internal transcribed spacer; NA, sequence not available.

Table 2 Gene IDs, sequence lengths, gene descriptions, and primer sequences for the nuclear ribosomal internal transcribed spacer (nrITS) and 13 nuclear genes used in this study

Gene ID	Sequence length (bp)	Gene description	Primer	Sequence (5′→3′)
nrITS	602–629	Nuclear ribosomal internal transcribed spacer	nrITS-4	TCCTCCGCTTATTGATATGC
			nrITS-5	GGAAGTAAAAGTCGTAACAAGG
rpl9	1157–1160	Ribosomal protein L9 gene	rpl9-F	GAGAGCGAAGATGAAGACG
			rpl9-R	TCAGTCTTCCCCGAGGATGG
cpi	645–649	Cysteine proteinase inhibitor gene	cpi-F	AACAGCCTCGAGATCGAAG
			cpi-R	GAACTCCTGCAACTCCTTG
ppi	778–781	Peptidyl-prolyl *cis–trans* isomerase gene	ppi-F	AAAATCACAGAGCCCAAGA
			ppi-R	CAAGCCATCGCTCCTATC
phi	1157–1168	Phosphatase inhibitor gene	phi-F	CTTCCTGGCGTTATGTGC
			phi-R	ATCTCCTGGTCCTGTTTGA
cci	1116–1129	Cytochrome b6-f complex iron-sulfur subunit gene	cci-F	CCACGAGCATCCCCGCTGATG
			cci-R	GCCCACCATGGAGCTTCTCCAG
idr	1367–1377	Acireductone dioxygenase gene	idr-F	TGGTACATGGATGATAGTG
			idr-R	TTCCTTGCTGGAAGGTGGTC
map	1459–1465	Microtubule-associated protein 1 light chain 3 gene	map-F	CCAAAGGCTCATTCAAG
			map-R	TGTTCTCCCCACTGTAAG
ndmd	904–949	NADP-dependent malate dehydrogenase gene	ndmd-F	GCTGCCAGTCAAAGAGGT
			ndmd-R	CTCCTGGAAGCATCGTGT
lia	806–832	Light-inducible protein ATLS1 gene	lia-F	CTCCTCCGTCGCCAAAATCATC
			lia-R	AGAACCGCGACTTGGGAACAGAA
sbd	955–1036	Saposin B domain-containing protein gene	sbd-F	TTTGGTGGACTATTATGC
			sbd-R	AAGATTCGGAAAGCCCTA
rpl33	714–720	Ribosomal protein L33 gene	rpl33-F	GGCTTTAGCAGGTCTATTG
			rpl33-R	TCCCCGTGAATTGTATGTTTG
tcl	1120–1154	Transcriptional corepressor LEUNIG gene	tcl-F	CTTGCGATGGAGATGGTG
			tcl-R	TCTTGTTCTCCGATATG
drp	1563–1589	DNA-binding-related protein gene	drp-F	ATGGCCGAACTATCAAAACC
			drp-R	TATTCGACCACATTCACCATCT

F, forward; R, reverse.

adjusted in SeqMan (version 7.10; DNAStar, London, UK). The phylogenetic trees were reconstructed using maximum parsimony (MP) and maximum likelihood (ML) analyses, as implemented in PAUP* 4.0b (Swofford, 1998). Parsimony analyses were carried out by using a heuristic search with tree bisection–reconnection branch swapping, the MulTrees option, accelerated transformation optimization, and 1000 random addition replicates. Indels were treated as the fifth state, and each indel with two or more base pairs was considered as a single mutational event. Standard measures of homoplasy such as consistency index (CI), retention index (RI), rescaled consistency index (RC), and bootstrap support (BS) values were calculated. Bootstrap analyses were carried out with 1000 replicates of the heuristic search with simple taxon addition and maxtrees was set to 500. *Duabanga grandiflora* was used to root the phylogenetic trees. For ML analysis, an appropriate nucleotide substitution model is needed to set the ML parameters for the combined dataset. Based on the Akaike Information Criterion in Modeltest 3.7 (Posada & Buckley, 2004), the best-fit model among the 56 models of nucleotide substitution is the equal-frequency Tamura–Nei model with a proportion of invariant sites (TrNef + I). Similar to the MP analysis, the ML analysis was carried out by using heuristic search with tree bisection–reconnection branch swapping, holding one tree at each step. The maxtrees was also set to 500. Node support was estimated with 1000 bootstrap replicates. We further concatenated the sequences of the nrITS and the 13 nuclear genes to resolve the phylogenetic position of *S. griffithii*, with *S. caseolaris* as the outgroup. The phylogenetic trees were reconstructed using the MP and ML analysis described above. For the ML analysis, the data were run under the transversion model with the proportion of invariant sites (Tvm + I) inferred by Modeltest 3.7.

Results

Phylogeny of *Sonneratia* based on sequences of nrITS and *rpl9*

The partition–homogeneity test analysis indicated that the ITS and *rpl9* gene sequences could be combined for phylogenetic analysis ($P = 0.689$). The alignments of the combined dataset consisted of 1791 nucleotide sites, of which 128 were parsimoniously informative. Phylogenetic analysis of this dataset yielded two most parsimonious trees of 414 steps (one is shown in Fig. 1; CI = 0.9058; RI = 0.8691; RC = 0.7873). In the MP trees, *Sonneratia* species fell into two clades, with *S. caseolaris* forming one clade, and the remaining four species forming the other (BS = 100% and 96%, respectively). The high

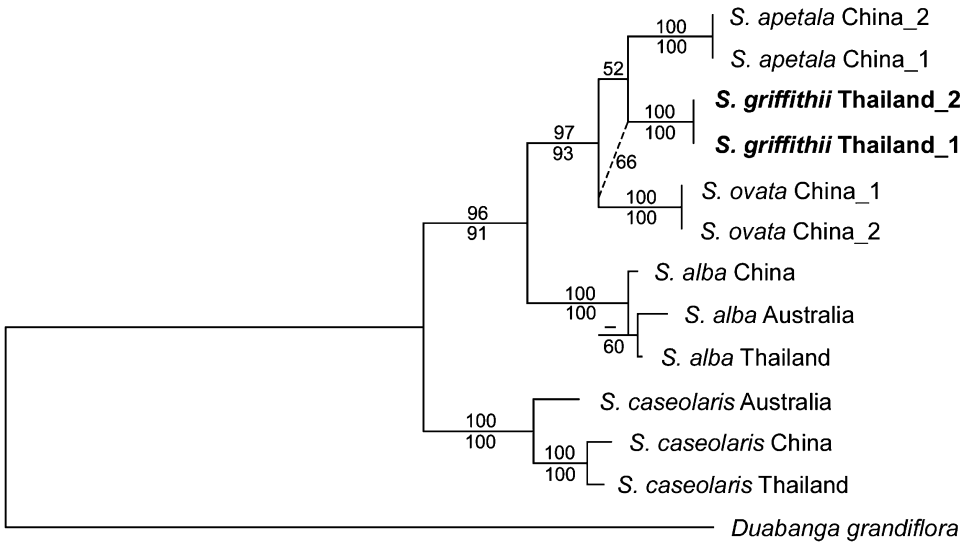

Fig. 1. One of the most parsimonious trees of *Sonneratia* inferred from the combined sequences of the nuclear ribosomal internal transcribed spacer and the nuclear gene *rpl9*. Samples from *S. griffithii* are shown in bold. Numbers above and below the branches indicate maximum parsimony and maximum likelihood bootstrap values (>50%), respectively. Dashed lines show different relationships in the maximum likelihood tree compared with the maximum parsimony tree. Countries and numbers after species names represent sample accessions. –, <50% bootstrap support.

BS values for the two clades suggest that *S. caseolaris* diverged first within *Sonneratia*. In the other clade, *S. alba* was sister to the three other species, with high BS (96%). However, the MP tree did not show well-resolved relationships for *S. ovata*, *S. apetala*, and *S. griffithii* (BS = 52% for clustering *S. griffithii* and *S. apetala* together).

The ML analysis generated an identical topology to that produced based on the parsimony principle except that *S. griffithii* was sister to *S. ovata* with very low BS (dashed lines in Fig. 1, BS = 66%). Thus, the phylogenetic relationships among *S. ovata*, *S. apetala*, and *S. griffithii* were not resolved based on the combined nrITS and *rpl9* sequence data.

Phylogenetic position of *S. griffithii* based on sequences of nrITS and 13 nuclear genes

Because *S. caseolaris* was the first to diverge in this genus, we used it as an outgroup in phylogenetic analyses based on the concatenated sequences of nrITS and 13 nuclear genes. The MP analysis generated one most parsimonious tree (tree length = 1022; CI = 0.8728; RI = 0.9006; RC = 0.7861), which was identical to the tree reconstructed using ML. We found that *S. alba* was sister to three other species, consistent with the results of the nrITS and *rpl9* sequences. Furthermore, the phylogenetic relationships among *S. griffithii*, *S. ovata*, and *S. apetala* were well resolved in this analysis (Fig. 2). Among these

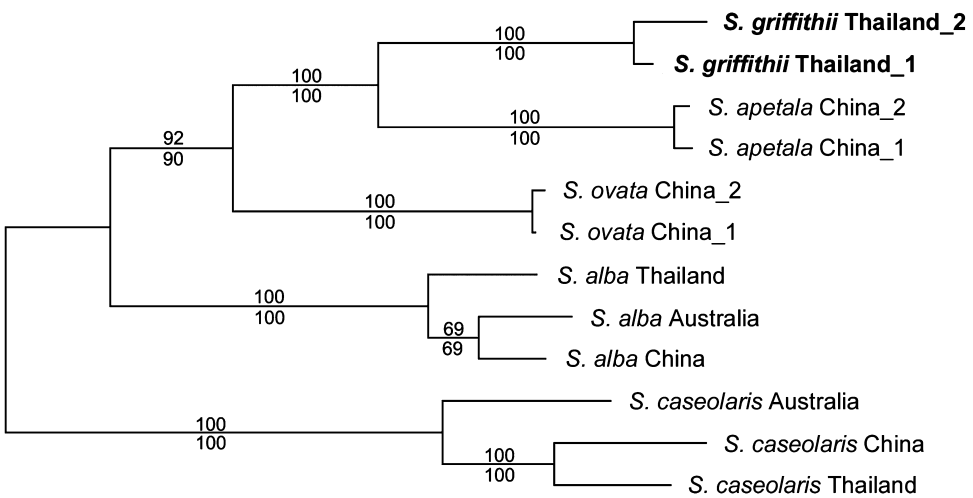

Fig. 2. Most parsimonious tree of *Sonneratia* using the combined sequences of the nuclear ribosomal internal transcribed spacer and 13 nuclear genes. Samples of *S. griffithii* are shown in bold. Numbers above and below branches indicate maximum parsimony and maximum likelihood bootstrap values, respectively. Countries and numbers after species names represent sample accessions.

three species, *S. griffithii* and *S. apetala* were the closest, with strong BS (100% and 100% for the MP and ML analyses, respectively). *Sonneratia ovata* was sister to *S. griffithii* and *S. apetala*, with 100% BS.

Discussion

Phylogenetic position of *Sonneratia griffithii*

As mentioned above, there have been conflicting views regarding the phylogenetic affinity of *S. griffithii* to other *Sonneratia* species based on morphological traits (Backer & van Steenis, 1951; Tomlinson, 1986; Aksornkoae et al., 1992); both *S. caseolaris* and *S. ovata* have alternately been considered its closest relative. Our phylogenetic analysis based on two DNA fragments, nrITS and *rpl9*, could not reach an unambiguous conclusion on the phylogenetic position of *S. griffithii*. We sequenced 12 additional genes for this. Based on sequences of the nrITS and 13 nuclear genes, the phylogenetic relationships among *S. ovata*, *S. griffithii*, and *S. apetala* were well resolved. *Sonneratia griffithii* and *S. apetala* are most closely related to each other, and *S. ovata* is sister to these two species. In fact, *S. griffithii* is morphologically different from *S. caseolaris* due to the former's lack of petals, white filament, the calyx not enclosing the fruit, and lanceolate leaves. Although *S. griffithii* resembles *S. ovata* because of its lack of petals and its white filament, its fruits are very hard even when mature, which is different from *S. ovata*. Although *S. griffithii* and *S. apetala* also show many morphological differences, they are distributed exclusively along the coasts of the Indian Ocean, whereas *S. ovata* is restricted to the West Pacific Ocean. Sister relationships between *S. griffithii* and *S. apetala* may imply that their relatively recent divergence may have occurred in the Indian Ocean. Thus, this provides a good start for speciation studies.

***Sonneratia* phylogeny and intrageneric classification**

A previous study on the phylogeny of the genus *Sonneratia* did not include the narrowly distributed species *S. griffithii*, and the phylogenetic relationships between *S. griffithii* and the other four species in this genus remain unknown. Moreover, the inclusion of two hybrids, *S.* × *hainanensis* and *S.* × *gulngai*, in that study may have resulted in topological changes that distorted the relationships among the other species analyzed (McDade, 1992). Our phylogenetic analysis includes five non-hybrid taxa of the genus *Sonneratia*. Based on the sequence data of the nrITS and the nuclear gene *rpl9*, *Sonneratia* falls into two clades, one consisting of *S. caseolaris* and the other comprising the remaining four species.

Sonneratia has been assigned intrageneric classifications by some authors on the basis of morphological characteristics (Ko, 1985, 1993; Wang & Chen, 2002). Based on the presence/absence of petals, *Sonneratia* was first divided into two sections: *Sonneratia* and *Pseudosonneratia* (Ko, 1985, 1993). Section *Sonneratia* consists of *S. alba* and *S. caseolaris*, and section *Pseudosonneratia* comprises *S. ovata*, *S. apetala*, and *S. griffithii*. Later, Wang & Chen (2002) reclassified *Sonneratia* into two sections with the same names, *Sonneratia* and *Pseudosonneratia*, based on the shapes of stigmas. Section *Pseudosonneratia*, which contains only *S. apetala*, has peltate stigmas, whereas section *Sonneratia*, which includes all the other *Sonneratia* species, has capitate stigmas. As shown in the phylogenetic tree (Fig. 2), paraphyletic relationships were revealed among the species of section *Pseudosonneratia* (Ko, 1985, 1993; Wang & Chen, 2002). Therefore, our molecular data do not entirely support the previous intrageneric classification of *Sonneratia* by Ko (1985, 1993) and Wang & Chen (2002).

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