Molecular phylogeny of Sonneratia

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ABSTRACT: Among the mangrove members grown in Okinawa Prefecture, Japan, Sonneratia alba Sm. placed in the family Sonneratiaceae has a specific character of yellowish green colored calyx, while the same species grown in Pan Yi River, Phang-naga, Thailand has the same species but have reddish colored calyx. To pinpoint whether they belong to different species, we conducted molecular phylogenetic study on the genus Sonneratia using DNA sequence information of chloroplast ribosomal protein L9 gene (rpl9) and nuclear cysteine proteinase inhibitor gene (cpi). Our results show that S. alba, S. apetala, S. griffithii, S. ovate, and S. caseolaris are distinct species, but S. lanceolata is a form of S. caseolaris. Sonneratia apetala is very closely related to S. griffithii and S. caseolaris is a diverse species showing considerable genetic differentiation. The Japanese populations or Thai populations of S. alba do not form a monophyletic lineage, suggesting that these populations belong to the same species. Natural hybridizations between S. alba and S. griffithii in Thailand were confirmed again and the hybrid is suggested to be given a name S. xalbo-griffithii. Either rpl9 or cpi or both can serve as DNA barcodes of Sonneratia.

KEYWORDS: DNA barcode, hybridization, mangrove, phylogeny, Sonneratia

The Sonneratiaceae is a dicot family occurring on tropical and subtropical sea-shore from East Africa and Madagascar to Southeast Asia, the Malay Archipelago to the Philippines, and tropical Australia to Pacific Islands, southern China and the Ryukyu Islands, Japan (Spalding et al. 2010). There are two genera, Sonneratia and Duabanga, and seven species in total in the family. Their flowers consist of numerous long stamens and small petals however, sometimes no petal. Sonneratia alba Sm. is a major mangrove tree. Its habits is muddy or sandy soil on the seashores, estuaries and tidal riversides of the front community, often forming upper stream forests surrounding islands. Its root system, conical pneumatophores stuck out from the ground, forms as ecological system supporting many marine organisms.

Morphology of Sonneratia alba is quite peculiar (Fig. 1). It is, for instance, a tree up to 10 m tall on Iriomote Island, Japan. It could be 20 m tall with diameter at breast height up to 80 cm at the age of more than 30 years old. Its branches spread out on the ground. Their leaves are opposite, entire, ovate or obovate, base round at base, broadly round and recurving on apex. Their flowers are ephemeral, usually bloom for one night. Each flower has six slender white petals and numerous white filaments 3 to 4 cm long with pale yellow anthers. The calyx is smooth, inside yellow green (Iriomote-jima type, Fig. 1b) or pinkish red (Thai type, Fig. 1e). Their fruits are 4-5cm diameter, calyx cup shaped, and contain over 200 seeds per fruit. It is distributed in tropical and subtropical areas from East Africa and Madagascar to Southeast Asia, the Malay Archipelago to the Philippines, and tropical Australia to Pacific Islands, southern China and the Ryukyu Islands (Spalding et al. 2010).

Sonneratia alba typically forms pure forests. However in Thailand, there are two kinds of mixed forests: One is the Andaman Sea side communities commonly associated with Avicennia alba, and the other is the Gulf of Thailand side communities associated with Avicennia marina. In this paper we will (1) test the genetic differentiation between the form of yellow-green calyx and pinkish red calyx of S. alba; and (2) phylogenetic relationships among species in Sonneratia using DNA sequence information.

MATERIALS AND METHODS

Eight populations of S. alba were sampled: two populations in Japan and six populations in Thailand (Table 1). To understand the relationship between S. alba and other species of Sonneratia, four other species and an interspecific hybrid species were also sampled (Table 2).

Young leaves were removed from twigs and quickly dried by burying in silica gel. Total DNA was extracted using mCTAB method (Li et al. 2012). Two pairs of primers were designed to amplify chloroplast ribosomal protein L9 gene (rpl9) (rpl9F gag ggg gtc agc atc aag gtg, rpl9R act tcc tga tat ctt tat ttt g) and nuclear cpi (cpiF tgc tcg ctt tgc tgg tga g, cpiR aag aag atc cac ggc ttc acc c). PCR amplifications were almost the same as Dong et al. (2012) except that the annealing temperatures were set at 55°C. PCR fragments of rpl9 were directly sequenced at Majorbio (Shanghai, China) using the PCR primers. The
PCR fragments of cysteine proteinase inhibitor gene (cpi) were subcloned to plasmids and eight colonies of each sample were sequenced at Majorbio. The sequences were checked and assembled with Sequencher v 5.2 (Gene Code Corporation, Ann Arbor, USA). The newly generated data were combined with those downloaded from GenBank to form rpL9 dataset and cpi dataset. Both datasets were aligned with Clustal X 2.0.5 (Larkin et al. 2007) and manually adjusted with Se-Al 20.a11 (Rambaut 1996) if necessary. Alleles were labelled with the first letter of its origin species epithet isolated by a bar (|), i.e., “|a” indicates from S. alba and “|g” indicated from S. griffithii. The two datasets were concatenated and subjected to phylogenetic analyses using PAUP* 4.0b10 (Swofford, 2002). Maximum parsimony analyses were conducted with starting tree from stepwise addition; 100,000 heuristic search replicates with random sequence addition; tree bisection-reconnection branch swapping, and saving multiple trees (MulTrees button selected), and saving no more than two trees score ≥5 from each replicate. Parsimony bootstrap percentages (BP) were based on 1000 replicates each with 100 random replicates. The trees were rooted using S. ovata as functional outgroups.

**RESULTS AND DISCUSSION**

Total of 82 sequences belong to six species and an interspecific hybrid were analyzed. The concatenated dataset had 1806 characters. Among them 29 variable characters are parsimony-uninformative. There are 132

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**Table 1. Population locations of Sonneratia alba studied in this study.**

| Population locality | Latitude        | Longitude        | Voucher   |
|---------------------|-----------------|------------------|-----------|
| Komi, Taketomi-cho, Yaeyama-Gun, Okinawa-Ken, Japan | 24°20'41.07"N | 123°55'41.45"E | BOP040937 |
| Komi, Taketomi-Cho, Yaeyama-Gun, Okinawa-ken, Japan | 24°19'21.70"N | 123°54'38.79"E | BOP040939 |
| Khanom River, Nakohn Si Thammarat, Thailand | 9°13'5.38"N | 99°50'37.56"E | BOP040571 |
| Pan Yi River, Phang-naga, Thailand | 8°19'45.06"N | 98°30'38.76"E | BOP040579 |
| Pan Yi River, Phang-naga, Thailand | 8°22'37.32"N | 98°30'48.24"E | BOP040584 |
| Pan Yi River, Phang-naga, Thailand | 8°24'12.96"N | 98°31'9.06"E | BOP040582 |
| Pan Yi River, Phang-naga, Thailand | 8°22'37.32"N | 98°30'48.24"E | BOP040584 |
| Pan Yi River, Phang-naga, Thailand | 8°21'29.28"N | 98°29'49.50"E | BOP040586 |
parsimony-informative characters. Heuristic searches
found the most parsimonious trees with tree length = 217,
CI= 0.751, and RI= 0.964. The strict consensus tree
indicates five clades corresponding to five species and a
interspecific hybrid species (Fig. 2). Sonneratia ovata was
set to be functional outgroup. There is not very much
hybridizations happen between S. alba × S. caseolaris,
but it has clearly diverged each other. There are some variations within S. griffithii. Sonneratia ovata and S. caseolaris
had not been verified true as indicated by calyx. However, either Japanese populations or Thai populations do not form a monophyletic lineage,
suggesting that these populations belong to the same
species. Which infraspecific rank should those morphotypes be given depends on our understanding of
geographical patterns of the morphotypes. Sonneratia caseolaris is a diverse species showing considerable
genetic differentiation. It deserves further investigation at
population level. Sonneratia lanceolata Blume is a
questionable species. It served as a parent of S. ×urama N.
C. Duke together with S. alba. Our data (Fig. 2) indicates
for sure that it is just a form of S. caseolaris.

There are some hybrid species in Sonneratia. Natural
hybridizations happen between S. alba × S. caseolaris (=S.
×gulngai N. C. Duke) in northwestern Bornea (Muller and
Hou-Liu 1966) and northeastern Australia (Duke 1984),
between S. alba × S. ovata (= S. ×hainanensis W. C. Ko,
E. Y. Chen et W. Y. Chen) in Hainain, China (Ko 1985),
between S. alba × S. lanceolata (= S. ×urama N. C.
Duke) in northeastern Australia and southern New Guinea
(Duke 1994), and between S. alba × S. griffithii in Asia
(Qiu et al. 2008). Considering S. lanceolata is a synonym of
S. caseolaris, S. ×urama becomes a synonym of S.
×gulngai. Hybridization events between S. alba × S.
griffithii happen in Thailand are confirmed again in this
study and the hybrid is suggested to be given a name S.
×albo-griffithii.

Identification of species is the first step toward
biological studies. It is often not as easy jab, especially for
cases of fruits, seeds, seedlings, etc. Therefore, DNA
barcoding is increasingly adopted to identify plant
materials. To find better genes for identification of
Sonneratia species, we evaluated 13 genes, i.e., cysteine
proteinase inhibitor gene (cpi), cytochrome B6-F complex
iron-sulfur subunit 2 gene, DNA-binding related protein
gene, internal transcribed spacer (ITS), iron-deficiency-
responsible protein gene, macrophage migration inhibitory

Fig. 2. Strict consensus tree of Sonneratia inferred using PAUP from the concatenating rpL9 and cpi. Numbers on
branches represent bootstrap support values from maximum parsimony analysis. (See the next page)
factor family protein gene, *matK*, microtubule-associated protein 1 light chain 3 gene, peptidyl-prolyl cis-trans isomerase gene, phosphatase inhibitor gene, ribosomal protein L9 gene (*rpL9*), saposin B domain-containing protein gene, tRNA-Leu *trnL* gene and *trnL-trnF* intergenic spacer, and found that *cpi* and *rpL9* resolve more haplotypes although they are not the most variable genes. ITS is the most variable gene and resolves the highest number of haplotypes. Unfortunately it has multiple copies, which needs more work in cloning. Either *cpi* or *rpL9* is sufficient for identifying all five species. However, *rpL9* would mistakenly assign hybrid species *S. alba* × *S. griffithii* to *S. alba*, and *cpi* requires cloning because there are 17 sites of difference and one indel between the two parents. In conclusion, either *rpL9* or *cpi* or both can serve as DNA barcodes of *Sonneratia*.

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**LITERATURE CITED**

Dong, W., Liu, J., Yu, J., Wang, L. and Zhou, S. 2012. Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. *PLoS ONE* 7(4): e35071.

Duke, N. C. and Jackes, B. R. 1987. A systematic revision of the mangrove genus *Sonneratia* (Sonneratiaceae) in Australasia. Blumea 32: 277–302.

Duke, N. C. 1984. A mangrove hybrid, *Sonneratia xgaengai* (Sonneratiaceae) from north-eastern Australia. Austrobailey 2: 103–105.

Duke, N. C. 1994. A mangrove hybrid, *Sonneratia xurama* (Sonneratiaceae) from northern Australia and southern New Guinea. *Australian Syst. Bot.* 7: 521–526.

Ko, W.C. 1985. Notes on the genus *Sonneratia* (Sonneratiaceae) in S. E. Asia. *Acta Phytotaxonomica Sinica* 23: 311–314.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. 2007. Clustal W and clustal X version 2.0. *Bioinformatics* 23: 2947–2948.

Li, J. L., Wang, S., Yu, J., Wang, L. and Zhou, S. 2013. A modified CTAB protocol for plant DNA extraction. *China Bull. Bot.* 48(1): 72–78.

Miyawaki, A., Okuda, S., Suzuki, K., Fujiwara, K., Nakamura, Y., Murakami, Y., Ohno, K., Suzuki, S. and Sabhasri, S. 1985. Phytoecological studies of mangrove vegetation in Thailand. Ecological studies on the vegetation of mangrove forests in Thailand. 1-100. (in Japanese) Yokohama National University, Yokohama.

Muller, J. and Hou-Liu, S.Y. 1966. Hybrids and chromosomes in the genus *Sonneratia* (Sonneratiaceae). Blumea 14: 337–343.

Qui, S., Zhou, R. C., Li, Y. Q., Havanond, S., Jaengjai, C. and Shi, S.H. 2008. Molecular evidence for natural hybridization between *Sonneratia alba* and *S. griffithii*. *Jour. Systematics and Evolution* 46(3): 391–395.

Rambaut, A. 1996. Se-Al: Sequence Alignment Editor. version 2.0. Oxford: University of Oxford, Department of Zoology.

Santisk, T. 1983. Taxonomy and distribution of terrestrial trees and shrubs in the mangrove formations in Thailand. *Natural History Bulletin of the Siam Society* 31(1): 63–91.

Santisk, T. 1992. *Sonneratiaceae*. Flora of Thailand 5(4) 434–444. The forest Herbarium Bangkok.

Spalding, M., Kainuma, M. and Collins, L. 2010. *World Atlas of Mangroves*. A collaborative project of ITTO, ISME, FAO, UNEP-WCMC, UNESCO-MAB, UNU-INWEH and TNC. 280–281. Earthscan London.

Swofford, D. 2002. *PAUP*®. Phylogenetic Analysis Using Parsimony (and other methods). Version 4.0b10. Sunderland, Massachusetts, Sinauer.

Tomlinson, P. B. 1986. The botany of mangroves. 367–374. Cambridge University Press Cambridge.