Molecular evidence for natural hybridization between
Sonneratia alba and S. griffithii

Suo QIU 1Ren-Chao ZHOU 2Yun-Qin LI 3Sonjai HAVANOND
Chanop JAENGJAI 1Su-Hua SHI 1

1(State Key Laboratory of Biocontrol and Key Laboratory of Gene Engineering of the Ministry of Education, Sun Yat-Sen University, Guangzhou 510275, China)
2(Experimental Center of Fundamental Teaching, Zhuhai Campus, Sun Yat-Sen University, Zhuhai 519002, China)
3(Department of Marine and Coastal Resources, Phayathai, Bangkok 10400, Thailand)

Abstract
Interspecific hybridization has been frequently observed in the mangrove genus Sonneratia. However, no natural hybridization has been reported between Sonneratia alba and S. griffithii to date, despite their overlapping distribution in the coast of Andaman Sea. In this study, cysteine proteinase inhibitor gene (cpi) from the nuclear genome, and two intergenic spacers (trnL-trnF and trnV-trnM) from the chloroplast genome, were sequenced to determine whether natural hybridization took place between the two species. Our results revealed two distinct types of cpi sequences from the putative hybrid matching those acquired from S. griffithii and S. alba, respectively. Sequencing of the chloroplast trnL-trnF and trnV-trnM regions showed that S. alba differed from S. griffithii by one nucleotide in each region, and the putative hybrid had the identical sequences with S. griffithii. Molecular data demonstrated clearly that there indeed existed natural hybridization between S. alba and S. griffithii, and that S. griffithii was the maternal parent in this hybridization event.

Key words chloroplast DNA, mangroves, natural hybridization, nuclear gene, Sonneratia.

Natural hybridization is ubiquitous in flowering plants and plays a significant role in plant evolution and diversification (Arnold, 1997). Among mangroves, interspecific hybrids have been reported in four genera, namely, Rhizophora L. (Duke & Bunt, 1979; Parani et al., 1997; Lo, 2003), Bruguiera Lamarck (Ge, 2001), Lumnitzera Willd. (Tomlinson et al., 1978; Tomlinson, 1986), and Sonneratia L. f. (Duke, 1984, 1994; Tomlinson, 1986; Duke & Jackes, 1987; Wang et al., 1999; Zhou et al., 2005). Sonneratia (Lythraceae sensu lato), a typical mangrove genus comprising about six species (Duke & Jackes, 1987; Tomlinson, 1986), is widely distributed from eastern Africa through Indo-Malaya to northeastern Australia and some islands in the west Pacific Ocean. There are frequent reports of natural hybridization between species of Sonneratia across the Indo-West Pacific region. Natural hybridization in Sonneratia was first reported by Muller and Hou-Liu (1966) in northeastern Borneo, where two hybrids, S. alba×S. ovata and S. alba×S. caseolaris, were postulated based on a study of morphology and cytology. However, they were not formally named. Later, the taxon S. alba×S. caseolaris was observed in northeastern Australia as having widespread distribution and exhibiting consistent morphological characteristics, and it was named S. ×gulngai N. C. Duke (Duke, 1984). S. ×gulngai has also been found in China and Sri Lanka (Ko, 1993; Jayatissa et al., 2001). The other hybrid taxon, S. alba×S. ovata, was also found in Hainan, China, and named as S. ×hainanensis W. C. Ko, E. Y. Chen & W. Y. Chen (Ko, 1985; Wang et al., 1999). A third hybrid, S. ×urama N. C. Duke, was described in northeastern Australia and southern New Guinea (Duke, 1994). It was proposed as a distinct hybrid entity originating from S. alba and S. lanceolata. Hybrid origins of S. ×gulngai and S. ×hainanensis have been documented by molecular data (Zhou et al., 2005).

During our field survey in the western coast of Thailand, we found that S. alba J. Smith and S. griffithii Kurz coexisted in Ranong Mangrove Forest Center, Ranong. In Sonneratia, S. alba is the most widespread species, occurring in almost the whole range of Sonneratia, whereas S. griffithii is restricted to the coast of Andaman Sea, from the upper Malay Peninsula to Bengal (Tomlinson, 1986). Both species grow in low intertidal zones of downstream estuaries (Duke et al., 1998). However, we found that S. griffithii was slightly upward in the habitat relative to S. alba in our field survey. Habitat differentiation between the two species may reflect their different levels of...
of salt tolerance, as observed in other species of *Sonneratia* (Duke et al., 1998; Zhou et al., 2007). *Sonneratia alba* has white petals while *S. griffithii* lacks any petal. The leaves of *S. alba* are oblong to obovate whereas those of *S. griffithii* are ovate to almost orbicular. A few morphologically intermediate individuals occur in the overlapping areas of *S. alba* and *S. griffithii*. These individuals usually have degenerated white petals and broadly ovate leaves. We consider that these individuals are probably hybrids between the two species. Although many hybrids have intermediate morphological features between their parents (Rieseberg & Ellstrand, 1993), morphological intermediacy is not invariably associated with hybrids (Morrell & Rieseberg, 1998; Wolfe et al., 1998a, b; Park et al., 2003). Therefore, the status of the putative hybrid requires further investigation.

Single or low-copy nuclear genes have been successfully used in identifying hybrids in plants (Sang & Zhang, 1999; Ferguson & Sang, 2001; Gaskin & Schaal, 2002; Pan et al., 2007). In addition, chloroplast DNA, usually maternally transmitted in angiosperms (Morgensen, 1996), can be used to determine the maternal parent of hybrids (e.g., Ferris et al., 1997; Moody & Les, 2002). In the present study, a nuclear cysteine proteinase inhibitor (*cpi*) gene from *S. alba*, *S. griffithii* and the putative hybrid was sequenced to determine the hybrid status of the morphologically intermediate taxon. Once its hybrid status was confirmed, we sequenced two intergenic spacers of chloroplast DNA (*trnL-trnF* and *trnV-trnM*) from the three taxa to determine the direction of hybridization.

1 Material and Methods

1.1 Plant materials

We collected *Sonneratia alba*, *S. griffithii* and the putative hybrid in Ranong Mangrove Forest Center, Ranong, Thailand (N 09°52′33″, E 98°35′87″). Leaves from two individuals of each taxon were collected in plastic bags with silica gels for DNA extraction. Table 1 listed the details of the samples collected. Voucher specimens were deposited in the Herbaria of Sun Yat-Sen University (SYS).

1.2 DNA extraction

Total cellular DNAs were extracted from dried leaf tissues using the CTAB method according to Doyle and Doyle (1987).

1.3 Sequencing of cysteine proteinase inhibitor gene (*cpi*)

Cysteine proteinase inhibitor genes (*cpi*) constitute a small multi-gene family in plants and are involved in plant defense against insects (Lim et al., 1996). There are three one-intron members within this family in both rice and arabidopsis (Martinez et al., 2005). Cysteine proteinase inhibitor gene (*cpi*) was amplified using primers cpi-F (′5′ AACAGCTCG-AGATCGAAG 3′) and cpi-R (′5′ GAACTCTGCA-ACTCCTGG 3′), which were designed according to the sequence of *cpi* from a cDNA library of *Sonneratia caseolaris* (Zhou et al., 2007). PCR was conducted with the following conditions: 94 ℃ (4 min); 30 cycles of 94 ℃ (1 min), 55 ℃ (1 min), 72 ℃ (1.5 min); and a final extension of 8 min at 72 ℃. PCR products were purified by electrophoresis through a 1.2% agarose gel followed by use of the Pearl Gel Extraction Kit (Pearl Bio-tech). While direct sequencing is feasible for both *S. alba* and *S. griffithii*, it produced chimeric or unreadable peaks in the chromatogram for the putative hybrid. Hence, cloning sequencing was performed subsequently for the putative hybrid. Purified PCR products were cloned into plasmids using the pMD18-T Vector System (Takara). Twenty positive clones were randomly selected for each amplification product and cultured for isolating plasmids. Positive clones with the inserts of correct size were confirmed by colony PCR. The plasmids with correct inserts were sequenced using universal M13-47 and RV-M primers. Sequencing was conducted in ABI 3730 DNA automated sequencer with Bigdye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Clones that clearly resulted from PCR-mediated recombination were excluded.

1.4 Chloroplast *trnL-trnF* and *trnV-trnM* sequencing

All samples from *Sonneratia alba*, *S. griffithii*, and the putative hybrid were used in the chloroplast *trnL-trnF* and *trnV-trnM* sequencing. Chloroplast *trnL-trnF* and *trnV-trnM* regions were amplified using universal primers trn-c and f (Taberlet et al., 1991), and trnV and trnM (Cheng et al., 2005), respectively. PCR products were purified and then directly sequenced using the methods mentioned above.

2 Results

2.1 Sequences of the *cpi* gene in *S. alba*, *S. griffithii*, and the putative hybrid

For both *S. alba* and *S. griffithii*, the *cpi* gene could be directly sequenced and clear sequences were obtained. Neither species showed sequence variation between accessions. The length of *cpi* gene of *S. alba* and
Table 1  Taxa of Sonneratia used in this study

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Voucher</th>
<th>Accession number</th>
<th>cpi</th>
<th>trnL-trnF</th>
<th>trnV-trnM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. alba 1</td>
<td>S. Shi 200709-11 (SYS)</td>
<td>EU418796</td>
<td>EU418841</td>
<td>EU418831</td>
<td></td>
</tr>
<tr>
<td>S. alba 2</td>
<td>S. Shi 200709-15 (SYS)</td>
<td>EU418797</td>
<td>EU418842</td>
<td>EU418832</td>
<td></td>
</tr>
<tr>
<td>S. griffithii 1</td>
<td>S. Shi 200709-01 (SYS)</td>
<td>EU418798</td>
<td>EU418845</td>
<td>EU418833</td>
<td></td>
</tr>
<tr>
<td>S. griffithii 2</td>
<td>S. Shi 200709-16 (SYS)</td>
<td>EU418799</td>
<td>EU418846</td>
<td>EU418834</td>
<td></td>
</tr>
<tr>
<td>The putative hybrid 1</td>
<td>S. Shi 200709-04 (SYS)</td>
<td>EU418800-EU418816</td>
<td>EU418843</td>
<td>EU418835</td>
<td></td>
</tr>
<tr>
<td>The putative hybrid 2</td>
<td>S. Shi 200709-09 (SYS)</td>
<td>EU418817-EU418830</td>
<td>EU418844</td>
<td>EU418836</td>
<td></td>
</tr>
</tbody>
</table>

SYS = Sun Yat-Sen University.

Table 2  cpi haplotypes of the putative Sonneratia hybrid revealed by cloning sequencing

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Clone number</th>
<th>Sequence length</th>
<th>cpi haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>The putative hybrid 1</td>
<td>1, 3, 5, 7, 10, 12, 13, 14, 17, 19</td>
<td>645 bp</td>
<td>SA</td>
</tr>
<tr>
<td></td>
<td>2, 6, 9, 11, 15, 16, 20</td>
<td>641 bp</td>
<td>SG</td>
</tr>
<tr>
<td>The putative hybrid 2</td>
<td>1, 4, 7, 10, 11, 15, 18, 20</td>
<td>645 bp</td>
<td>SA</td>
</tr>
<tr>
<td></td>
<td>2, 3, 6, 9, 16, 17</td>
<td>641 bp</td>
<td>SG</td>
</tr>
</tbody>
</table>

SA, haplotype that is identical or highly similar to S. alba; SG, haplotype that is identical or highly similar to S. griffithii.

Table 3  Variable sites of the nucleotide sequences of chloroplast trnL-trnF and trnV-trnM regions in the three taxa of Sonneratia

<table>
<thead>
<tr>
<th>Taxon</th>
<th>trnL-trnF</th>
<th>trnV-trnM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (bp)</td>
<td>Variable sites</td>
</tr>
<tr>
<td>S. alba</td>
<td>1000</td>
<td>T$_{127}$</td>
</tr>
<tr>
<td>S. griffithii</td>
<td>1000</td>
<td>G$_{127}$</td>
</tr>
<tr>
<td>The putative hybrid</td>
<td>1000</td>
<td>G$_{127}$</td>
</tr>
</tbody>
</table>

The number subscript the variable sites are the positions of variable sites.

was 645 bp, but 641 bp for S. griffithii. There were 18 nucleotide substitutions and a 4-bp indel between S. alba and S. griffithii. By contrast, direct sequencing of the cpi gene generated chimeric or unreadable sequences for both accessions of the putative hybrid. In the subsequent cloning sequencing, we obtained two distinct types (designated as Type SA and Type SG, respectively) of cpi sequences from both accessions of the putative hybrid after excluding nine clones clearly resulting from PCR-mediated recombination. The two distinct types of sequences corresponded to that of S. alba and S. griffithii, respectively. There were ten clones of Type SA and seven clones of Type SG for the first accession. For the second accession of the putative hybrid, there were eight clones of Type SA and six clones of Type SG (Table 2). Most clones of Type SA shared identical sequence with S. alba, and most clones of Type SG shared the same sequence as S. griffithii. In the other few clones, one or two nucleotide point mutations were observed. These minor variations may be due to PCR error caused by Taq DNA polymerase or unsampled intraspecific polymorphism.

2.2 Sequences of the chloroplast trnL-trnF and trnV-trnM in S. alba, S. griffithii, and the putative hybrid

The chloroplast trnL-trnF and trnV-trnM regions in Sonneratia alba, S. griffithii, and the putative hybrid exhibited limited variation. Sequence lengths are consistently 1000 bp in the chloroplast trnL-trnF region, and 840 bp in the trnV-trnM region for the three taxa. Sequences of both accessions from each taxon were identical. In total, there were two nucleotide substitutions between S. alba and S. griffithii, one in the trnL-trnF region and the other in the trnV-trnM region (Table 3). Both accessions of the putative hybrid had the same trnL-trnF and trnV-trnM sequences as S. griffithii.

3 Discussion

So far there has been no report describing natural hybridization between S. alba and S. griffithii. The putative hybrid studied here possesses two types of cpi sequence, each corresponding to that of S. alba
and \( S. \text{griffithii} \), and chloroplast haplotypes of \( S. \text{griffithii} \). Our results provide compelling evidence for natural hybridization between \( S. \text{alba} \) and \( S. \text{griffithii} \). Thus there are four interspecific hybrids that have been reported in \( Sonneratia \) and three have been documented by molecular data. With the exception of \( S. \text{apetala} \), all other species in \( Sonneratia \) are involved in interspecific hybridization. Interestingly, we find that \( S. \text{alba} \) is involved in all four cases of interspecific hybridization. There are at least two factors that contribute to frequent occurrence of interspecific hybridization in \( Sonneratia \).

One factor is that \( Sonneratia \) species often have partially overlapping geographic distribution and slightly overlapping habitats. For example, \( S. \text{alba} \) and \( S. \text{caseolaris} \) are sympatric across almost the whole range of \( S. \text{caseolaris} \), from India to China and Australia. \( Sonneratia \text{grifithii} \) and \( S. \text{alba} \) coexist in the coast of Andaman Sea. All species in \( Sonneratia \) grow within estuaries (Duke et al., 1998). The physiological tolerance of each species to salinity determines its habitat. \( Sonneratia \text{alba} \) occurs on the more salty seaward side of mangrove forests, whereas \( S. \text{caseolaris} \) grows on the less salty inland side (Duke et al., 1998). \( Sonneratia \text{alba} \) and \( S. \text{griffithii} \) occupy similar habitats, but \( S. \text{griffithii} \) was slightly upward along streams in comparison with \( S. \text{alba} \). There is considerable overlap in geographic distribution and habitats between species of \( Sonneratia \), providing spatial chances for hybridization.

The other factor that may contribute to natural hybridization is the long and partially overlapping flowering periods, and shared pollinators between species of \( Sonneratia \). Most species of \( Sonneratia \) taxa flower more than six months each year and have a long overlap of flowering season. For example, \( S. \text{alba} \) flowers almost throughout the year. All the species of \( Sonneratia \) are mainly pollinated by bats (Tomlinson, 1986), and this also provides great chances for interspecific hybridization in this genus. \( Sonneratia \text{alba} \) has the widest range and the longest flowering period among species of \( Sonneratia \), which is probably the reason why it is involved in all cases of hybridization.

Despite frequent hybridization in \( Sonneratia \), it seems that all hybrids are simple \( F_1 \)s. All the individuals of \( S. \times \text{gulngai} \) and \( S. \times \text{hainanensis} \) have been identified as \( F_1 \)s by AFLP markers (Zhou et al., 2005). With respect to the hybrid \( S. \text{alba} \times S. \text{griffithii} \), direct sequencing of five other nuclear genes also produced chimeric or unreadable sequences (data not shown). It is reasonable to speculate that \( S. \text{alba} \times S. \text{griffithii} \) are heterozygous at all the six nuclear genes examined. Maintenance of biparental sequences at all six randomly selected nuclear loci in the hybrid individuals implies that they are likely to be simple \( F_1 \)s, because the chance of randomly sampling six heterozygous loci from a non-\( F_1 \) hybrid is very low \( [P \leq (1/2)^6] \).

The restriction of hybrids to \( F_1 \)s appears to be a general phenomenon in \( Sonneratia \) and can be explained by strong postzygotic isolation between hybridizing species or hybrid breakdown. For example, the proportion of sterile pollen in either \( S. \times \text{gulngai} \) (95.6%) or \( S. \times \text{hainanensis} \) (54.4%) is much higher than in the parental species, \( S. \text{alba} \) (8.8%), \( S. \text{caseolaris} \) (5.7%), and \( S. \text{ovata} \) (3.3%) (Wang et al., 1999). Since all interspecific hybrids of \( Sonneratia \) appear to be \( F_1 \)s, these species seem to be able to maintain genetic integrity in spite of interspecific hybridization. Therefore, these non-hybrid species in \( Sonneratia \) should be considered as well defined biological species. As species in \( Sonneratia \) are very likely subject to parapatric speciation (Zhou et al., 2007), the hybrid zones of \( Sonneratia \) are probably primary hybrid zones (not secondary ones by allopatric divergence and subsequently secondary contact). Thus, the occurrence of hybridization and strong postmating isolation between parental species suggest that these species may be at the last stage of speciation and can achieve complete reproductive isolation via reinforcement.

Maternal inheritance of the chloroplast DNA in \( Sonneratia \) has been identified using DAPI (4′, 6-diamidino-2-phenylindole) staining technology (R. Zhou, unpublished data). Based on sequence data of chloroplast \( trnL-trnF \) and \( trnV-trnM \), \( S. \text{griffithii} \) was identified as the maternal parent of the hybrid, at least in the two individuals of the hybrid. \( Sonneratia \text{alba} \) is thus the paternal parent. If the trend continues, the direction of hybridization should be unidirectional.

Acknowledgements We thank Shong HUANG for his help during the field survey. This study was supported by grants from the National Basic Research Program of China (2007CB815701), the National Natural Science Foundation of China (30730008, 30470119), the Chang Hung-Ta Science Foundation of Sun Yat-sen University and the Young Teacher Foundation of Sun Yat-Sen University (2006-33000-1131357).

References
York: Oxford University Press.


