Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 35 (2005) 595–601
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular criteria for determining new hybrid species—An application to the *Sonneratia* hybrids

Renchao Zhou [a], Suhua Shi [a,*], Chung-I Wu [b]

[a] *State Key Laboratory of Biocontrol and Key Laboratory of Gene Engineering of the Ministry of Education, Zhongshan University, Guangzhou 510275, People's Republic of China*
[b] *Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637, USA*

Received 26 June 2004; revised 7 March 2005
Available online 11 April 2005

Abstract

The possible hybrid origin of new species can usually be corroborated by molecular means. Here, we suggest that the segregation patterns of the molecular markers be further analyzed. A true hybrid species should show the patterns under continuous breeding among its members, at least beyond the F2 generation. We applied the guidelines to the putative hybrid species of *Sonneratia*, a widespread mangrove genus, and concluded that all the observed hybrids in this genus are simple F1's. Thus, *S.* × *gulngai* and *S.* × *hainanensis* are not true hybrid species. The segregation patterns of molecular markers should be heeded in interpreting the existence of hybrid species.

Keywords: AFLP; Internal transcribed spacer; Mangrove; Natural hybridization; *Sonneratia*; Speciation

1. Introduction

Formation of new species by hybridization or introgression is a most interesting phenomenon (Coyne and Orr, 2004; Rieseberg, 1997; Wu, 2001). Plant hybridization often results in the formation of hybrid zone, but the formation of hybrid zone is not always accompanied by hybrid speciation. It is important to differentiate the process of hybrid speciation from the maintenance of stable hybrid zones (Ungerer et al., 1998).

A good deal of research concerns whether a particular taxon is of hybrid origin (see Arnold, 1997). The answer is usually a demonstration that the hybrids in question indeed possess the genetic materials from the said parental species. Moreover, it has to be shown that the admixture resulted from secondary hybridization, rather than from shared polymorphisms inherited from the common ancestors.

In some other cases, the hybrid status of the specimen can be strongly inferred on the ground of morphological analysis and ecological context. For example, the collection may show morphological intermediacy and was found in the zone of contact between the two putative parental species. While such a collection has sometimes been given a species designation, it is necessary to show that the specimen represents a self-sustaining species of hybrid origin. In other words, it has to be shown that the putative hybrid species has the genetic materials from the two parental species and those genetic materials can assort among themselves in a single genome. If the hybrids collected are more advanced than, say, the F3 generation, then the hybrids could potentially be self-sustaining. Otherwise, they are merely regenerated from the parental species repeatedly and should not be considered new species.

The mangrove genus *Sonneratia* (Lythraceae *sensu lato*) (Graham et al., 1993), comprising about five to nine species (Duke and Jackes, 1987; Ko, 1985, 1993; Wang and Chen, 2002), is widely distributed in the

* Corresponding author. Fax: +86 20 34022356.
E-mail address: lssssh@zsu.edu.cn (S. Shi).

1055-7903/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2005.03.012

Indo-West Pacific region. In China, *Sonneratia* is naturally distributed on Hainan Island and includes three indigenous species, *S. alba*, *S. ovata*, and *S. caseolaris* and an exotic species *S. apetala* introduced from Bengal in the 1980s. Two new species, *S. hainanensis* and *S. paracaseolaris*, were recently reported on the basis of the morphological features in flower and leaf that are remarkably different from other *Sonneratia* species (Ko, 1985, 1993). However, they were both proposed as natural hybrids *S.* × *hainanensis* and *S.* × *gulngai*, derived from hybridization between *S. alba* and *S. ovata*, *S. alba* and *S. caseolaris*, respectively (Duke, 1984; Wang et al., 1999). All the species and the putative hybrids in *Sonneratia* are diploid, with the same number of chromosomes $2n = 22$ (Wang et al., 1998).

An interesting phenomenon in *Sonneratia* hybrid zones is that there is little morphological variation among *S.* × *gulngai* or *S.* × *hainanensis* individuals. The observation suggests that all hybrid individuals are likely F1s and no advanced hybridization is taking place. We wish to test this hypothesis in this study. To that end, we cloned and sequenced the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA from the hybrids and their putative parental species. We further used AFLP markers to determine the extent of admixture between the parental genomes. Molecular criteria are proposed for such a determination. The absence of hybrid species in *Sonneratia* (and mangroves in general) is discussed in light of mechanisms of reproductive isolation and ecological speciation.

2. Materials and methods

2.1. Plant materials

In Hainan, *S. alba* and *S. caseolaris* overlap geographically in Qionghai and Wenchang where two hybrid zones are formed. *S. alba* and *S. ovata* coexist only in Wenchang, and altogether only four *S.* × *hainanensis* individuals were found in our field survey. Three indigenous species *S. alba*, *S. caseolaris*, and *S. ovata*, an introduced species *S. apetala*, and two putative hybrids *S.* × *gulngai* and *S.* × *hainanensis* were sampled from Qionghai and Wenchang. Eight to 10 individuals were collected for each taxon except *S.* × *hainanensis*. Leaves harvested from individual trees were dried with silica gel until DNA extraction. Reference specimens were deposited in Herbarium of Sun Yat-Sen University (SYS).

2.2. DNA extraction, nr ITS cloning, and sequencing

Total genomic DNAs were extracted from dried leaf tissues using CTAB method according to Doyle (1991). For ITS cloning experiments, two accessions were randomly selected for each taxon. Complete ITS region (including ITS-1, 5.8 S rRNA gene and ITS-2) was amplified using ITS-4 and ITS-5 primers (White et al., 1990). PCR products were purified by electrophoresis through 1.2% agarose gel followed by use of E. Z. N. A. Gel Extraction Kit (Omega). Purified PCR products were cloned into plasmids using the pGEM-T Easy Vector System (Promega). Six to eight positive clones were selected for each amplification product and cultured for isolating plasmids. Isolated plasmids were digested with *Eco*RI to determine whether they contained the inserts of correct size. The plasmids with correct inserts were sequenced using universal T7-promoter and SP6-promoter primes. Sequencing was conducted in ABI 3700 DNA automated sequencer with Big Dye Taq Terminator Cycle Sequencing Kit (Applied Biosystems). The boundaries of the ITS sequences were determined by comparison with those of carrot (X17534). All the sequences had been submitted to the GenBank with Accession Nos. AY680865–680950.

2.3. Phylogenetic analyses

ITS sequences were aligned in Clustal X with some corrections manually. Those clones with identical sequence within a taxon were treated as one operational taxonomic unit (OTU) in the phylogenetic analyses. We analyzed two data sets, which were constructed for the two putative hybrids, respectively. Parsimony, as implemented in PAUP 4.0b (Swofford, 1998), was used to infer phylogenies based on nucleotide substitutions in aligned sequences. Parsimony analyses were performed by using heuristic search with TBR swapping, MULTREES option, ACCTRAN optimization, and 100 random addition replicates for the two data sets. Indels were treated as missing data. Standard measures of homoplasy such as consistency index (CI), retention index (RI), and level of internal support (bootstrap values) were calculated. Bootstrap analyses were carried out with 1000 replications of heuristic search with simple taxon addition and maxtrees set to 500. *Duabanga grandiflora* (AF208695), *Lagerstroemia speciosa* (AF201688), and *Trapa maximowiczii* (AY035757), which are closely related to *Sonneratia* (Huang and Shi, 2002; Shi et al., 2000), were used as outgroup species.

2.4. AFLP assay and data analysis

The AFLP technique is based on the selective PCR amplification of restriction fragments from total digests of genomic DNA (Vos et al., 1995). AFLP fragments are distributed over all genomic locations in the way restriction fragments are. Therefore, AFLP fragments generally correspond to unique and random positions in the genome. By using different combinations of primers

and a series of AFLP amplifications, we can screen a sizable fraction of the whole genome.

The AFLP assay was conducted according to Vos et al. (1995), except that *Eco*RI selective primers were labeled with fluorescent 6-carboxy fluorescein (6-FAM) on the 5′ nucleotide. The selective amplification primer pairs in this study were E1M1 (E-AAG, M-CAG), E1M7 (E-AAG, M-CAT), E3M8 (E-AGC, M-CTT), E4M7 (E-ACA, M-CAT), E5M4 (E-AAC, M-CAA), and E6M4 (E-ACC, M-CAA). The amplified products were run on an ABI Prism 377 DNA sequencer using 4.25% denaturing sequencing polyacrylamide gel in 1× TBE running buffer.

Total amplified bands, common bands, and monomorphic parent-specific bands were scored. Co-migrating bands within a gel between different individuals were considered to be homologous. Bands were considered monomorphic species-specific if they were present in all samples of one species and absent in all samples of the other species. The inherited patterns of parent-specific bands in the putative hybrids were analyzed as reported by Teo et al. (2002).

3. Results and analyses

We first chose two individuals (accessions) from each of the putative hybrid species, *S.* × *hainanensis* and *S.* × *gulngai*, as well as two individuals from each of their possible parents *S. alba*, *S. caseolaris*, *S. ovata*, and *S. apetala* for phylogenetic analysis using cloning nr ITS sequences. Once their hybrid status was confirmed, we surveyed more individuals from the hybrids and their parents by the AFLP analysis. The objective of the AFLP analysis is to determine the degree of hybridization in the hybrids—i.e., intercross hybrids (F1, F2, and so on) or backcross hybrids at any generation. We conclude that all hybrids collected in the hybrid zones are F1's. There is no advanced hybridization among these *Sonneratia* species.

3.1. Phylogenetic analysis based on nr ITS sequences

Within each non-hybrid species, *S. alba*, *S. caseolaris*, *S. ovata*, and *S. apetala*, a high degree of similarity among clones was observed; many in fact have identical sequences. Minor variations exist because homogenization by concerted evolution is not complete. In contrast, two distinct classes of ITS sequences were obtained in both *S.* × *gulngai* and *S.* × *hainanensis*, each corresponding to those of each of their putative parental species.

The aligned sequences of *S.* × *gulngai* data set are 657 bp in length, of which 103 are parsimony-informative. Phylogenetic analysis of this data set yielded a single most parsimonious tree of 352 steps, with a consistency index (CI) of 0.861, and a retention index (RI) of 0.921 (Fig. 1). All clones of both accessions of each non-hybrid species form their own species clades, most with strong bootstrap support. *S. ovata* and *S. apetala* have the closest genetic relationship among these species of *Sonneratia*. Both accessions of *S.* × *gulngai* have two distinct types of ITS sequences. One type forms a well-supported monophyletic group with *S. alba*, and the other type was clustered with *S. caseolaris*. *S.* × *gulngai* is clearly a hybrid between these two parental species.

The aligned sequences of *S.* × *hainanensis* data set were 654 bp in length, of which 106 were parsimony-informative. Phylogenetic analysis gave rise to a single most parsimonious tree of 359 steps, with a consistency index (CI) of 0.872, and a retention index (RI) of 0.930 (Fig. 2). Again, two distinct types of ITS sequences were obtained from both accessions of *S.* × *hainanensis*, each clustered with either the *S. alba* or *S. ovata* clade.

3.2. AFLP pattern in *S.* × *gulngai* and in the parental species, *S. alba* and *S. caseolaris*

The results are shown in Table 1. AFLP assay generated 340 bands from the three taxa using six primer combinations. Among them, 82 bands were shared by all the samples of the three taxa. The patterns of amplified bands using primer pair E1M7 are shown in Fig. 3. In total, there were 113 monomorphic species-specific bands present in *S. alba* samples and 114 monomorphic species-specific bands in *S. caseolaris* samples. No taxon-specific bands existed in any of the eight samples of *S.* × *gulngai*. All these samples of *S.* × *gulngai* exhibited the 113 *S. alba*-specific bands and 104–112 (mean = 107.0) of the *S. caseolaris*-specific bands.

3.3. AFLP pattern in *S.* × *hainanensis* and in the parental species, *S. alba* and *S. ovata*

The results are shown in Table 1. Six primer combinations produced 286 bands from *S.* × *hainanensis*, *S. alba*, and *S. ovata*. One hundred and two bands were common to all the samples of the three taxa. There were 91 monomorphic species-specific bands present in *S. alba* and 82 monomorphic species-specific bands in *S. ovata*. No taxon-specific band existed in any of *S.* × *hainanensis* samples. All four samples of *S.* × *hainanensis* possessed 85–86 (mean = 85.8) *S. alba*-specific bands and 78–82 (mean = 80.8) *S. ovata*-specific bands.

3.4. Analysis

The results of Table 1 unambiguously support the hypothesis of hybrid origin of both *S.* × *gulngai and S.* × *hainanensis*. All bands from either hybrid taxon were derived from the putative parents; there were no hybrid-specific bands. Moreover, both hybrid taxa

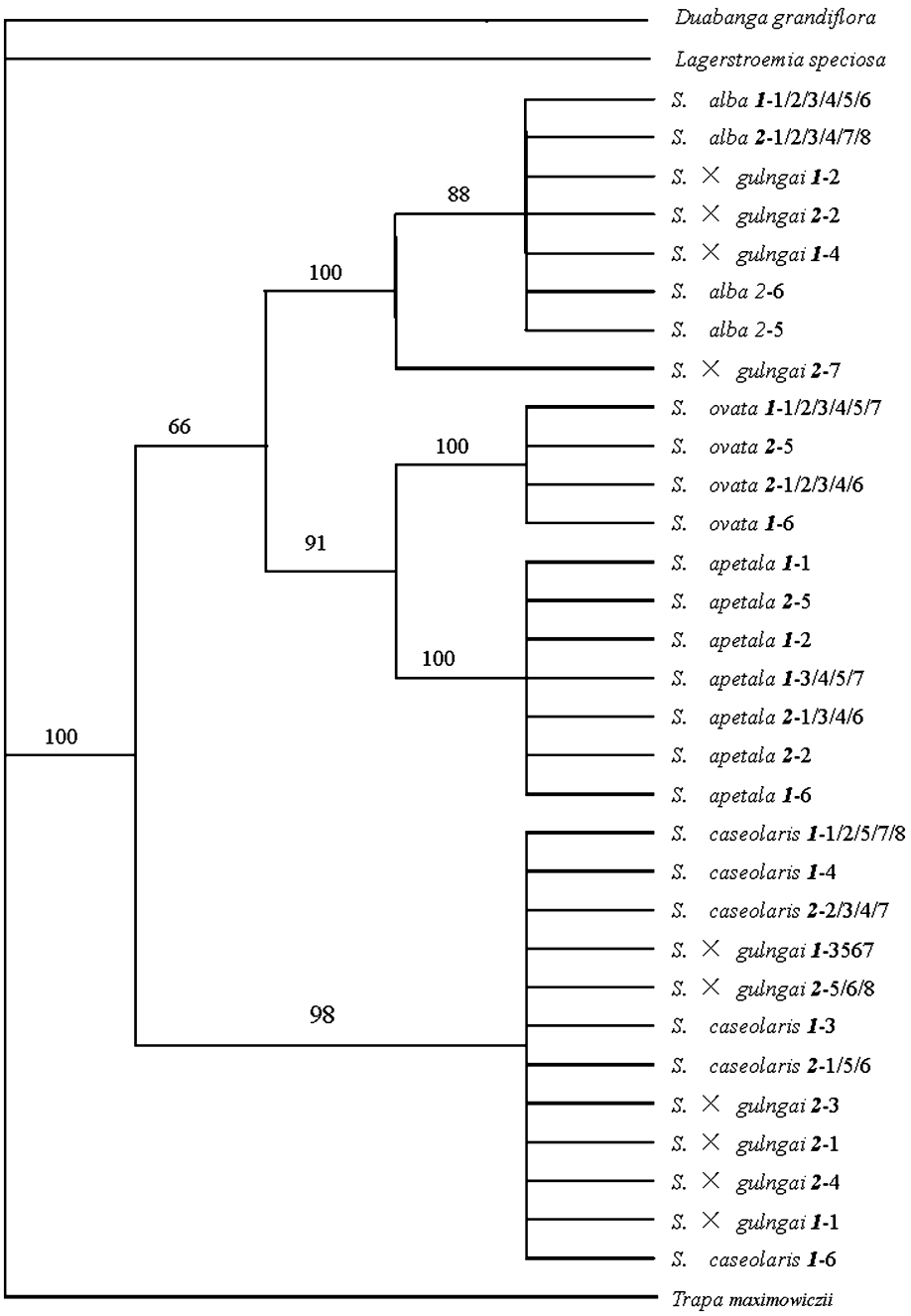

Fig. 1. A most parsimonious tree (352 steps) constructed from nr ITS sequences (CI = 0.861; RI = 0.921) to show the phylogenetic position of *S.* × *gulngai*. The numbers above the branches are bootstrap values. The numbers following the taxon name are the accession number and clone number(s), respectively.

appear to be the F1's between the parental species (*S. alba* and *S. caseolaris* for *S.* × *gulngai*; *S. alba* and *S. ovata* for *S.* × *hainanensis*). No hybrid individuals represent a stage of hybridization beyond the simple F1's, as reasoned below.

Let us designate the genetic contribution to the hybrids from either parental species as p and q ($p + q = 1$), respectively. If there is no backcross to either parent, then $p = 0.5 = q$. With one backcross, $p = 0.75$ and $q = 0.25$. p and q can range between 0 and 1 for more complex scenarios. In the F2 generation, the expected proportion of bands in the hybrids that are homozygous for either parent is 0.25. Homozygosity for the allele from parent 1 means the absence of the band from parent 2. For example, if the *S.* × *gulngai* samples were F2's, we expect $113 \times 0.25 = 28.3$ bands from *S. alba* and $107 \times 0.25 = 26.8$ from *S. caseolaris* would be missing in *S.* × *gulngai*. The results of Table 1 show

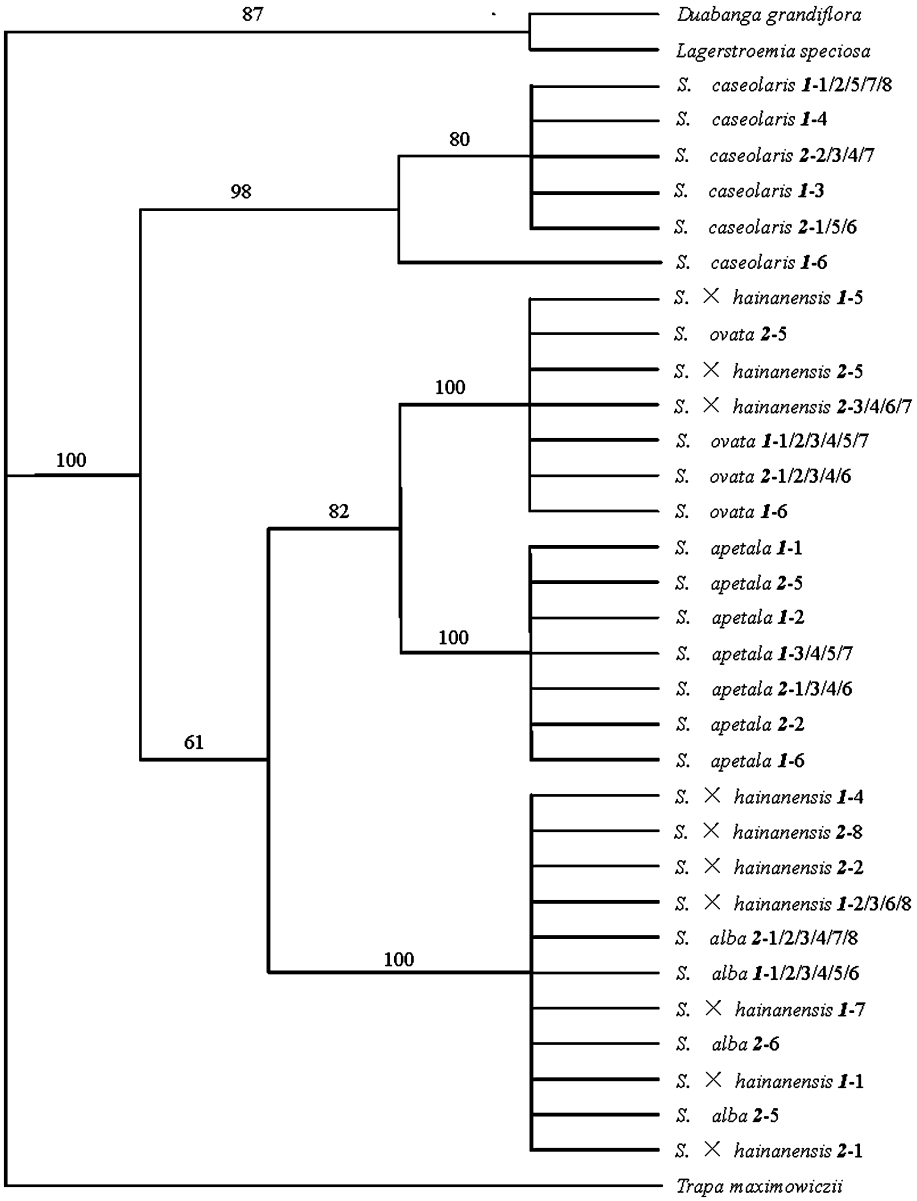

Fig. 2. A most parsimonious tree (359 steps) constructed from nr ITS sequences (CI = 0.872; RI = 0.930) to show the phylogenetic position of *S.* × *hainanensis*. The numbers above the branches are bootstrap values. The numbers following the taxon name are the accession number and clone number(s), respectively.

Table 1
The AFLP patterns in the parental species (P1 and P2) and the individuals of the putative species hybrids (H)

	P1 = *S. alba* P2 = *S. caseolaris* H = *S.* × *gulngai*	P1 = *S. alba* P2 = *S. ovata* H = *S.* × *hainanensis*
P1-P2 shared	82	102
P1-P2-H shared	82	102
P1 (unique)	113	91
P1-H shared	113	85.8 (85–86)
P2 (unique)	114	82
P2-H shared	107.0 (104–110)	80.8 (79–82)
H (unique)	0	0

the numbers to be 0 and 7, respectively ($P < 0.001$ by the binomial test). Clearly, the samples from neither *S.* × *gulngai* nor *S.* × *hainanensis* are F2's.

We shall consider the more general cases where the hybrids have an arbitrary degree of genetic contribution from either parent. They have also crossed among themselves for a number of generations to achieve a degree of inbreeding, F, where F is the inbreeding coefficient between 0 and 1 (Crow and Kimura, 1970). In those cases, the proportion of homozygotes for alleles from parent 1, heterozygotes, and homozygotes for alleles from parent 2 would be, respectively,

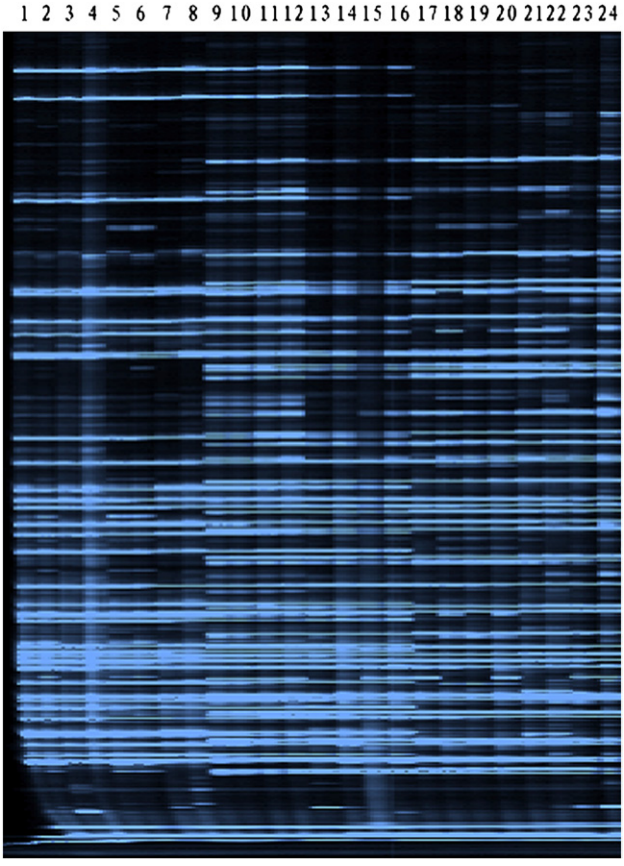

Fig. 3. Amplified bands of *Sonneratia* × *gulngai* and its parental species *S. alba* and *S. caseolaris* using primer pair E1M7. Lanes 1–8, *S. alba*; lanes 9–16, *S.* × *gulngai*; and lanes 17–24, *S. caseolaris*.

$$[p^2(1-F)+pF] : [2pq(1-F)] : [q^2(1-F)+qF].$$

Therefore, the total proportion of missing bands would be half the sum of the two proportions for homozygotes:

$$[(1-F)(p^2+q^2)+F]/2. \quad (1)$$

The division by 2 is due to the fact that one of the two parental bands would be missing at each homozygous locus.

We should note that the specific nature of AFLP bands does not affect the validity of Eq. (1). In other words, whether the allelic form of a species-specific band is a missing band, or another band on the gel, does not matter. As long as the two parental species have comparable numbers of species-specific bands, then at each homozygous locus, half of the total number of species-specific bands would be missing. Indeed, there is a small fraction of bands, at less than 10% in each of the three parental species, which are not fixed within species. We excluded these possibly heterozygous bands from our analysis and scored only monomorphic, species-specific bands. Within each species, these monomorphic, species-specific bands represent homozygous loci (or sites).

In the case of *S.* × *gulngai*, the proportion of missing species-specific bands is 3.1% [(0 + 7)/(113 + 114)]. For *S.* × *hainanensis*, the proportion is 3.7%. In contrast, the smallest number from Eq. (1) is 25% for F2's, when $p = q = 0.5$ and $F = 0$. Because the total proportion of missing bands is too small for gene assortment under any degree of hybridization, we can safely conclude that neither *S.* × *gulngai* nor *S.* × *hainanensis* can be anything other than F1 hybrids.

4. Discussion

Natural hybrids are relatively common in flowering plants (Arnold, 1997; Rieseberg and Ellstrand, 1993). In this report, we suggest the molecular criteria for determining the species status of the putative hybrids. Both ITS sequences of nr DNA and AFLP markers provide compelling evidence for the hybrid origins of *S.* × *gulngai* and *S.* × *hainanensis*. However, all hybrid individuals in *Sonneratia* collected appear to be F1 plants and no advanced hybridization events have been observed. Neither hybrid type appears to be a self-sustaining entity to deserve the species status.

While we corroborated the F1 status of all morphologically hybrids collected in the wild, there is a distinct possibility that some backcross hybrids may resemble either parent morphologically and may have escaped detection. Nevertheless, we have analyzed in total 190 individuals from eight *S. alba* populations by the AFLP assay. We have not observed any sample that is morphologically *S. alba* but is molecularly a hybrid with many *S. alba* bands missing. The presence of F1 hybrids, and only F1 hybrids, in nature has also been observed elsewhere. When we analyzed the *S.* × *gulngai* samples from Daintree River (Australia) using AFLP markers, all hybrid individuals were again found to be F1s (data not shown).

Sonneratia hybrids are restricted in the overlapping areas of parental species' geographic distribution. In China, individuals of *S.* × *gulngai* can only be found in Qionghai and Wenchang where *S. alba* and *S. caseolaris* coexist. No individual of *S.* × *gulngai* is found in Sanya, Lingshui, and Wanning where only one of the parents can be found. *S. ovata* exists only in Wenchang, so does *S.* × *hainanensis*. No individual of *S.* × *hainanensis* is found in places where only *S. alba* occurs. The geographic distributions are consistent with the hypothesis that hybrids are merely regenerated from parental species and cannot expand their range by their own seed dispersal.

Why are the *Sonneratia* hybrids not able to propagate? Some highly sterile F1 hybrids become successful species by adopting a vegetative mode of reproduction (Brysting et al., 2000; Sang et al., 1995), but in *Sonneratia* as in many other species, the mode of asexual reproduction is absent. Homoploid hybrid speciation in nature may be a rare phenomenon, and fewer than 10

cases have been rigorously documented in plants (Ferguson and Sang, 2001; Rieseberg, 1997). In many cases, postmating reproductive isolation plays a large role in preventing hybridization to proceed beyond F1 or F2. This is, however, only partially true in these two hybrids. The proportion of sterile pollen in either *S.* × *gulngai* (95.62%) or *S.* × *hainanensis* (54.43%) is indeed much higher than that of the parental species, *S. alba* (8.76%), *S. caseolaris* (5.68%), and *S. ovata* (3.25%) (Wang et al., 1999). Furthermore, the seed germination rate for *S.* × *gulngai* and *S.* × *hainanensis* is 22.9 and 64.0%, respectively, lower than that of *S. alba* (90.0%), *S. caseolaris* (94.0%), and *S. ovata* (74.0%) (Wang et al., 1999).

On the other hand, partial fertility does permit the production of F2 and backcross progeny and allow introgression of genetic materials across nascent species boundaries (Wu, 2001). At least between *S. alba* and *S. ovata* which produce the relatively fit *S.* × *hainanensis*, advanced hybridization seems plausible. Its absence may thus be connected to the narrow ecological niches in mangrove's habitat. Since these hybrids may often be ecologically maladapted, even when they are physiologically fit, reproductive isolation between *Sonneratia* species may be considered a case of ecological speciation (Schluter, 1998). In studying interspecific hybrids, it is necessary to analyze the segregation patterns of the molecular markers in order to determine the stage of hybridization (F1, F2, etc.). We suggest that other putative hybrid species may also benefit from analyses of this type.

Acknowledgments

We thank Fengxiao Tan and Shuguang Jian for technical help in DNA isolation and AFLP assay, Hanghang He, Xuejun Ge, and Cairong Zhong for helping us in sampling, and Yuguo Wang, Linghui Wu for valuable discussions on the manuscript. This study was supported by grants from the National Natural Science Foundation of China (30230030, 30470119), the Chang Hungta Science Foundation of Sun Yat-Sen University, the Qiu Shi Science and Technology Foundation, and the National Key Project for Basic Research (973) Grant 2003CB715904.

References

Arnold, M.L., 1997. Natural Hybridization and Evolution. Oxford University Press, New York.

Brysting, A.K., Holst-Jensen, A., Leitch, I., 2000. Genomic origin and organization of the hybrid *Poa jemtlandica* (Poaceae) verified by genomic in situ hybridization and chloroplast DNA sequences. Ann. Bot. 85, 439–445.

Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Sunderland, MA.

Crow, J.F., Kimura, M., 1970. An Introduction to Population Genetics Theory. Harper and Row Publishers, New York.

Doyle, J., 1991. DNA protocols for plants—CTAB total DNA isolation. In: Hewitt, G.M., Johnston, A. (Eds.), Molecular Techniques in Taxonomy. Springer, Berlin, pp. 283–293.

Duke, N.C., 1984. A mangrove hybrid, *Sonneratia* × *gulngai* (Sonneratiaceae) from north-eastern Australia. Austrobaileya 2, 103–105.

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

Ferguson, D., Sang, T., 2001. Speciation through homoploid hybridization between allotetraploids in peonies (*Paeonia*). Proc. Natl. Acad. Sci. USA 98, 3915–3919.

Graham, S., Crisci, J., Hoch, P., 1993. Cladistic analysis of the Lythraceae sensu lato based on morphological characters. Bot. J. Linn. Soc. 113, 1–33.

Huang, Y., Shi, S., 2002. Phylogenetics of Lythraceae sensu lato: a preliminary analysis based on chloroplast rbcL gene, psaA-ycf3 spacer, and nuclear rDNA internal transcribed spacer (ITS) sequences. Int. J. Plant Sci. 163, 215–225.

Ko, W., 1985. Notes on the genus *Sonneratia* (Sonneratiaceae) in Southeast Asia. Acta Phytotax. Sin. 23, 311–314.

Ko, W., 1993. Notes on some *Sonneratia* (Sonneratiaceae) of China. J. Trop. Subtrop. Bot. 1, 11–13.

Rieseberg, L.H., 1997. Hybrid origins of plant species. Annu. Rev. Ecol. Syst. 28, 359–389.

Rieseberg, L.H., Ellstrand, N.C., 1993. What can molecular and morphological markers tell us about plant hybridization?. Crit. Rev. Plant Sci. 12 213–241.

Sang, T., Crawford, D.J., Stuessy, T.F., 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. USA 92, 6813–6817.

Schluter, D., 1998. Ecological causes of speciation. In: Howard, D.J., Berlocher, S.H. (Eds.), Endless Forms: Species and Speciation. Oxford University Press, Oxford, pp. 114–129.

Shi, S., Huang, Y., Tan, F., He, X., Boufford, D.E., 2000. Phylogenetic analysis of the Sonneratiaceae and its relationship to Lythraceae based on ITS sequences of nr DNA. J. Plant Res. 113, 253–258.

Swofford, D.L., 1998. PAUP* (version 4.0b). Phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland, MA.

Teo, C.C., Kiew, R., Set, O., Lee, S.K., Gan, Y.Y., 2002. Hybrid status of kuwini, *Mangifera odorata* Griff. (Anacardiaceae) verified by amplified fragment length polymorphism. Mol. Ecol. 11, 1465–1469.

Ungerer, M.C., Baird, S.J., Pan, J., Rieseberg, L.H., 1998. Rapid hybrid speciation in wild sunflowers. Proc. Natl. Acad. Sci. USA 95, 11757–11762.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van der Lee, T., Hornes, M., Frijters, A., Pot, J., Wheeler, T., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.

Wang, R., Chen, Z., 2002. Systematics and biogeography study on the family Sonneratiaceae. Guihaia 22, 214–219.

Wang, R., Chen, Z., Huang, X., 1998. Chromosome counts on Chinese mangroves. J. Trop. Subtrop. Bot. 6, 40–46.

Wang, R., Chen, Z., Chen, E., Zheng, X., 1999. Two hybrids of the genus *Sonneratia* (Sonneratiaceae) from China. Guihaia 19, 199–204.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Wu, C.-I., 2001. The genic view of the process of speciation. J. Evol. Biol. 14, 851–865.