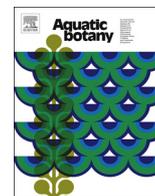




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Short communication

# Hybridization with natives augments the threats of introduced species in *Sonneratia* mangroves

Wei Xie<sup>a</sup>, Cairong Zhong<sup>b</sup>, Xinnian Li<sup>a</sup>, Zixiao Guo<sup>a,\*</sup>, Suhua Shi<sup>a</sup><sup>a</sup> State Key Laboratory of Biocontrol, Guangdong Key Lab of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510275, China<sup>b</sup> Hainan Dongzhai Harbor National Nature Reserve, Haikou, 571129, China

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## ABSTRACT

Hybridization between introduced and native species is highly likely as human introduction removes spatial barriers. The mangrove species *Sonneratia apetala* has been introduced to South China from Bangladesh and frequently planted as part of reforestation efforts over the previous three decades. *Sonneratia apetala* has been shown to suppress the growth of native mangrove species and has become invasive in several mangrove forests of South China. In this study, we report an additional competitive advantage of *S. apetala* via cross-species hybridization with the native species *S. alba*, using morphological and DNA polymorphism data. We further show that the native *S. alba* is the maternal parent in this hybridization, thus becoming even more vulnerable to the introduction on both the ecological and genetic fronts. Given that hybridization accompanies some of the most serious invasions on record, we recommend monitoring hybridization events before any wide-spread introduction.

## 1. Introduction

Introduced exotic species can cause severe damage to native ecosystems, local economy and human health. Particularly destructive examples in plants are *Mikania micrantha* (Yang et al., 2017) and *Spartina alterniflora* (Wang et al., 2006). Introduced species may evolve rapidly as they encounter new abiotic and biotic environments, putting evolutionary pressure on native species through competitive exclusion, niche displacement, hybridization and introgression, and predation (Mooney and Cleland, 2001). Particularly, hybridization with native relatives is highly likely as human introduction removes spatial barriers between formerly isolated taxa (Hufbauer and Torchin, 2007). In turn, hybridization may increase the invasiveness of the exotic species by reducing the opportunity for conspecific fertilization or by genetic introgression and assimilation (Todesco et al., 2016). Thus, for the conservation of valuable and endangered native species, the evolutionary influence of introduced species should be carefully studied, apart from autecological processes.

Mangroves, a collection of polyphyletic woody or shrubby species that have adapted to tropical and subtropical coastal intertidal zones, are of great conservation importance for their ecological services in ameliorating the impact of flooding, support of coastal food webs, carbon sequestration, and affect on global biogeochemical cycles (Xu et al., 2017). However, mangroves are susceptible to both

anthropogenic disturbances and climate change. Recent active mangrove restoration projects helped mangrove forest area to recover in some countries (López-Portillo et al., 2017). However, these efforts sometimes involve the use of exotic species, for example *S. apetala* in China (Ren et al., 2009).

*Sonneratia apetala* is naturally restricted to southern India, Bangladesh, and Sri Lanka (Duke, 2013), but was introduced to Dongzhai harbor, Hainan Island, China from Bangladesh in 1985 (Ren et al., 2009). It has been frequently transplanted from Dongzhai harbor to other South China coasts (including Guangdong, Guangxi and Fujian provinces) for mangrove restoration since 1991 (Ren et al., 2009). Despite its effectiveness as a pioneer restoration species and benefits in improving soil fertility, the use of *S. apetala* for mangrove reforestation has long been controversial (Peng et al., 2012; Ren et al., 2009). The increasing concerns focus on possible invasiveness it showed in some locations due to its competitive advantage including rapid growth, massive seed production and high spreading ability (Ren et al., 2009). *Sonneratia apetala* inhabits a niche similar to its congener *S. caseolaris* (Chen et al., 2013). When planted together, *S. apetala* suppresses growth of native mangrove species such as *Kandelia obovata*, *Avicennia marina*, and *Aegiceras corniculatum*, indicating higher competitiveness (Peng et al., 2012).

In addition to its autecological influence, evolutionary processes, including hybridization, may represent a further threat from *S. apetala*.

\* Corresponding author.

E-mail address: [guozx8@mail.sysu.edu.cn](mailto:guozx8@mail.sysu.edu.cn) (Z. Guo).<https://doi.org/10.1016/j.aquabot.2019.103166>

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**Fig. 1.** Morphological diagnostics of the putative hybrids and their parents. Leaves of *Sonneratia alba* (up-left), putative hybrid (up-middle) and *S. apetala* (up-right). The ruler unit is millimeter. Flowers of *S. alba* (bottom-left), putative hybrid (bottom-middle), and *S. apetala* (bottom-right). The photos were taken by the authors, except that photo of *S. alba* flower is adapted from the World Mangrove ID (Duke, 2013).

Natural hybridization is common within the *Sonneratia* genus, which consists of six species (Duke, 2013). For example, *S. alba* can mate with *S. ovata* to produce *S. × hainanensis* and *S. alba* can mate with *S. caseolaris* to produce *S. × gulgai* (Ragavan et al., 2014; Zhou et al., 2005). In Dongzhai harbor, the first location it was planted in China, *S. apetala* grows together with all these native congeners and hybrids. We found two individuals with morphological characters intermediate between *S. apetala* and *S. alba* in field surveys at this location. To clarify whether these two individuals are hybrids, we employed molecular diagnostics using nine nuclear and three chloroplast genes. We confirmed that hybridization between *S. apetala* and the native congener species has indeed occurred in the wild, providing an important data point for predicting the invasiveness of *S. apetala* in China, as well as management decision-making. The proper selection of mangrove species for restoration is essential to avoid additional threats to the native ecosystem.

## 2. Materials and methods

### 2.1. Plant material

The introduction of *S. apetala* from Bangladesh to Dongzhai harbor (Hainan, China, 19°56'N/110°34'E) is well documented (Ren et al., 2009). Propagules of *S. apetala* were collected in Bangladesh and planted in Dongzhai harbor in 1985. In the field survey, we recognized the six living trees originally introduced following the planting record. Leaves of these six *S. apetala* individuals and two randomly chosen descendants located close to the putative hybrids were collected. The two putative hybrids as well as two *S. alba* individuals randomly chosen near the hybrids were sampled. Two *S. ovata* individuals from the same location were also included as the outgroup. Leaves of each sample were collected in plastic bags with silica gel before DNA extraction. Voucher specimens of the two putative hybrids were deposited in the Herbarium of Sun Yat-Sen University (SYS) with identifiers CHN20170329 and CHN20171021.

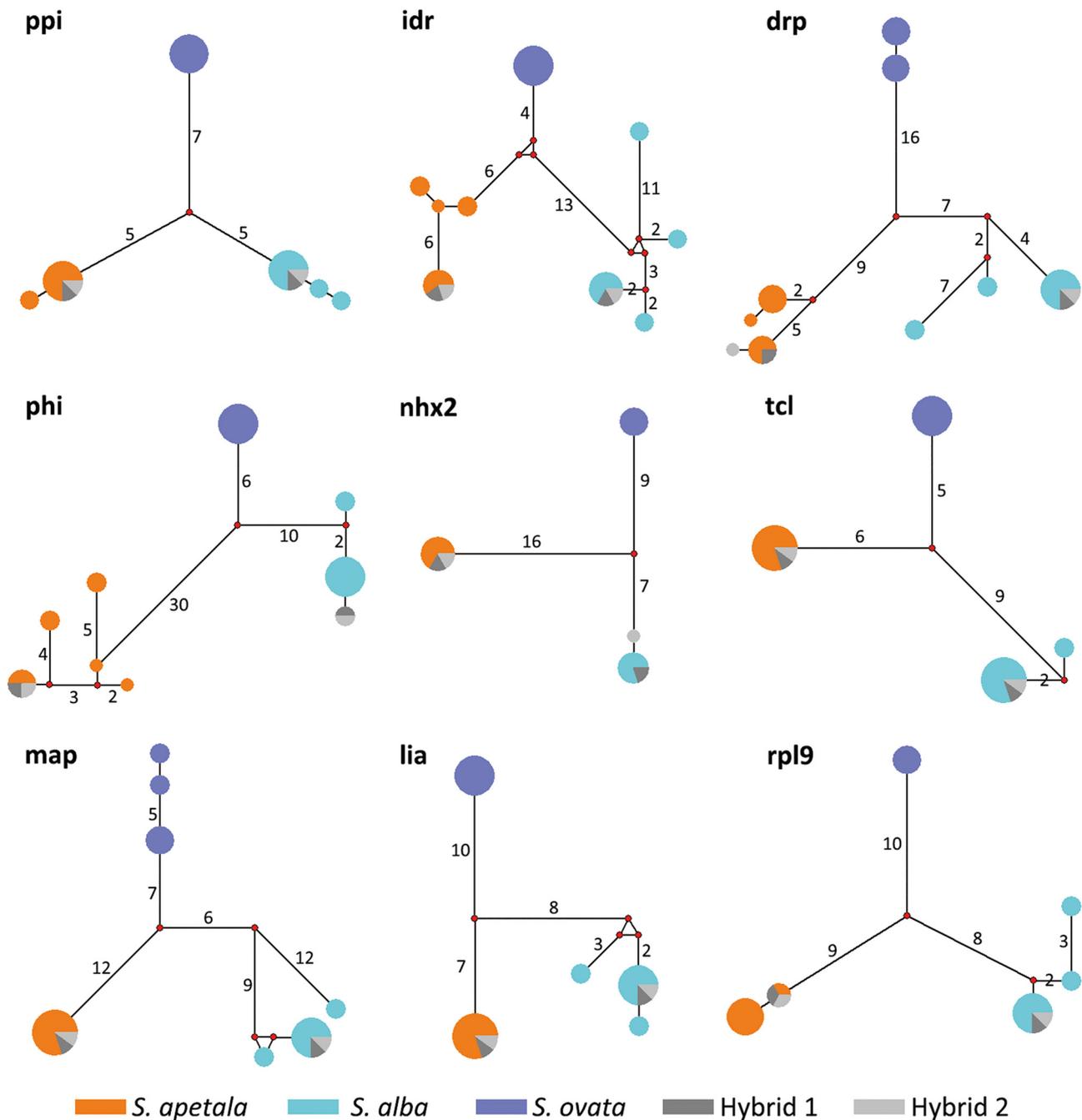


Fig. 2. Haplotype networks. The haplotypes were inferred from sequences of *Sonneratia alba*, *S. apetala*, *S. ovata*, and the two putative hybrids. Numbers on lines indicate mutation steps; single mutations are not indicated.

## 2.2. DNA extraction and sequencing

Total genomic DNA was extracted from dried leaf tissue using the CTAB method. Polymerase chain reactions (PCR) were conducted (see the Supplementary Table S1 for primer sequences) to amplify nine single- or low-copy nuclear genes (*ppi*, *tcl*, *lia*, *rpl19*, *map*, *idr*, *drp*, *phi*, and *nhx2*) and three chloroplast regions (*trnF-L*, *rbcl*, and *psbA-trnH*). The PCR program is: 94°C (4 min); 30 cycles of 94°C (1 min), 55°C-60°C (1 min), 72°C (1.5 min); and a final extension of 8 min at 72°C. PCR products were purified by electrophoresis through a 1.2% agarose gel followed by treatment using the Pearl Gel Extraction Kit (Pearl Biotech). Extracted DNA was sequenced on an ABI 3730 sequencer. Heterozygosity of the putative hybrids caused partial failure of direct sequencing, thus we also used clone sequencing to phase haplotypes in

these individuals. The pMD18-T Vector System (Takara, Guangzhou, China) was used for cloning. Six to eight positive clones of each gene were sequenced. Adaptor sequences were trimmed. Variant sites for each haplotype were corrected manually and verified by examining at least three clones. All sequences generated were deposited in GenBank with accession numbers MG751042-MG751061, MH583029-MH583040, MK267182-MK267238, and MK281633 (supplementary Table S2).

## 2.3. Sequence and flow-cytometry analyses

Published *S. alba*, *S. apetala*, and *S. ovata* sequences from different locations (Yang et al., 2015) were also included in the alignment. Polymorphic sites were checked manually. Haplotypes were inferred

using DNASP (Librado and Rozas, 2009). Gaps were not considered when inferring haplotypes. Lastly, we used the NETWORK 5.0.0.3 software (Fluxus Technology Ltd.) to compute haplotype networks. Accession numbers of sequences used here are summarized in the electronic Supplementary table S2.

To test whether polyploidization occurred after hybridization, the relative nuclear DNA content of *S. apetala*, *S. alba*, and the two putative hybrids was estimated using flow cytometry according to the protocol published by Doležel et al. (2007). The measurements were performed on a BD FACSVerser device with BD FACSuite software. *Oryza sativa ssp. japonica* was used as the internal standard.

### 3. Results

#### 3.1. Morphological characteristics of the putative hybrids

Leaves of *S. alba* are obovate to oval, with the apex broadly rounded and even emarginated (Fig. 1). In contrast, *S. apetala* leaves are narrow, tapering gradually toward the apex (Fig. 1). The leaves of the two hybrids are oblong with obtuse apices. They are narrower and longer than those of *S. alba* but broader and shorter than *S. apetala* (Fig. 1). The mushroom-shaped stigmas of the two hybrids are similar to but smaller than those of *S. apetala*, and larger than the punctate stigma of *S. alba* (Fig. 1). Calyces of *S. alba* have six (rarely seven) lobes, while those of *S. apetala* have four. Of the 40 flowers we collected from one putative hybrid, 11 have four lobes, seven have six, one has seven, and the remaining 21 flowers have five lobes. This morphological evidence suggests that the two putative hybrids are offspring from hybridization events between *S. alba* and *S. apetala*.

#### 3.2. Molecular validation of the putative hybrids

We confirmed the hybrid status of these specimens by comparing DNA sequences of nine nuclear loci from our two putative hybrids to *S. apetala* and *S. alba* individuals, following the guidelines presented in an *Avicennia* study (Huang et al., 2014). The test relies on fixed DNA sequence differences between parental species (homozygous within, but different between species; Supplementary Table S3). Particularly, the six individuals introduced originally from Bangladesh are homozygous at almost all sites, indicating that they are pure *S. apetala*. In contrast, the hybrids are heterozygous at all informative sites, with one nucleotide from each parental taxon in all the nuclear genes we assayed (Supplementary Table S3). This molecular evidence clearly indicates that the hybridization occurred after the introduction of *S. apetala* to China.

We next inferred haplotypes of *S. alba*, *S. apetala*, the putative hybrids, and their close relative *S. ovata* and constructed haplotype networks. Haplotypes from each of the three species form distinct groups at all nine loci (Fig. 2). In contrast, the hybrids harbor both haplotypes from the two parents. Each of the two hybrids has two haplotypes, one shared with *S. alba* and the other with *S. apetala*. Hybrid-derived sequences from three genes (*drp*, *phi*, and *nhx2*) diverged slightly from their parents. The rest of the loci were exactly identical to the species they came from (Fig. 2).

#### 3.3. Maternal parent identification

Chloroplast matrilineal inheritance has been confirmed in *Sonneratia* species (Zhou et al., 2008), allowing us to use chloroplast markers to infer which species were the maternal parent. We used three loci: *rbcl* (residing in a gene), and two intergenic regions *trnL-trnF* and *psbA-trnH*. The two parental species differ by one mutation at *rbcl*, four at *trnL-trnF*, and three at *psbA-trnH*. As shown in the Supplementary Table S4, both hybrids carry *S. alba* alleles, identifying it as the maternal parent.

#### 3.4. Absence of a polyploidization event

Both *S. alba* and *S. apetala* are diploid, with a karyotype of  $2n = 22$  (Wang et al., 1998). If polyploidization occurred as a result of hybridization, the two hybrids are expected to carry twice or more the amount of DNA contained in cells from the parental species. Using flow cytometry, we estimated the genome size of *S. apetala* and *S. alba* at 360 MB and 290 MB respectively. In comparison, the genome sizes of the hybrids are 320MB and 340MB, values that are between the two parental species. This result indicates that this hybridization event did not result in polyploidization.

### 4. Discussion

Mangrove forests are important coastal ecosystems but are dramatically affected by human disturbances. As conservation activities gained in urgency, multiple reforestation projects succeeded in increasing the area covered by mangrove forests in China (López-Portillo et al., 2017). In these projects, exotic species such as *S. apetala* were introduced to China and planted together with native congeners. Although *S. apetala* was once considered the best reforestation species, there is now concern that it may become invasive. The apparent hybridization between *S. apetala* and *S. alba* we observe may contribute to this invasiveness. *Sonneratia apetala* males may compete for female *S. alba*, reducing reproductive opportunities for the native congener. Hybridization may also contaminate *S. alba* genomes through genetic introgression and assimilation. Additionally, hybridization may increase invasiveness of *S. apetala* by introducing genetic variation and evolutionary novelty from a species already adapted to the local environment (Hufbauer and Torchin, 2007). There is precedent for such a scenario in the case of *M. micrantha* whose invasion into Asia is facilitated by genetic admixture between different source populations (Yang et al., 2017).

It is striking that hybridization occurred in such a short time since the first introduction of *S. apetala* to Dongzhai harbor (33 years, 1985–2018). However, the observed hybridization may have accelerated the invasion. Commonly, a long lag time between colonization and the successful invasion of exotic species is expected, 170 years in trees and 131 years in shrubs (Kowarik, 1995). Hence, this hybridization event so soon after introduction should call attention to its potential threat to local mangrove ecosystems where the exotic *S. apetala* was planted. The transplanting and expanding of *S. apetala* should be managed properly. Native mangrove species may be a better choice for mangrove restoration.

In the notorious case of *Spartina*, *Spartina alterniflora* was introduced from north America to the UK by accident in the 19<sup>th</sup> century, and then hybridized with the native *Spartina maritima* to form *Spartina × townsendii* (Ainouche et al., 2009). The intermediate then experienced polyploidization to form the fertile *Spartina anglica*, which is now among the top 100 most harmful invasive species (Ainouche et al., 2009). The absence of polyploidization accompanying this hybridization event may mitigate the threat of the hybrids themselves.

The introduction of exotic mangrove species has long been popular. As early as 1902, *Rhizophora mangle* was introduced to Hawaii where there were no mangroves before (Chimner et al., 2006). Since then, several additional species have been introduced with negative consequences for Hawaiian ecology and economy (Chimner et al., 2006). Mangrove species are discontinuously distributed in the Indo-West Pacific (IWP) and Atlantic-East Pacific (AEP) region, but some introductions crossed this discontinuity. For example, the IWP species *Bruguiera gymnorrhiza* and *Lumnitzera racemosa* were introduced to Florida, USA (Fourqurean et al., 2010), while the AEP species *Laguncularia racemosa* was introduced to China (Zhong et al., 2011). In addition to a cautionary view of restoration with this species, we recommend hybridization monitoring before widespread planting in other introduction cases.

In addition to this cautionary view of restoration with this species, our discovery also provides important insights into hybridizations within the *Sonneratia* genus. In all the reported hybridization events within this genus, *S. alba* was found to hybridize with *S. caseolaris*, *S. ovata*, *S. griffithii*, and *S. lanceolata* (Qiu et al., 2008; Ragavan et al., 2014; Zhou et al., 2005). Obviously, *S. alba* is essential for hybridization in this genus. Interestingly, *S. alba* is usually the maternal parent (Zhou et al., 2008), with a single exception of crossing with *S. griffithii* (Qiu et al., 2008). Our report complements the last case of hybridization between *S. alba* and other *Sonneratia* species. These interesting parental origins suggest that further research might elucidate the causes of these observations.

### Data accessibility

All data produced or used in this study can be downloaded from Genbank with accession numbers indicated in the electronic Supplementary Table S2.

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### Author contributions

S. Shi and Z. Guo conceived the study; C. Zhong, X. Li and W. Xie collected the samples and did the morphological identification. W. Xie performed the molecular experiments. Z. Guo and W. Xie analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

### Statement

All authors have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere. The authors have no conflict of interest to declare.

### Declaration of Competing Interest

The authors declare no competing interests.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.aquabot.2019.103166>.

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