Molecular evidence for double maternal origins of the diploid hybrid *Hippophae goniocarpa* (Elaeagnaceae)

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Received 28 March 2006; accepted for publication 21 August 2007

Homoploid hybrid plant species are rare, and the mechanisms of their speciation are largely unknown, especially for homoploid hybrid tree species. Two contrasting hypotheses have been proposed to explain the origin of *Hippophae goniocarpa*: (1) it is a diploid hybrid originating from *H. rhamnoides* ssp. *sinensis* × *H. neurocarpa* ssp. *neurocarpa*, and (2) it originated via marginal differentiation from *H. rhamnoides* ssp. *sinensis*. Regardless of which of these hypotheses is true (if either), previous studies have suggested that *H. rhamnoides* ssp. *sinensis* is the only maternal donor for this hybrid species. In this study, we aim to elucidate the maternal composition of *H. goniocarpa* and to test the two hypotheses. For this purpose, we sequenced the maternal chloroplast DNA *trnL-F* region of 75 individuals representing *H. goniocarpa*, *H. rhamnoides* ssp. *sinensis*, and *H. neurocarpa* ssp. *neurocarpa* in two co-occurring sites of the taxa. Seven haplotypes were identified from three taxonomic units, and their phylogenetic relationships were further constructed by means of maximum parsimony, maximum likelihood, and network analyses. These seven haplotypes clustered into two distinct, highly divergent lineages. Two haplotypes from one lineage were found in *H. rhamnoides* ssp. *sinensis*, and five (representing the other lineage) in *H. neurocarpa* ssp. *neurocarpa*. *Hippophae goniocarpa* shared four common haplotypes from both lineages, but the haplotypes detected from the two populations differed to some extent, and in each case were identical to local haplotypes of the putative parental species. Thus, both *H. rhamnoides* ssp. *sinensis* and *H. neurocarpa* ssp. *neurocarpa* appear to have together contributed to the maternal establishment of *H. goniocarpa*. These results clearly demonstrate that the marginal origin hypothesis should be rejected, and support the hybrid origin hypothesis. *Hippophae goniocarpa* exhibits a sympatric distribution with its two parent species, without occupying new niches or displaying complete ecological isolation. However, this species has effectively developed reproductive isolation from its sympatric parent species. Our preliminary results suggest that *H. goniocarpa* may provide a useful model system for studying diploid hybrid speciation in trees. © 2008 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2008, 156, 111–118.

ADDITIONAL KEYWORDS: chloroplast DNA sequence – haplotype – speciation – taxonomy.

INTRODUCTION

It is now widely accepted that hybridization plays a crucial role in plant evolution, contributing to both intraspecific variation through introgression (Anderson, 1948) and the establishment of new lineages through homoploid or polyploid hybrid speciation (Stebbins, 1950; Grant, 1981; Rieseberg, 1997; Gross, Schwarzbach & Rieseberg, 2003). By contrast with abundant records of natural allopolyploid hybrid speciation, diploid hybrid species are rarely found and, to date, no more than ten such hybrid species have been definitively verified using molecular markers (Rieseberg, 1997). Unlike allopolyploid speciation, in which postzygotic sterility can be rapidly overcome through genome doubling (Grant, 1981), the development of a fertile diploid hybrid segregator
depends on the recombinant pairing of two different sets of chromosomes, stochastic sorting of genetic sterility factors that differentiated the parental species, and ecological or spatial prezygotic isolation from both parental species (Gallez & Gottlieb, 1982; Buerkle et al., 2000; Abbott, 2003). In evolutionary terms, homoploid hybrid speciation is less likely to occur in trees than in herbs because of their large genomes, long life histories, and wide distributions of potential parent species. Nevertheless, there is still robust evidence indicating that *Pinus densata*, an alpine pine that occurs on the Qinghai-Tibetan Plateau, has diploid hybrid origins (Wang, Szmidt & Savolainen, 2001). In addition, it has been demonstrated that allopolyploid species commonly have multiple origins (Soltis & Soltis, 1993). Few diploid hybrid species have been assessed to determine whether they have single or multiple origins. However, all such species examined to date – including *Pinus densata*, *Argyranthemum sundingii*, *Helianthus anomalus*, and *Helianthus deserticola* (Brochmann, Borgen & Stabøtorp, 2000; Wang et al., 2001; Schwarzbach & Rieseberg, 2002; Gross et al., 2003) – appear to have multiple origins. In the study presented here, we aim to examine the maternal origin(s) of another putative diploid hybrid species, *Hippophae goniocarpa* Y.S. Lian et al. ex Swenson & Bartish.

*Hippophae* is a small genus of the Elaeagnaceae, comprising between five and seven species (Rousi, 1971; Bartish et al., 2002). The species of this genus are dioecious and wind pollinated, with gender being determined genetically (Rousi, 1971; Lian, Chen & Lian, 1998; Bartish et al., 2000). They occupy a wide range of habitats in Asia and Europe, from seashores to high mountains (c. 5000 m in altitude). All the species and infraspecific varieties currently known are diploid with 2n = 24 (Rousi, 1971; Lian et al., 1998). As a result of its nitrogen-fixing characteristics and its importance in forest restoration, medicine, and food, this genus has received a great deal of attention in diverse research contexts (Lian et al., 1998). *Hippophae goniocarpa* was described from a few specimens originating from Qinghai and Sichuan, China, and two subspecies, ssp. *goniocarpa* and ssp. *litangensis*, were recognized (Lian, Chen & Sun, 1995). However, Bartish et al. (2002) showed that this unpublished species is not monophyletic, and suggested that the two putative subspecies probably originated from different hybrid events: from *H. rhamnoides* ssp. *sinensis* Rousi × *H. neurocarpa* ssp. *neurocarpa* S.W. Liu & T.N. He (referred to here as *H. neurocarpa*) and *H. rhamnoides* ssp. *ynnanensis* Rousi × *H. neurocarpa* ssp. *stellatopilosa* Y.S. Lian, respectively. Therefore, they validly published them as two separate species, *H. goniocarpa* Y.S. Lian et al. ex Swenson & Bartish and *H. litangensis* Y.S. Lian & X.L. Chen ex Swenson & Bartish, and this taxonomic treatment was followed in the present study. However, Lian, Chen & Sun (1997) and Lian et al. (2003) insisted that both *H. goniocarpa* and *H. litangensis* together originated from the marginal differentiation (through the divergence of the isolated populations in the distributional edges) of *H. rhamnoides*, and should be combined as a single species. They provided two lines of independent evidence to support this hypothesis. First, the flowering period of this species does not overlap with those of its putative parents. Second, chromosome pairing and segregation at meiosis are normal. In fact, this ‘evidence’ should be treated cautiously, as the flowering period of this species does partially overlap with that of one of its putative parent species, *H. neurocarpa* (Lian et al., 1995), and ‘normal behaviour’ of the chromosomes during meiosis is common in most stabilized diploid hybrid species (Arnold, 1997; Rieseberg, 1997).

All morphological characters of *H. goniocarpa* are intermediate between those of the assumed parent species (Lian et al., 1997). Fruits of both *H. goniocarpa* and *H. rhamnoides* ssp. *sinensis* are fleshy, whereas those of *H. neurocarpa* are dry. In addition, *H. goniocarpa* differs from *H. rhamnoides* ssp. *sinen- sis* in that its ripe fruits are black–brown or deep green, without any bright yellow coloration, terete, and ridged; these features are similar to those of *H. neurocarpa* (Lian et al., 1995, 1998, 2003). In addition, the species’ hybrid origin has been unequivocally demonstrated by random amplification of polymorphic DNA (RAPD), chloroplast DNA (cpDNA), and ITS data (Bartish et al., 2000, 2002; Sun et al., 2003). Furthermore, Bartish et al. (2002) demonstrated that cpDNA is maternally inherited in *Hippophae* and, on the basis of the clustering of the cpDNA haplotypes of the two putative hybrid species (*H. goniocarpa* and *H. litangensis*), that they were mothered by *H. rhamnoides* ssp. *sinen- sis* and *H. rhamnoides* ssp. *ynnanensis*, respectively. Despite the disagreement regarding the mode of origin of *H. goniocarpa*, this species is usually considered to have a single maternal origin, based on a combination of morphological and molecular evidence presented by various authors (for example, Lian et al., 1998, 2003; Bartish et al., 2000, 2002; Chen et al., 2003; Sun et al., 2003).

The study presented here was designed to examine the maternal origins of *H. goniocarpa* s.s. according to Bartish et al. (2002). The genetic makeup of this species was expected to show one of two possible patterns, as suggested by previous authors (Lian, 2000; Bartish et al., 2002). The first anticipated pattern was that all individuals of this species in both populations would have haplotypes that cluster only with *H. rhamnoides* ssp. *sinen- sis*. This pattern would support two alternative hypotheses: (1) that *H. gonioc-
carpa was mothered by *H. rhamnoides* ssp. *sinensis* during its original hybridization (if the haplotypes were identical), or (2) that its origin was marginal (if the two species had closely related, but differentiated haplotypes). The second anticipated pattern was that haplotypes of this species would either cluster with or be identical to those of both assumed parent species. This pattern would undoubtedly support the hybrid origin hypothesis. We analysed the *trn*L-*F* region sequence of the maternally inherited cpDNA to characterize 75 individuals of the three species. This region has been shown to be highly variable and useful for studying infraspecific phylogeny and interspecific relationships (for example, Liu *et al.*, 2002, 2006; Stappen *et al.*, 2002; Denda & Yokota, 2003; Meerow, Lehmiller & Clayton, 2003; Sauquet *et al.*, 2003; Wang, Yang & Liu, 2005; Zhang *et al.*, 2005). Our results suggest that *H. goniocarpa* has double maternal origins from both assumed parent species, and further that the homoploid hybrid speciation of this species is at an early stage.

**MATERIAL AND METHODS**

**PLANT MATERIALS**

In September 2002, leaf samples were collected from the three target species at two locations in Qilian County, Qinghai, China. Only female plants with distinguishable fruits were sampled. The male trees are difficult to ascribe to a particular species because of the lack of accurate diagnostic characters. Therefore, we did not include male trees in our analysis. The two sites are spaced about 20 km apart (the first at 38°01.833′ N, 100°36.077′ E, 3090 m altitude, and the second at 38°16.965′ N, 100°50.306′ E, 3010 m altitude; Table 2). We examined most female trees with fruits that were present, and found no more than 30 female individuals of *H. goniocarpa* at either site. Because this species, like its putative parents, exhibits strong clonal growth, some of these female individuals are likely to have originated from clonal reproduction, resulting in identical genotypes. Therefore, we sampled only one tree of any pair or group located less than 100 m apart, so that only 11 and 12 trees were sampled at each of the two sites. In addition, samples were collected from 11 and 41 female trees of *H. rhamnoides* ssp. *sinensis* and *H. neurocarpa*, respectively. All sampled trees were located more than 100 m apart, and clearly belonged to different clones. A total of 75 individuals of the three species were sampled. Voucher specimens of each sampled tree were collected and deposited in the Herbarium, North-west Plateau Institute of Biology, Chinese Academy of Sciences (HNWP). Silica gel was used to dry fresh leaves rapidly in the field.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, following Doyle & Doyle (1987). The *trn*L-*F* region was amplified using the primers c (5′-CGGAATTGGTAGACGCTACG) and f (5′-ATTGAAGTCGGTACACAGAG), according to Taberlet *et al.* (1991). Polymerase chain reactions (PCRs) were performed in 25-μL reaction mixtures containing 19 μL of sterile, double-distilled water, 2.5 μL of 10 × Taq polymerase reaction buffer, 1 μL each of 5 pmol of primer ‘c’ and ‘f’, 1 unit of TaqDNA polymerase, and about 10–40 ng of plant DNA. The temperature programme included a denaturation step at 94 °C for 2 min, followed by 38 cycles of 94 °C for 50 s, 51 °C for 45 s, 72 °C for 1 min 30 s, and, finally, an extension step of 72 °C for 8 min. PCR products were purified using a CASpure PCR Purification Kit following the manufacturer’s instructions (Casarray, Shanghai, China). The sequenced samples were run on a Megabase 500 Automated DNA Analysis System using dye-terminator chemistry, according to the manufacturer’s protocol.

**ANALYSIS OF SEQUENCE DATA**

Sequences were aligned using the software CLUSTAL X (Thompson *et al.*, 1997), and edited manually. Seven haplotypes were identified from the final alignment of all sampled sequences. The DNA sequences of haplotypes in the three species, respectively, and one outgroup were submitted to GenBank under accession numbers AY913811–AY913821 and DQ334671.

The phylogenetic relationships between the identified haplotypes were assessed by means of maximum parsimony and maximum likelihood analyses using PAUP 4.0b10 (Swofford, 2000), with one sample of *H. tibetana* as an outgroup. In all analyses, gaps were treated as missing and indels were coded as one variable character relative to the other corresponding sequences. Modeltest3.06 (Posada & Crandall, 1998) was used to select parameters and assumptions for maximum likelihood analysis. Both maximum parsimony and maximum likelihood heuristic search parameters were random sequence additions (1000 replicates) with tree bisection–reconnection (TBR) branch swapping, MULTREES, and COLLAPSE options switched on. Bootstrap values were estimated to assess the relative support for relationships between haplotypes (1000 replicates) (Felsenstein, 1985). In addition, the parsimonious network of the genetic relationships amongst all the haplotypes was constructed using the program Network (http://www.fluxus-engineering.com) (Bandelt, Forster & Öhl, 1999). It uses parsimony criteria to identify median vectors. The default settings were used for all other parameters. It runs using all haplotypes.
RESULTS

The aligned trnL-F region data set of the 75 individuals consisted of 837 positions. These individuals had seven distinct haplotypes (HA, HB, HC, HD, HE, HF, and HG; Table 1). Parsimony analysis of these haplotypes, when gaps were excluded, produced a single most parsimonious tree with 32 steps, a consistency index of 1.000, and a retention index of 1.000 (Fig. 1). The maximum likelihood tree topology under the selected model (GTR + G) is similar to the most parsimonious tree shown in Figure 1. These two phylogenetic analyses arranged the seven haplotypes into two distinct groups with strong support: the first contained HA and HB, and the second contained the remaining five haplotypes (Fig. 1). A similar relationship was demonstrated by the most parsimonious network (Fig. 2): HA and HB differed from the other haplotypes by containing one long 98-bp indel, one short 3-bp indel, and two 1-bp indels that the others lacked, and 14 substitutions (seven transitions and seven transversions). By contrast, HA and HB differed at only one singleton transition site (T → C). Four indels or mutations differentiated the remaining five haplotypes (HC, HD, HE, HF, and HG) that formed the second group.

Hippophae rhamnoides ssp. sinensis was found to possess HA and HB types, although HA was predominant in both populations (Table 2). The remaining five haplotypes (HC, HD, HE, HF, HG) were found in H. neurocarpa: all five in the first population (HD in half of the individuals sampled), but only HD in the second population. Four different haplotypes (HA, HB, HC, and HD) were detected in the putative hybrid species H. goniocarpa ssp. goniocarpa. In the first population, three haplotypes (HA, HB, HC) were present, and, in the second, only HA and HD were found. Clearly, therefore, the distribution of the haplotypes in the hybrids in the two populations is closely correlated with the haplotypes recovered locally from both putative parent species. For example, HA and HB were recovered at the first site from H. rhamnoides ssp. sinensis, and HC also appeared in individuals of H. neurocarpa collected from this site.

DISCUSSION

In this study, we have shown that H. goniocarpa is highly genetically diverse with different maternal haplotypes. Four haplotypes of this species were also recovered in both assumed parent species with sympatric distributions. These findings conflict with the hypothesis that this species originated through differentiation from marginal populations of H. rhamnoides (Lian et al., 1997, 2003). If this were the case,
the maternal haplotypes of *H. goniocarpa* should be relatively uniform and closely related to, but with a discernible divergence from, *H. rhamnoides*. However, our results unequivocally support the hypothesis that *H. goniocarpa* has hybrid origins, as suggested by previous RAPD and internal transcribed spacer (ITS) analyses (Bartish *et al.*, 2000, 2002; Sun *et al.*, 2003). The major difference between our results and those of the cited studies is that we have demonstrated that both *H. rhamnoides ssp. sinensis* and *H. neurocarpa*, rather than only the former (as suggested by Bartish *et al.*, 2002 and Sun *et al.*, 2003), contributed to the maternal establishment of this assumed hybrid species.

A single maternal origin with subsequent introgression could explain why one population of a hybrid species possesses haplotypes of both parents (Gross *et al.*, 2003). However, with this scenario, both populations would be expected to have the same dominant haplotype, with low frequencies of different haplotypes resulting from local introgression from the parent species. By contrast, in the first population, only six of the 12 sampled individuals contained haplotype HA of *H. rhamnoides ssp. sinensis*, whereas, in the second, haplotype HD of *H. neurocarpa* was more common than any other haplotype (7/11). A probable explanation for this haplotype distribution is that both populations had multiple maternal origins from both *H. rhamnoides ssp. sinensis* and *H. neurocarpa*. Furthermore, evidence of multiple maternal origins has been found in most diploid hybrid species examined to date, for example, *Pinus densata*, *Argyranthemum sundingii*, *Helianthus anomalus*, and *Helianthus deserticola* (Brochman *et al.*, 2000; Wang *et al.*, 2001; Schwarzbach & Rieseberg, 2002; Gross *et al.*, 2003). As the two populations studied are situated less than 20 km apart, it is also plausible that their haplotypes originated separately, and then mixed as a result of infraspecific gene flow. Another common feature of stable hybrid diploid species is that the range of some populations appears to have expanded during or after lineage sorting and initial speciation, leaving distinctive genetic signatures with the predominance of specific haplotypes in these populations (Arnold, 1997). However, we did not find

**Figure 1.** The single most parsimonious tree (length, 32; consistency index, 1.000; retention index, 1.000) based on the phylogenetic analyses of seven chloroplast DNA haplotypes recovered from the three species *Hippophae goniocarpa*, *H. rhamnoides ssp. sinensis*, and *H. neurocarpa*. Numbers above and below the branches are bootstrap values (percentages) for 1000 replicates based on maximum parsimony and maximum likelihood analyses. *Hippophae tibetana* was designated as the functional outgroup. Solid bars represent identified indels.

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**Figure 2.** Most parsimonious network of the seven chloroplast DNA haplotypes. Haplotypes are given within or beside each circle. The size of each circle roughly corresponds to the frequency of a given haplotype across all three species. The proportional representation of each species is indicated by the pie charts within each circle. Mutational steps are indicated by bars between haplotypes.
a clearly dominant haplotype in either population of *H. goniocarpa*, although the samples did not encompass all of the extant female individuals. All other previously documented diploid hybrid species are ecologically or spatially separate from their parents (Gallez & Gottlieb, 1982; Rieseberg, 1997; Brochmann et al., 2000; Wang et al., 2001). By contrast, all recorded populations of *H. goniocarpa* are sympatrically distributed with their two parents (Lian et al., 1995, 2003). Although it is reproductively isolated from *H. rhamnoides ssp. sinensis*, most of the flowering period of *H. goniocarpa* overlaps with that of *H. neurocarpa* (Lian et al., 1997). These lines of evidence suggest that *H. goniocarpa* is a newly developed lineage, at an early stage of speciation through reciprocal hybridization between local haplotypes of both parents (Arnold, 1997; Barton, 2001).

However, it is difficult to differentiate the offspring of a new lineage from hybrid swarms (Rieseberg, 1997). The persistence of hybrid swarms is probably dependent on a number of factors, including high fitness of hybrids compared with that of the parent species in intermediate habitats, continuous formation of hybrids, but with subsequent negative selection (Barton & Hewitt, 1985), and nonsexual maintenance of a few randomly produced hybrids (Arnold, 1997). In addition, Milne, Terzioğlu & Abbott (2003) found abundant F₁ hybrids of two *Rhododendron* species in Turkey, but no genetic introgression, which they attributed to postgermination failure of the F₁ hybrid derivatives. However, a number of observations support the hypothesis that *H. goniocarpa* represents a distinct lineage, differentiated from both parent species, rather than a hybrid swarm. First, the morphological identification and maternal molecular components are highly congruent in both parental species, *H. rhamnoides ssp. sinensis* and *H. neurocarpa*. This suggests that *H. goniocarpa* is not a bridge promoting genetic introgression from one species to the other, as in hybrid swarms of other trees (for example, Tsukaya, Fukuda & Yokoyama, 2003). Second, despite the identical *trnL-F* sequences of *H. goniocarpa* and the two parental species revealed here, Bartish et al. (2002) found that the single sampled individual of *H. goniocarpa* that clustered closely with *H. rhamnoides ssp. sinensis* had at least nine automatic mutations in a survey of eight fragments of the chloroplast genome. A further sequencing of more fragments may reveal more genetic differentiation. This finding suggests that the *H. rhamnoides ssp. sinensis* maternal haplotypes in *H. goniocarpa* may have generated the diverged mutations. Third, according to a previous investigation (Lian et al., 1997) and our unpublished data, more than 90% of the seeds of *H. goniocarpa* germinate successfully, whereas only 20% of the seeds of *H. neurocarpa* are viable. The higher germination rate of *H. goniocarpa* seeds suggests that the hybrid breakdown observed in hybrid swarms of other species does not occur in *H. goniocarpa*. In addition, the difference in germination rates between this species and one of the assumed parents suggests that their reproduction systems have differentiated. Similarly, the flowering periods of *H. rhamnoides ssp. sinensis* and *H. goniocarpa* are nonoverlapping. Such reproductive isolation should provide an effective reproductive barrier between this newly developed lineage and its sympatric parent species. It remains largely unknown whether hybridization triggered the higher germination rate of *H. goniocarpa* and whether this new lineage has a higher level of fitness than its sympatric parents. In herbs, the fitness and speciation of hybrid species can be tested by comparing artificially produced hybrids and their resultant progeny under controlled conditions (Schwarzbach & Rieseberg, 2002; Gross et al., 2003). It is difficult, however, to conduct similar experiments on most hybrid tree species because of the long time taken to reach sexual maturity. However, available data suggest that *H. neurocarpa* provides another model system for studying hybrid speciation in trees, because it is now at the early speciation stage. It is

| Table 2. Distribution of seven haplotypes (HA–HG) in two sites of three *Hippophae* species |
|----------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|
|                                  | HA     | HB     | HC     | HD     | HE     | HF     | HG     | Total  |
| **First site**                   |        |        |        |        |        |        |        |        |
| *H. rhamnoides ssp. sinensis*    | 6      | 1      | –      | –      | –      | –      | –      | 7      |
| *H. goniocarpa*                  | 6      | 3      | 3      | –      | –      | –      | –      | 12     |
| *H. neurocarpa*                  | –      | –      | 5      | 10     | 3      | 1      | 1      | 20     |
| **Second site**                  |        |        |        |        |        |        |        |        |
| *H. rhamnoides ssp. sinensis*    | 3      | 1      | –      | –      | –      | –      | –      | 4      |
| *H. goniocarpa*                  | 4      | –      | –      | 7      | –      | –      | –      | 11     |
| *H. neurocarpa*                  | –      | –      | –      | 21     | –      | –      | –      | 21     |

possible, therefore, to compare fitness, competition, and resource acquisition acquirements of this hybrid lineage and its sympatric parent species in the same habitats.

ACKNOWLEDGEMENTS

Support for this research was provided by the National Natural Science Foundation of China (30430560), the Chinese Academy of Sciences (Key Innovation Plan KSCX2-SW-106 and Special Fund for Outstanding PhD Dissertation), and FANEDD 200327.

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