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RESEARCH ARTICLE

Confirmation of natural hybrids between *Gentiana straminea* and *G. siphonantha* (Gentianaceae) based on molecular evidence

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Abstract A few individuals with intermediate morphology always appeared in the sympatric distributions of *Gentiana straminea* and *G. siphonantha*. These intermediate individuals were hypothesized to be the hybrids of two species after a careful evaluation of their morphological characteristics. To test this hypothesis, sequence comparison of the internal transcribed spacer (ITS) regions of the nuclear ribosomal and *trn*S (GCU)-*trn*G (UCC) intergenic spacer region of the chloroplast DNA from *Gentiana straminea*, *G. siphonantha* and the putative hybrids was performed. The results suggest that most intermediate individuals were the natural hybrids between *G. straminea* and *G. siphonantha*. In addition, we examined the sequence variation among the individuals of both parent species and analyzed the possibility leading to the incongruent identification in some individuals based on morphologic and molecular evidences, respectively. The intraspecific diversification of DNA fragments within both parent species and their high variability in hybrid swarms probably resulted from chloroplast genome recombination and incomplete lineage sorting during the early stages of speciation origin of the parent species.

Keywords *Gentiana straminea*, *G. siphonantha*, hybrids, internal transcribed spacer (ITS), *trn*S-G

1 Introduction

Hybridization, well known as an important creative force in evolution, can produce a considerable number of new lineages by exchanging genetic materials between closely related species, thus playing a key role in biological adaptation to novel or extreme habitats. There are two kinds of hybridization during speciation and formation of a new lineage: homoploid and polyploid. The former refers to those hybrid lineages without a change in chromosome number, whereas the latter involves the full duplication of a hybrid genome (allopolyploidy). In addition, autopolyploids also prevail in such a scenario that polyploids form within a morphologically stable species. However, allopolyploid is considered the most important mechanism to create new species and produce the current species diversity (Arnold, 1997; Arnold et al., 2003; Rieseberg and Carney, 1998). Though reproductive isolation and independent genetic lineage are one of basic characteristics of "species" (Rieseberg et al., 2006), hybridization is very common in natural species due to an incomplete reproductive isolation among closely related species (Stebbins, 1959; Grant, 1981). Research on speciation through hybridization, hybrids and hybrid swamps in sympatric distributions of different species have become hotspots in plant phylogeny and evolution over the past few years (Ellstrand et al., 1996; Rieseberg et al., 2003). It should be noted that a few morphological intermediates may form through convergent evolution or environmental selection, not by hybridization. Otherwise, these intermediate individuals with morphological traits of both parental species may originate from direct or introgressive hybridization (Rieseberg, 1995; Rieseberg et al., 1999; Schwarzbach et al., 2001; Lexer et al., 2003; Rauscher, 2002). Recently, a large number of research have confirmed natural hybrids or hybrid speciation by using molecular methods (Koch et al., 2003; Tsukaya et al., 2003). In addition, the increasing knowledge of gene flow and hybrid swamps based on these case studies provides more in-depth understanding of the isolation mechanism of speciation (Arnold, 1997)

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Gentiana, a large genus in the Gentianaceae mainly distributed in the alpine area of southwest China, contains 362 species from 15 sections (Ho and Liu, 2001). Up to now, only a few studies on interspecific hybridization from this genus have been reported (Pringle, 1966; Mansiona and Struwe, 2004). Both *G. straminea* Maxim. and *G. siphonantha* Maxim. ex Kusn. belong to sect. *Cruciata* Gaudin and are regarded as two closely related species due to their similar morphological traits. The former has a thyrose and lax cyme, a yellow-green and funnelform corolla with calyx-tubes 15–28 mm long; while in the latter, flowers are arranged in dense, terminal clusters or axillary whorls, with dark-blue corolla and campanulate, and calyx tubes 4–6 mm long (Ho and Liu, 2001). Furthermore, *G. straminea* mainly occurs in the alpine meadows, slopes and dry shrubs throughout Tibet, Qinghai, Gansu, and Sichuan with altitudes from 2000 to 4950 m, while *G. siphonantha* occurs in the degenerating grasslands, alpine meadows, shrubs and riversides in northwest Sichuan, Gansu, Qinghai and southwest Ningxia at altitudes between 1800 and 4500 m. Our field investigation revealed that a few individuals with intermediate morphology always appeared in sympatric distributions of these two species. Moreover, though the flowering time of *G. straminea* was earlier than *G. siphonantha*, their flowering period always overlapped (unpublished data). Further investigation of intermediate individuals suggested that even though their morphologies varied greatly, each owned a few important morphological diagnostic traits of both *G. straminea* and *G. siphonantha*. For example, their inflorescence was between thyrose and lax and densely clustered and the length of calyx tubes was 8–12 mm. In particular, their flower color was neither dark-blue nor yellow-green, but purple. There were no other relative species within sect. *Cruciata* distributed in the study area and the other gentian species had different flowering periods from these intermediate individuals. Furthermore, the intermediate individuals obviously had the morphological diagnostic traits of sect. *Cruciata*: conspicuous remains of fibrous leaf bases on the caudex, numerous adventitious roots combined into a contiguous or fused and twisted robust structure and well-developed vegetative rosette leaves (Ho and Liu, 2001). Based upon these lines of evidence, we hypothesized that the intermediate individuals were the natural hybrids of *G. straminea* and *G. siphonantha*. Meanwhile, another recent study in our lab revealed that sect. *Cruciata* experienced radiative speciation which might result in incomplete reproductive isolation between species and further promote occasional hybridization between two different species.

Comparison of genomic composition, differentiation and phylogenetic lineage between the hybrids and parental species has greatly increased documentations of hybridization events and confirmed allopolyploids and autopolyploids (Koch et al., 2003). Arnold (1997), Arnold et al. (2003), Rieseberg and Carney (1998), and Rieseberg et al. (2003) summarized the advances in this research field in two main aspects. First, maternal inheritance markers can be used to identify the putative parents and confirm the matriarchal origins of hybrids. Because of the maternal inheritance of the chloroplast genomes in most angiosperms (Mogensen, 1996; Ferris et al., 1997), molecular markers based on this genome variation were widely used as matriarchal markers (Rieseberg et al., 2003) to identify maternal resources of the putative hybrids from one or two species (Arnold, 1997). Most hybrid swarms or species were found to be mothered by two parental species (Rieseberg et al., 2003). In addition, because of the relatively short history of the divergent species that probably gave rise to the hybrids, most chlorotypes in the hybrids were found to be completely the same as those found in their parents (Arnold, 1997). Second, the other markers, i.e., ISSR, AFLP and nuclear genes or DNA fragments inherited from both parents, were often used to confirm origins of hybrids together with the maternal ones, and determine whether the putative hybrids own the same molecular footprints of the other parent (Rieseberg et al., 2003). Due to the development of universal primers and ease of amplification and sequencing, chloroplast segments and nuclear internal transcribed spacer (ITS) sequences have been broadly used for studying intraspecific variation and interspecific relationship in plants (Wendel et al., 1995; Wang et al., 2005; Liu et al., 2006; Wang et al., 2004; Yuan et al., 2004; Chen et al., 2005; Zhang et al., 2005). Therefore, these molecular markers are complemented with each other for illuminating the genetic composition of hybrids. In the present study, we utilized population genetics to analyze the variation of both nuclear ITS and chloroplast *trn*S-G sequences of *G. straminea*, *G. siphonantha* and the sympatric intermediates. We aimed to test: (1) whether the intermediate individuals were the hybrids; (2) if it were true, whether gene or DNA fragment recombination occurred due to the gene leakages of uniparental genetic inheritance traits during hybridization or gene flows between intermediate individuals and their parents.

2 Materials and methods

2.1 Population sampling

All materials used in this study were collected from Qumalai (N: 34° 06. 152′; E: 96° 09. 841′; Alt: 4400 m), Qinghai Province, China in 2002. A total of 55 individuals were sampled, including 15 *G. straminea*, 22 *G. siphonantha* and 18 morphological intermediates. Fresh leaves were dried and stored in silica gel immediately. While multiple collections were conducted at one site, plants were chosen with a distance of at least 10 m apart from

each other. Voucher specimens from each population were collected and deposited in the Herbarium of the Northwest Plateau Institute of Biology (HNWP), Chinese Academy of Sciences, China.

2.2 DNA extraction, amplification and sequencing

Total genomic DNA was isolated following the CTAB method of Doyle and Doyle (1987). While performing the polymerase chain reaction (PCR), we used primers "ITS 1a"and "ITS 4" for the ITS region (White et al., 1990), and primers "*trn*S" and "*trn*G" for *trn*S-G region of cpDNA (Hamilton, 1999). PCRs were performed using a 25 μL system containing 25 ng plant DNA, 50 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 2 μM of each primer and 0.75 U of Taq polymerase. The reaction program was: 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 54°C for 50 s, 72°C for 1 min and a final extension at 72°C for 7 min. PCR products were purified using a CASpure PCR purification kit following the protocol recommended by the manufacturer (Casarray, Shanghai, China). Sequencing primers used for both *trn*S-G and ITS were the same as the PCR primers mentioned above. Sequencing reactions were carried out in a Biometra thermocycler using a DYEnamic dye terminator cycle sequencing kit (Amersham) following the manufacturer's protocol. Sequencing products were analyzed on a MegaBACE 500 DNA analysis system. Both strands of DNA were sequenced using forward and reverse primers. Sequence boundaries of the ITS region were determined by comparing to published sequences of *G. frigida* (Gentianaceae) downloaded from the GenBank database, and boundaries of *trn*S-G were determined with reference to Hamilton (1999).

In order to avoid the misidentified collections, PCR mismatches and other experimental errors, we conducted more than two repeat sequencing for haplotypes that occurred only in one individual and/or one population containing two or more haplotypes. All repeated results confirmed each other. Furthermore, we cloned the ITS PCR products of intermediate individuals 961–5 into the vector pGEM-T (Promega, Madison, Wis), and ten clones were chosen and sequenced using the universal primers of "T7" and "SP6".

2.3 Data analysis

Sequences were aligned using the default parameters of CLUSTAL W (Thompson et al., 1994) and refined manually. All sequences were deposited in the GenBank database under accession numbers DQ497573–DQ497593. Both datasets of ITS and *trn*S-G were subjected to maximum-parsimony (MP) analyses. Phylogenetic analyses were performed using PAUP 4.010a (Swofford, 2000) with indels (gaps) coded as binary states, present or not present. Heuristic parsimony searches were conducted with 100 replicates, in combination with ACCTRAN character optimization, MULPARS + TBR branch-swapping and STEEPEST DESCENT options on. Moreover, bootstrap percentage was analyzed to assess the relative support of the branches. Bootstrap values (BS) were calculated from 1000 replicates using a heuristic search with simple addition of sequences and TBR branch swapping on.

3 Results

3.1 *trn*S-G

The *trn*S-G sequences of all *G. straminea* were 553 bp in length, defined as the St haplotype. However, there were three different sequences within *G. siphonantha*, defined as haplotypes Si, St-si and Si-st. Haplotype Si, the main haplotype of *G. siphonantha*, was found in 14 individuals. Its length was 525 bp and it showed only two indels' differences from St, one 25-bp indel from positions 41 to 65 and another 3-bp indel from positions 165 to 167. St-si was found in 4 individuals, with only one substitution difference from the St. Si-st was found also in 4 individuals. However, it was different from the other haplotypes with more variations (Table 1).

Among the 18 intermediate morphological individuals, they contained all 4 haplotypes of both *G. siphonantha* and *G. straminea*. 11 individuals belonged to St, 4 individuals to St-si, 1 to Si and 2 to Si-st.

3.2 Internal transcribed spacer (ITS)

Within the *G. siphonantha*, all ITS sequences analyzed were the same with no variation or additive sites. But for the *G. straminea*, there were three distinct genotypes (ITS-st-A, B, C) divided by 3 variations on positions 58, 188 and 549. Both ITS-st-A and ITS-st-B had no additive sites, while ITS-st-C owned three additive sites. For example, position 58 of both the ITS-st-A and ITS-st-B was either C or T, but for ITS-st-C it was additive site Y (T + C). Besides these variations, there were also 5 substitutions and one 3-bp indel difference between *G. siphonantha* and *G. straminea* (Table 2).

ITS regions of the morphological intermediates were considerably variable: 961-4 belonged to ITS-st-A, and 961–6 was exactly the same as ITS-st-C on all variable sites except for position 188, which was the same as ITS-st-B at this site (Table 2). The remaining individuals owned at least 3 additive sites among the 5 substitution sites, especially on positions 58, 188 and 549, which characterized the diagnostic traits of *G. straminea*. In addition, most intermediates had the 3-bp ITS indel found in *G. straminea* and the 961 individuals (1, 9, 12, 13) had different sequences (Table 2). Two different sequences from cloning one intermediate were verified and the additive sites of two putative parental species were separated.

Table 1 Variable sites of chloroplast *trn*S-G fragment, recovered haplotypes and the distribution of each haplotype in the morphological delimitations

haplotype	individual number	parents and hybrids	variable sites							
			41–45	46–48	49–54	55–65	165–167	278	444	452
St	964 (1–12)	*G. straminea*	**********				AAA	G	C	G
	961 (1, 3, 4, 6, 7, 9–12, 14, 16)	hybrid								
St-si	961 (2, 5, 8, 18)	hybrid	**********				AAA	T	C	G
	963 (2, 6, 10, 12)	*G. siphonantha*								
Si	961 (13)	hybrid	----------				---	T	A	T
	963(3–5, 7–9, 11, 14–19, 21)	*G. siphonantha*								
Si-st	961(15, 17)	hybrid	--------	TAT	-------	^^^^^^^^	---	T	A	T
	963(1, 13, 20, 22)	*G. siphonantha*								

**********: ATGGATATGTACACATAGATATTAT; ^^^^^^: ATAGATATTAT.

For example, at site 184, *G. siphonantha* and *G. straminea* were T and G, respectively (Table 2).

3.3 Phylogenetic analyses

Using *G. cruciata* as the outgroup, the aligned *trn*S-G dataset produced 6 informative sites when indels were coded as informative sites. Heuristic search yielded one parsimonious tree (Fig. 1) with 9 steps length, consistency index (CI) = 0.778 and retention index (RI) = 0.667. St-si from *G. siphonantha* and the morphological intermediates and the common St in *G. straminea* clustered into one clade, while Si and Si-st formed the other one. Obviously St-si of the *G. siphonantha* originated from the common St in *G. straminea*. Although Si-st and Si in *G. siphonantha* clustered into one clade in MP analysis, the bootstrap percentage was very low (67%). Moreover, 4 of 6 informative sites supported Si-st and Si to form a monophyletic clade, while the remaining 2 mutations also supported Si-st to have a paralleling relationship with the clade comprising St and St-si.

When the indels were included, there were 8 informative sites for ITS sequence dataset using *G. cruciata* as outgroup l. One most parsimonious tree (CI = 1.000, RI = 1.000, Steps = 22) was produced by heuristic search and it consisted of two clades (Fig. 2). One clade contained all the morphological intermediates and *G. straminea*, while the other comprised all individuals from *G. siphonantha*.

4 Discussion

4.1 Morphological intermediates are hybrids between *G. siphonantha* and *G. straminea*

The morphological intermediates are suggested to originate from direct hybridization or introgressive hybridization of two distinguished species (Grant, 1981). However,

Table 2 Variable sites of ITS, recovered sequences and the distribution of each sequence in the morphological delimitations

individual number	parents and hybrids	variable sites										
		42	58	113–115	116–118	119	123–125	184	188	226	228	549
963 (1–22)	*G. siphonantha*	C	C	GTG	---	C	CAA	T	G	T	T	C
964 (1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 14) ITS-st-A	*G. straminea*	A	C	GTG	TAT	G	CAA	G	G	A	G	C
964 (7, 13, 15) ITS-st-B	*G. straminea*	A	T	GTG	TAT	G	CAA	G	T	A	G	T
964 (5) ITS-st-C	*G. straminea*	A	Y	GTG	TAT	G	CAA	G	K	A	G	Y
961 (6)	hybrid	A	Y	GTG	TAT	G	CAA	G	T	A	G	Y
961 (4)	hybrid	A	C	GTG	TAT	G	CAA	G	G	A	G	C
961 (2, 5, 8, 15, 16, 17, 18)	hybrid	M	C	GTG	TAT	S	CAA	K	G	W	K	C
961-5-clone 1	hybrid	C	C	GTG	TAT	C	CAA	T	G	T	T	C
961-5-clone 2	hybrid	A	C	GTG	TAT	G	CAA	G	G	A	G	C
961 (1, 9, 13)-i	hybrid	M	C	---	TAT	S	CAA	K	G	W	K	C
961 (1, 9, 13)-ii	hybrid	M	C	GTG	TAT	S	---	K	K	W	K	C
961 (12)-i	hybrid	M	Y	GTG	TAT	S	---	K	K	W	K	Y
961 (12)-ii	hybrid	M	Y	---	TAT	S	CAA	K	K	W	K	Y
961 (3, 10, 11, 14)	hybrid	M	T	GTG	TAT	S	CAA	K	K	W	K	Y
961 (7)	hybrid	M	C	GTG	TAT	S	CAA	K	K	W	K	Y

M: A/C; Y: T/C; S: G/C; K: T/G, W: A/T. i and ii stand for different sequences at more than two times sequencing.

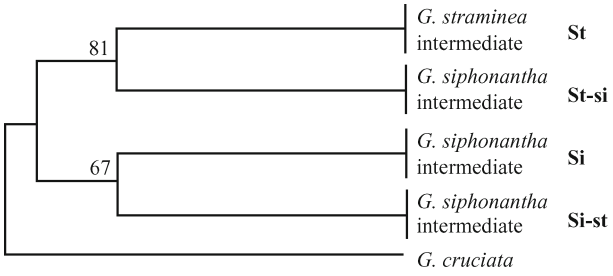

Fig. 1 Single MP tree based on *trn*S-G sequences. Numbers above branches are bootstrap values based on 1000 replicates.

based only on the morphological traits, it is insufficient to prove this and more evidence are needed, particularly from molecular research (Arnold, 1997). Our results based upon analyses of chloroplast *trn*S-G variation demonstrated that the intermediate individuals originated from hybridization between G. *siphonantha* and *G. straminea*, and either could be their maternal donors.

By comparison of ITS sequence variations (ITS1, the 5.8S gene, and ITS2) between *G. siphonantha* and *G. straminea*, there were 5 substitutions and a 3-bp indel in total (Table 1). Considering the ITS inheritance in both species, if the morphological intermediates originated from direct hybridization or introgressive hybridization, their ITS sequences in direct sequencing should have shown additive sites due to incomplete concert evolution of multiple copies from both parental species (Wendel et al., 1995). The result of direct sequencing shows that, except for 961–4 which was the same as genotype ITS-st-A, and 961–6, which combined variances of genotype ITS-st-B with ITS-st-C, 16 of a total

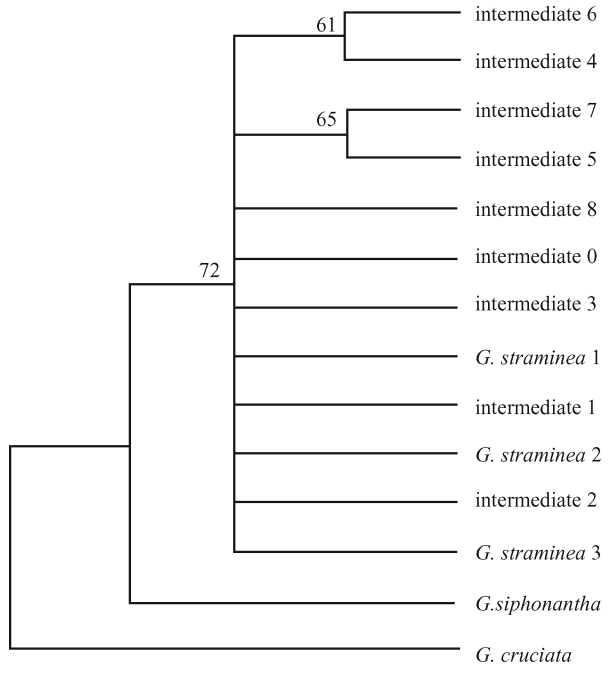

Fig. 2 Single MP tree based on ITS sequences. Numbers above branches are bootstrap values based on 1000 replicates

of 18 individuals exhibited traits of hybridization and owned at least 3 additive sites among the 5 total substitutions. In addition, our clone sequencing of one intermediate individual (961–5) also supported the hybridization origination of these intermediate individuals.

Combination analysis of the chloroplast gene and nuclear marker revealed that all individuals except two owned the molecular traits of two parental species. Taken together with the intermediate morphological traits, it indicated that these intermediate individuals were hybrids of *G. siphonantha* and *G. straminea*. Two individuals (961–4 and 961–6) did show intermediate morphological traits. However, we failed to find molecular footprints of the other parents' species. Their maternal species was probably *G. straminea*, thus no variation for the chloroplast *trn*S-G gene was detected. In addition, they might have experienced repeated backcrosses with *G. straminea* and therefore a concert evolution may result in ITS sequences to the maternal species *G. straminea*. Nevertheless, more independent evidences are needed to prove this hypothesis. Furthermore, unlike the multiple copies and concert evolution for the ITS region, single copy genes or DNA fragments are needed to identify whether the intermediate individuals are F1 or F2 hybrids and the congruence between morphological traits and genetic markers.

4.2 Variations of DNA sequences between the two parental species

Besides the intermediate individuals, some individuals of *G. straminea* also owned additive ITS sites on three positions (55, 188 and 549). Although such a scenario could occur for diverse reasons (for example, PCR mismatch and experimental errors), our results from multiple individuals and repeated experiments suggested the same finding. However, these individuals and the other ones of *G. straminea* show no variation in the *trn*S-G sequence. In addition, no additive site was found between the morphological individuals of *G. siphonantha*. These lines of evidence seem to reject the hypothesis that the additive sites within the morphological individuals of *G. straminea* were acquired from interspecific introgressions between two species through the hybrid individuals. Apparently, further evidence is also needed to explain this incongruence.

Although ITS shows no variation between all the individuals of *G. siphonantha*, *trn*S-G sequence dataset identified different haplotypes within this species: the most common one was Si; St-si was similar to the common St found in *G. straminea* with only one base difference on position 278; St-si was the same as Si on this site (Table 1). Similarly, the appearance of these different haplotypes in *G. siphonantha* may be due to the following three reasons. First, they may be remnants of ancient haplotypes due to incomplete lineage sorting at the early stage of speciation of *G. siphonantha* and *G. straminea*. Second, St-Si and Si-st may have experienced convergent evolution after the

divergence of the two species. Finally, hybridization may have induced the chloroplast recombination and the latter two haplotypes may firstly occur in the hybrids and then cross back with *G. siphonantha*. The backcross and the following evolution resulted in the retention of these two haplotypes within a few individuals that have morphological appearance similar to *G. siphonantha*, and their ITS sequences were also concerted into this species without leaving any hybrid signature. Further analyses, especially from the population genetics of the two species in allopatric distribution, are needed to confirm these hypotheses.

In conclusion, our results confirmed that the morphological intermediates are hybrids of two putative parental species. We also discovered different genotypes or haplotypes within the morphological parental species. The diverse variances between individuals of both species may result from either incomplete lineage sorting or chloroplast genome recombination due to hybridization. In addition, we found that morphological traits and molecular markers are not always in congruence with each other. This incongruence probably resulted from multiple backcrosses and/or introgressive hybridizations. These scenarios also existed in other species with hybrid histories (Tsukaya et al., 2003). Our research also suggests that it is insufficient to identify hybrids and recover hybridization events based exclusively on morphological and molecular evidence. In addition, these findings are also important for systematic studies. In reconstructing phylogenetic trees, researchers usually use one individual to represent one species. However, our results suggest that such a sampling method may distort the last phylogenic tree. Therefore, in such studies, it is better to sample more individuals from allopatric distributions.

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