

Hybridization and asymmetric introgression between *Cypripedium tibeticum* and *C. yunnanense* in Shangrila County, Yunnan Province, China

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Hybridization and introgression are thought to be important for speciation and adaptation in many plants. However, little is known about the hybridization and introgression among *Cypripedium* species. To investigate the evidence for hybridization and the pattern of introgression between *Cypripedium yunnanense* and *C. tibeticum* in Shangrila County, Yunnan Province, China, morphological characters and amplified fragment length polymorphism (AFLP) data for both the species and their putative hybrids were studied. Hand pollination was also performed to verify the crossability of the putative parents. Principal coordinate analysis based on morphological characters and the AFLP data suggested that the putative hybrids were true hybrids of these two *Cypripedium* species. Analysis with the NewHybrids software indicated that the putative hybrids were F1 generation individuals and backcrosses to *C. yunnanense*, but no F2 generation was found. Analysis with the Structure software demonstrated asymmetric introgression from *C. tibeticum* to *C. yunnanense*. We conclude that natural hybridization and introgression can occur between these two species and that in situ conservation of the parental species is required before fully assessing the evolutionary potential of hybrids.

Natural hybridization occurs widely among vascular plants and is a potentially creative evolutionary process (Tsukaya et al. 2003, Tagane et al. 2008). However, at the same time hybridization may erode the integrity of species and result in the local extinction of many populations and species (Allendorf et al. 2001, Kevin et al. 2008). In some cases, the extinction of the parental species can occur in only a few generations (Wolf et al. 2001). Whether hybridization remains limited to a narrow zone or leads to the extinction of the parental species is largely determined by the strength and type of reproductive barriers between the species involved. It is difficult to maintain a species threatened by hybridization in the wild if substantive reproductive barriers are lacking (Fredrickson and Hedrick 2006).

Impact of human activities on natural environments is common in most biomes, and recent increases in the number of species threatened by hybridization and introgression are largely the result of habitat disturbance (Fredrickson and Hedrick 2006, Mercure and Bruneau 2008). Hybridization and its consequences can be studied with experimental approaches (Lexer et al. 2003). The extent of hybridization varies widely among taxa, and the hybrids may predominantly comprise F1 individuals (Milne et al. 2003), backcrossed individuals (Lexer et al. 2005), or only advanced-generation individuals (Chung et al. 2005). Knowledge of the components of the hybrid

zone can help us to understand the processes and consequences of hybridization and introgression.

Most orchid species have specialized pollination systems that are dependent on a relatively narrow range of closely related animals, or even on a single pollinator species (Li et al. 2008). These specialized pollination systems contribute greatly to speciation and reproductive isolation in orchids. The genus *Cypripedium* contains 49 species, which are distributed in the temperate and alpine zones of the Northern Hemisphere (Zhang et al. 2008). *Cypripedium* species are pollinated by various wild bees (Bänziger et al. 2005), and most of the studied *Cypripedium* species attract pollinators through deception (Li et al. 2006). However, the extent of the specialization of particular *Cypripedium* species to particular bee species remains unclear, with a few important exceptions. For example, *C. calceolus* L. was visited by more than 50 insect species, although females of *Andrena haemorrhoa* Fabricius were the principal pollinators (Li et al. 2008). A large range of visitors or pollinators may sometimes lead to the breakdown of a specialized pollination system and the occurrence of hybridization, especially in disturbed habitats. Natural hybrids have been described between several species of *Cypripedium*, for example between *C. parviflorum* Salisb. and *C. candidum* Muhl. ex Willd and between *C. calceolus* and *C. macranthos* (Knyasev et al. 2000a, Bänziger et al. 2008).

Cypripedium yunnanense and *C. tibeticum* are relatively similar in their vegetative appearance, but *C. tibeticum* is characterized by a large, broad labellum and dark purple flowers, whereas *C. yunnanense* has a smaller, narrower labellum and a lilac flower. *Cypripedium yunnanense* is endemic to China, distributed in southwest and west Sichuan, southeast Xizang, and northwest Yunnan, whereas *C. tibeticum* has a wider distribution. In Shangrila County of Yunnan Province, *C. yunnanense* and *C. tibeticum* co-occur at some sites with overlap in flowering time. In the field, we found some individuals with a morphology intermediate between the two species, and speculated that they were hybrids. The labellum of the putative hybrid is purple, the color of its sepal, petal and labellum is darker than that of *C. yunnanense*. Based on the flower colour, they can be distinguished from the putative parents.

In this study, we present the results of an analysis of molecular and morphological data from these two species of slipper orchid (*C. tibeticum* and *C. yunnanense*) and their putative naturally occurring hybrids. Our main goals were to answer the following questions: 1) did the individuals with intermediate morphological traits originate by natural hybridization?, 2) what is the hybridization and introgression pattern between *C. tibeticum* and *C. yunnanense*?, 3) what are the factors affecting this hybridization and introgression pattern?

Material and methods

Study area

The study sites (Fig. 1) were located in Shangrila County of Yunnan Province, southwestern China. In Tianshengqiao (TSQ), approximately 200 individuals of *C. yunnanense* exist, but no *C. tibeticum* was found. However, about 30 individuals exhibited intermediate morphology between *C. yunnanense* and *C. tibeticum* in this site. Parts of the habitat have been destroyed by a highway, and the remainder is severely affected by grazing. In Napahai (NPH),

C. tibeticum and *C. yunnanense* coexist and there was large number of *C. tibeticum* individuals in this site, but only two clusters of *C. yunnanense* were found. In Wufengshan (WFS), large numbers of *C. yunnanense* exist, but no *C. tibeticum* was found. In Xianrendong (XRD), not more than one hundred individuals of *C. tibeticum* exist and no *C. yunnanense*. In Nixi (NX), the number of *C. yunnanense* was about sixty and no *C. tibeticum* exist. To check the possibility of hybridization, we used the sampling strategy for an amplified fragment length polymorphism (AFLP) analysis shown in Table 1. Leaf material was sampled in June 2009 and dried with silica gel for subsequent DNA extraction.

Morphological measures and pollination experiment

In the field, we identified the putative hybrids mainly based on flower color, and selected 15 putative hybrids in TSQ for analysis. To further distinguish the putative parental species and the hybrids, we measured flower size characters and did not reuse the identifying features such as flower color. Fifteen morphological characters (Table 2) were measured in the field during the flowering period in 2009. In TSQ, the morphological characters of *C. yunnanense* were not typical, so we sampled 14 *C. yunnanense* individuals from WFS for measurement. No *C. tibeticum* individuals were found in TSQ, so 15 *C. tibeticum* individuals from NPH were selected for measurement.

A hand pollination experiment was performed in the field in the flowering period of 2009 to detect the crossability of the putative parents. The anthers of 15 flowers from each species were emasculated, and pollen freshly collected from a flower of the desired population was applied to the stigma of the emasculated flower with forceps. Insects were excluded with fine cloth bags.

Fluorescent AFLP experiment

Fluorescent AFLP analysis with automated capillary electrophoresis was applied as follows. DNA was extracted

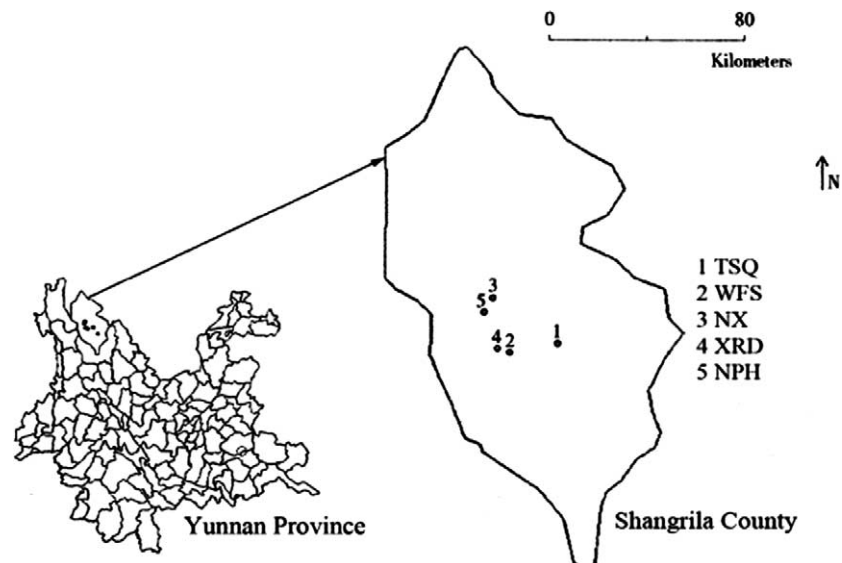


Figure 1. Map of the study sites in China.

Table 1. Study sites and sampling strategy for AFLP analysis.

Site (population)	Sampling species	Number of samples
Tianshengqiao (TSQ)	putative hybrid	15
Wufengshan (WFS)	<i>C. yunnanense</i>	16
Nixi (NX)	<i>C. yunnanense</i>	10
Xianrendong (XRD)	<i>C. tibeticum</i>	14
Napahai (NPH)	<i>C. tibeticum</i>	17

from each leaf sample (10–20 mg) using the AxyPrep Multisource Genomic DNA Miniprep Kit. The DNA restriction–ligation reactions and preselective and selective amplifications were performed as described by Vos et al. (1995). All the samples were amplified with two different primer combinations (E-AAC/M-CAG and E-ACC/M-CAA). The selective primers used were fluorescently labeled. Fragment separation and detection were performed by capillary electrophoresis using a genetic analysis system (CEQ 8000). The AFLP electropherograms were converted to binomial (presence/absence) data by setting a fluorescence threshold just above the background fluorescence. When ambiguous electropherograms were detected, the AFLP procedures were repeated to test for reproducibility.

Principal coordinates analysis

Principal coordinate analysis (PCoA) is a commonly used method for studying hybridization (Pellegrino et al. 2005, Travis et al. 2008). PCoA on the morphological data was performed using NTSYS-pc 2.1 (Rohlf 2000) and PCoA on the AFLP data of all samples from WFS, TSQ and NPH was performed using GenALEX 6.2 (Peakall and Smouse 2006) to define the interspecies relationships.

NewHybrids software analysis

Hybrid and backcross genotype posterior probabilities were estimated using the program NewHybrids 1.0 (Anderson and Thompson 2002). This method clusters individuals with no a priori knowledge of the parental allele frequencies, and has the advantage of specifically assuming a mixture of

parental and various hybrid classes in its probability model (Gagnaire et al. 2009). NewHybrids calculates the posterior probability that the sampled individual falls into one of a set of hybrid categories (parent 1, parent 2, F1, F2, backcross (BC) to parent 1, BC to parent 2, thus including the parents and two generations of offspring) by a Markov Chain Monte Carlo (MCMC) method in a Bayesian framework. The probabilities sum to 1, so if a given genotype has a probability of 0.8 for one category, the probability of the other five classes together is only 0.2 (Smulders et al. 2008). AFLP data were analyzed for all samples from NPH, WFS and TSQ populations, these individuals represent the putative parents and hybrids.

Admixture analysis

We used admixture analysis to determine whether introgression had occurred in the parental populations around TSQ. A model-based Bayesian procedure, implemented in Structure 2.2 (Pritchard et al. 2000) was used. Two subpopulations of *C. tibeticum* (NPH and XRD) and two subpopulations of *C. yunnanense* (WFS and NX) were sampled, and the AFLP data ($n = 57$ individuals) were analyzed. An a priori model assumption of $K = 2$ was used to account for the two parental species (Lorenzen et al. 2006, Zalapa et al. 2009). The results are based on simulations of 100 000 burn-in steps and 100 000 MCMC replicates under the admixture model. These runs were repeated five times to ensure the consistency of the results (Lorenzen and Siegmund 2004). The individuals were classified as pure species (*C. tibeticum* and *C. yunnanense*) or hybrids, based on a somewhat relaxed criterion of 90% posterior probability, which allowed the classification of some slightly introgressed individuals as pure species. Individuals with a probability between 40–60% were considered hybrids, and individuals with a probability between 11–39% or 61–89% were considered introgressants (Albaladejo and Aparicio 2007). AFLP data were analyzed for all samples from NPH, XRD, WFS and NX populations, these populations representing two putative parents were used to analyze whether or not introgression occurred.

Table 2. Morphological characters of *Cypripedium tibeticum* and *C. yunnanense*, and their putative hybrids.

	<i>C. yunnanense</i>		Putative hybrids		<i>C. tibeticum</i>	
	average (cm)	SE	average (cm)	SE	Average (cm)	SE
Sepal length	2.89	0.09	4.03	0.10	5.06	0.10
Sepal width	1.99	0.06	2.56	0.13	3.15	0.10
Synsepal length	2.61	0.08	3.82	0.19	4.61	0.10
Synsepal width	1.31	0.07	1.80	0.11	2.62	0.11
Petal length	3.24	0.08	4.40	0.16	5.88	0.14
Petal width	1.03	0.05	1.45	0.09	2.25	0.12
Labellum length	2.72	0.06	3.54	0.11	4.76	0.07
Labellum width	1.65	0.07	2.44	0.11	3.70	0.11
Labellum mouth width	0.76	0.03	1.00	0.06	1.47	0.04
Stigma length	0.63	0.02	0.80	0.02	1.03	0.02
Stigma width	0.42	0.01	0.54	0.02	0.72	0.02
Staminode length	1.01	0.06	1.09	0.04	1.80	0.03
Staminode width	0.61	0.02	0.86	0.04	1.22	0.03
Flower height	3.95	0.30	6.36	0.43	8.00	0.36
Flower width	3.88	0.27	4.84	0.53	8.21	0.34

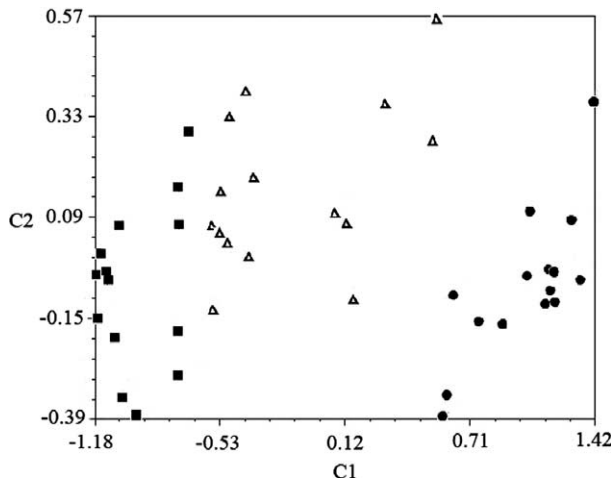


Figure 2. Scatterplot of a PCoA based on the morphological traits. Squares: *C. yunnanense*, triangles: putative hybrids, circles: *C. tibeticum*.

Results

Morphological traits of putative parents and hybrids

All the data for the 15 examined morphological traits showed that the putative hybrids were intermediate in size between the putative parents (Table 2). The PCoA of the morphological data showed that the putative hybrids fell between the two putative parents in the plot, which clearly illustrates the morphological relationships between *C. tibeticum*, *C. yunnanense* and their putative hybrids (Fig. 2). The variance in the first two principal coordinates accounted for 79.78% of the total variation. The first and second axes accounted for 71.31% and 8.48% of the variation, respectively. The intermediacy in the PCoA plot indicates that the individuals with intermediate morphological traits are of hybrid origin.

Crossability of the putative parents

One month after the pollination experiment, we investigated the fruiting rate and found that all individuals that were pollinated by hand set fruit. In September, we found that some capsules had been destroyed by grazing and insect pest. We collected 10 mature crossing fruits on *C. yunnanense* and 6 mature crossing fruits on *C. tibeticum*. The pollination experiment suggested that *C. tibeticum* and *C. yunnanense* were strongly cross-compatible, which means that it is possible for them to hybridize in the wild.

PCoA of the AFLP data

Two selective PCR primer pairs generated 230 loci in 72 individuals. Of the 230 loci, 220 were polymorphic, and only 10 were homomorphic. 31 *tibeticum*-specific bands were found, of which one band was present in 77.42% of the individuals in this species. Other bands varied between 3.23–64.52% frequency in *C. tibeticum* populations. In *C. yunnanense*, 18 *yunnanense*-specific bands were observed, of which two bands were present in 84.26% and 73.1% of

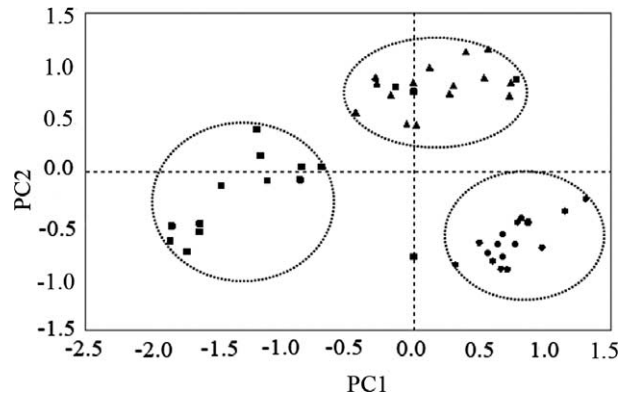


Figure 3. Scatterplot of a PCoA based on AFLP data for the putative parents and hybrids. Squares: *Cypripedium yunnanense*, triangles: putative hybrids, circles: *C. tibeticum*.

the samples of this species; other bands varied between 3.85–61.54% frequency. There were four bands specific to the putative hybrid with the frequency ranging from 6.67% to 13.33%. An ideal species-specific band is present in all individuals of one species and in none of the other. However, we did not detect any bands exclusive to all individuals of one species, perhaps because of the close relationship or introgression between the two *Cypripedium* species. But according to the 70% criterion described by Tagane et al. (2008), we detected one species-specific band in *C. tibeticum* populations and two species-specific bands in *C. yunnanense* populations.

The results of the PCoA of the AFLP data also showed that the putative hybrids were positioned between the two putative parents on the plot (Fig. 3). In this PCoA 37.13, 22.77 and 15.16% of the variation was loaded on the first, second, and third axes, respectively. The results of the PCoA based on the AFLP data were generally similar to the results of the morphological analysis. All the individuals from the NPH population clustered closely, but some of the *C. yunnanense* individuals clustered with the putative hybrids, which may indicate that the population of *C. yunnanense* in WFS is affected by hybridization or introgression.

Newhybrids analysis

Determination of the hybrid status of the individuals using the NewHybrids program revealed the occurrence of 13 F1 individuals with a posterior probability greater than 90%, 1 BC_{yun} individual with a posterior probability greater than 90%, and 1 BC_{yun} individual with a posterior probability of

Table 3. NewHybrids software categories of the putative parents and hybrids. tib = parental *Cypripedium tibeticum*, yun = parental *C. yunnanense*, F1 = tib × yun, F2 = F1 × F1, BC_{tib} = tib × F1, and BC_{yun} = yun × F1.

	No. of individuals					
	tib	F1	F2	BC _{tib}	BC _{yun}	yun
<i>C. tibeticum</i>	17					
Putative hybrids		13			2	
<i>C. yunnanense</i>		4			5	7

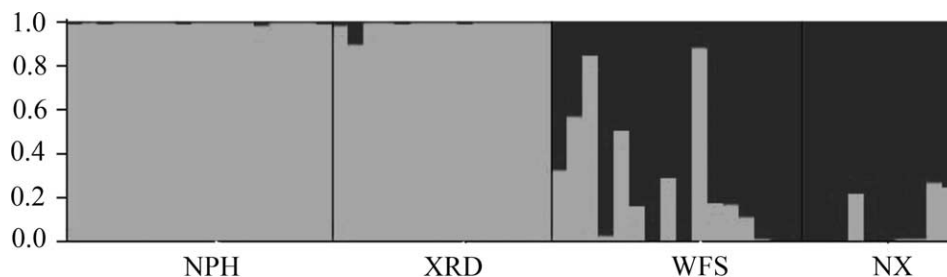


Figure 4. Bar plot of the results obtained with the Structure software using $K=2$. Each individual is represented as a vertical line partitioned into colored segments, the length of which is proportional to the individual's estimated K cluster membership coefficient. Gray bars: *Cypripedium tibeticum* samples, dark bars: *C. yunnanense* samples.

39.5% in TSQ (Table 3). In NPH, all the *C. tibeticum* individuals were identified as pure parents at the 90% posterior probability level. In the WFS population, four F1 individuals and five BC_{yun} individuals were detected.

Admixture analysis

Bayesian admixture analysis was performed using Structure 2.2, assuming $K=2$. Population assignments were made for each individual. Introgression was apparently unidirectional (Fig. 4) because it occurred at markedly different rates towards the two parental types. The introgression rates towards *C. tibeticum* were extremely low because all individuals of *C. tibeticum* were assigned to the parental type. However, for *C. yunnanense*, 43.8% (7/16) of individuals in the WFS population displayed a multi-locus genotype that could be considered hybrid or introgressant, and 30% (3/10) of individuals in the NX population could be considered introgressants.

Discussion

Evidence of hybridization

In the family Orchidaceae, many hybrids have been created in cross-pollination experiments, which means that their postzygotic isolation mechanisms are weak. Our pollination experiment between these two *Cypripedium* species also supported their crossability. Therefore, it is possible that hybridization has occurred by mispollination in the field. Commonly, the specialization of pollinators is the main reproductively isolating mechanism in co-occurring orchids. However, this mechanism is not absolutely effective. Recent molecular analyses have confirmed hybridization in several orchid genera, including *Cypripedium* (Worley et al. 2009), *Ophrys* (Stokl et al. 2008) and *Liparis* (Chung et al. 2005). *Cypripedium yunnanense* and *C. tibeticum* attract different pollinators, but field observations have also shown that some non-pollinating visitors enter the labellum of different co-occurring *Cypripedium* species (Bänziger et al. 2008), and these non-specialized visitors might contribute to hybridization.

Our morphological data indicate that the putative hybrids, identified by floral colour, were indeed intermediate between *C. tibeticum* and *C. yunnanense* in all measured characters (Table 2). The AFLP markers and morphological traits were highly consistent and supported the occurrence

of hybridization between *C. yunnanense* and *C. tibeticum* (Fig. 2, 3). The results of the NewHybrids analysis confirmed that most of the putative hybrids in TSQ were the F1 generation although backcrossing to *C. yunnanense* had occurred. Of the 15 individuals with intermediate morphological traits, most were assigned to the F1 category and no individual was assigned to the F2 category (Table 3). Therefore, the F2 individuals may be assumed to have low fitness or crosses between the F1 hybrids may produce non-viable seeds. Hybrid sterility is a common postzygotic isolating mechanism. A previous study has shown that crosses between F1 hybrid individuals can result in lower fruit production and lower seed viability in orchids (Scopece et al. 2008). Another study has shown that *C. ventricosum* is a species of hybrid origin and that most of the individuals are F1 hybrids; backcrosses occur very rarely and the formation of next-generation hybrids is hindered by marked pollen sterility in *C. ventricosum* (Knyasev et al. 2000a, 2000b). Although we detected introgression between *C. yunnanense* and *C. tibeticum*, most individuals were still morphologically distinct, indicating that some reproductive isolation mechanisms still exist between these species.

Asymmetric introgression

Asymmetric hybridization in plants is relatively common (Wu and Campbell 2005). Gemma et al. (2010) detected the introgression of *Pyrola grandiflora* single-nucleotide polymorphic (SNP) alleles into apparently morphologically 'pure' *P. minor* plants, but the opposite scenario was not observed. Similarly, we detected introgression from *C. tibeticum* into *C. yunnanense*, but the opposite introgression was very weak (Fig. 4), so that the introgression appear functionally unidirectional or asymmetric from *C. tibeticum* to *C. yunnanense*. Various causes of asymmetric hybridization have been suggested (Zha et al. 2010) and there are several possible reasons for the asymmetric introgression in our study. First, in Shangrila County, there is a substantial overlap in the flowering periods of *C. tibeticum* and *C. yunnanense*. The flowers of *C. tibeticum* are bigger than those of *C. yunnanense* and the pollinators of *C. tibeticum* are larger, and when the pollinator exits through the basal orifice of the labellum of *C. tibeticum*, it first passes the stigma and picks up a portion of a pollinium. If it enters the labellum of *C. yunnanense* by mistake, interspecies pollination is completed as the pollinator of *C. tibeticum* struggles in the labellum of *C. yunnanense*. On the contrary, it is easy for the

pollinators of *C. yunnanense* to creep through the labellum of *C. tibeticum* without touching the stigma. Second, the direction of introgression strongly depends on the relative frequency of the parental species in the studied stands, and the first-generation hybrids will also more likely mate with the more abundant species, leading to asymmetric introgression (Lepais et al. 2009). In our study, there was no *C. tibeticum* in TSQ or WFS. However, *C. yunnanense* was present at both sites, and was especially numerous in WFS. Therefore, it was easy for the F1 hybrids to mate with *C. yunnanense* first. Third, microhabitat selection or the relative competitive ability of hybrids between the F1 individuals and *C. tibeticum* can also lead to asymmetric introgression. There are several other possible explanations to asymmetric hybridization, such as differences in fruit abortion, in pollen germination rates, in floral morphology, or in pollinator behavior (Archibald et al. 2004), or the hybrids may cross more easily with *C. yunnanense* individuals. There is plenty of work on hybridization and introgression, but it is not common that hybrid populations are dominated by the F1 generation and that no F2 generation is detected (Table 3). This means that there is a certain degree of reproductive isolation between the two *Cypripedium* species studied here. However, further studies are required to determine the causes of this observed asymmetric introgression, and it will be helpful for conservation to determine the causes for why F1 hybrids dominate in mixed populations of these two species.

Implications for conservation and future studies

Human activity results in an artificial increase in hybridization rates through habitat disturbance (Mercure and Bruneau 2008). The extinction of species through hybridization is receiving increasing attention as a legitimate conservation problem (Ferdy and Austerlitz 2002, Rubidge and Taylor 2004, Hegde et al. 2006). The here described study area has been greatly disturbed in recent decades, so it is important to protect the local environments of these *Cypripedium* species.

In the present study no F2 generation was observed. This suggests that hybrid breakdown is a kind of reproductive isolation mechanism between these two species. Our results also show that backcross exists to a certain degree. However, further work on pollen and seed fertility is required to fully assess the evolutionary potential of hybrids. Comparative ecophysiological studies could help determine whether the hybrids threaten the survival of *C. yunnanense* populations or instead form a source of adaptive genes that could facilitate continued existence.

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References

- Albaladejo, R. G. and Aparicio, A. 2007. Population genetic structure and hybridization patterns in the mediterranean endemics *Phlomis lychnitis* and *P. crinita* (Lamiaceae). – *Ann. Bot.* 100: 735–746.
- Allendorf, F. W. et al. 2001. The problems with hybrids: setting conservation guidelines. – *Trends Ecol. Evol.* 16: 613–622.
- Anderson, E. C. and Thompson, E. A. 2002. A model-based method for identifying species hybrids using multilocus genetic data. – *Genetics* 160: 1217–1229.
- Archibald, J. K. et al. 2004. Hybridization and gene flow between a day- and night- flowering species of *Zaluzianskya* (Scrophulariaceae s.s., tribe *Manuleeae*). – *Am. J. Bot.* 91: 1333–1344.
- Bänziger, H. et al. 2005. Pollination of a slippery lady slipper orchid in southwest China: *Cypripedium guttatum* (Orchidaceae). – *Bot. J. Linn. Soc.* 148: 251–264.
- Bänziger, H. et al. 2008. Pollination of wild lady slipper orchids *Cypripedium yunnanense* and *C. flavum* (Orchidaceae) in southwest China: why are there no hybrids? – *Bot. J. Linn. Soc.* 156: 51–64.
- Chung, M. Y. et al. 2005. Patterns of hybridization and population genetic structure in the terrestrial orchids *Liparis kumokiri* and *Liparis makinoana* (Orchidaceae) in sympatric populations. – *Mol. Ecol.* 14: 4389–4402.
- Ferdy, J.-B. and Austerlitz, F. 2002. Extinction and introgression in a community of partially cross-fertile plant species. – *Am. Nat.* 160: 74–86.
- Fredrickson, R. J. and Hedrick, P. W. 2006. Dynamics of hybridization and introgression in red wolves and coyotes. – *Conserv. Biol.* 20: 1272–1283.
- Gagnaire, P. A. et al. 2009. Natural selection influences AFLP intraspecific genetic variability and introgression patterns in Atlantic eels. – *Mol. Ecol.* 18: 1678–1691.
- Gemma, E. B. et al. 2010. Unidirectional hybridization at a species' range boundary: implications for habitat tracking. – *Divers. Distrib.* 16: 1–9.
- Hegde, S. G. et al. 2006. The evolution of California's wild radish has resulted in the extinction of its progenitors. – *Evolution* 60: 1187–1197.
- Kevin, S. B. et al. 2008. Interspecific seed discounting and the fertility cost of hybridization in an endangered species. – *New Phytol.* 177: 276–284.
- Knyasev, M. S. et al. 2000a. Interspecific hybridization in northern Eurasian *Cypripedium*: morphometric and genetic evidence of the hybrid origin of *C. ventricosum*. – *Lindleyana* 15: 10–20.
- Knyasev, M. S. et al. 2000b. On the interspecific hybridization in Eurasian species of *Cypripedium* (Orchidaceae) and taxonomic status of *C. ventricosum*. – *Bot. Zhurn. St Petersburg.* 85: 94–102.
- Lepais, O. et al. 2009. Species relative abundance and direction of introgression in oaks. – *Mol. Ecol.* 18: 2228–2242.
- Lexer, C. et al. 2003. Experimental hybridization as a tool for studying selection in the wild. – *Ecology* 84: 1688–1699.
- Lexer, C. et al. 2005. Barrier to gene flow between two ecologically divergent *Populus* species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression. – *Mol. Ecol.* 14: 1045–1057.
- Li, P. et al. 2006. Deceptive pollination of the Lady's Slipper *Cypripedium tibeticum* (Orchidaceae). – *Plant Syst. Evol.* 262: 53–63.
- Li, P. et al. 2008. Pollination of *Cypripedium plectrochilum* (Orchidaceae) by *Lasioglossum* spp. (Halictidae): the roles of

- generalist attractants versus restrictive floral architecture. – *Plant Biol.* 10: 220–230.
- Lorenzen, E. D. and Siegmund, H. R. 2004. No suggestion of hybridization between the vulnerable black-faced impala (*Aepyceros melampus petersi*) and the common impala (*A. m. melampus*) in Etosha National Park, Namibia. – *Mol. Ecol.* 13: 3007–3019.
- Lorenzen, E. D. et al. 2006. Hybridization between subspecies of waterbuck (*Kobus ellipsiprymnus*) in zones of overlap with limited introgression. – *Mol. Ecol.* 15: 3787–3799.
- Mercure, M. and Bruneau, A. 2008. Hybridization between the escaped *Rosa rugosa* (Rosaceae) and native *R. blanda* in eastern North America. – *Am. J. Bot.* 95: 597–607.
- Milne, R. I. et al. 2003. A hybrid zone dominated by fertile F1s: maintenance of species barriers in *Rhododendron*. – *Mol. Ecol.* 12: 2719–2729.
- Peakall, R. and Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. – *Mol. Ecol. Notes* 6: 288–295.
- Pellegrino, G. et al. 2005. Confirmation of hybridization among sympatric insular populations of *Orchis mascula* and *O. provincialis*. – *Plant Syst. Evol.* 251: 131–142.
- Pritchard, J. K. et al. 2000. Inference of population structure using multilocus genotype data. – *Genetics* 155: 945–959.
- Rohlf, F. J. 2000. NTSYS-pc: numerical taxonomy and multivariate analysis system, ver. 2.1. – Exeter Software.
- Rubidge, E. M. and Taylor, E. B. 2004. Hybrid zone structure and the potential role of selection in hybridizing populations of native westslope cutthroat trout (*Oncorhynchus clarki lewisi*) and introduced rainbow trout (*O. mykiss*). – *Mol. Ecol.* 13: 3735–3749.
- Scopece, G. et al. 2008. Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. – *Am. Nat.* 171: 315–326.
- Smulders, M. et al. 2008. Natural hybridisation between *Populus nigra* L. and *P. xcanadensis* Moench. Hybrid offspring competes for niches along the Rhine river in the Netherlands. – *Tree Genet. Genomes* 4: 663–675.
- Stokl, J. et al. 2008. Scent variation and hybridization cause the displacement of a sexually deceptive orchid species. – *Am. J. Bot.* 95: 472–481.
- Tagane, S. et al. 2008. Hybridization and asymmetric introgression between *Rhododendron eriocarpum* and *R. indicum* on Yakushima Island, southwest Japan. – *J. Plant Res.* 121: 387–395.
- Travis, S. et al. 2008. Disentangling the role of hybridization in the evolution of the endangered Arizona cliffrose (*Purshia subintegra*; Rosaceae): a molecular and morphological analysis. – *Conserv. Genet.* 9: 1183–1194.
- Tsukaya, H. et al. 2003. Hybridization and introgression between *Callicarpa japonica* and *C. mollis* (Verbenaceae) in central Japan, as inferred from nuclear and chloroplast DNA sequences. – *Mol. Ecol.* 12: 3003–3011.
- Vos, P. et al. 1995. AFLP: a new technique for DNA fingerprinting. – *Nucl. Acids Res.* 23: 4407–4414.
- Wolf, D. E. et al. 2001. Predicting the risk of extinction through hybridization. – *Conserv. Biol.* 15: 1039–1053.
- Worley, A. C. et al. 2009. Hybridization and introgression between a rare and a common lady's slipper orchid, *Cypripedium candidum* and *C. parviflorum* (Orchidaceae). – *Botany* 87: 1054–1065.
- Wu, C. A. and Campbell, D. R. 2005. Cytoplasmic and nuclear markers reveal contrasting patterns of spatial genetic structure in a natural *Ipomopsis* hybrid zone. – *Mol. Ecol.* 14: 781–792.
- Zalapa, J. E. et al. 2009. Patterns of hybridization and introgression between invasive *Ulmus pumila* (Ulmaceae) and native *U. rubra*. – *Am. J. Bot.* 96: 1116–1128.
- Zha, H. G. et al. 2010. Asymmetric hybridization in *Rhododendron agastum*: a hybrid taxon comprising mainly F1s in Yunnan, China. – *Ann. Bot.* 105: 89–100.
- Zhang, S. et al. 2008. Variation of photosynthetic capacity with leaf age in an alpine orchid, *Cypripedium flavum*. – *Acta Physiol. Plant.* 30: 381–388.

Supplementary material (available as Appendix NJB 918 at <www.oikosoffice.lu.se/appendix>). Appendix A1.