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CITATION:

Nagasawa, Koki ...[et al]. Genetic purity of a rear-edge population of Carex podogyna Franch. et Sav. (Cyperaceae) maintained under interspecific hybridization. Genes & Genetic Systems 2022, 97(2): 93-99

ISSUE DATE:

2022-04-01

URL:

http://hdl.handle.net/2433/278397

Genes Genet. Syst. (2022) **97**, p. 93–99

Genetic purity of a rear-edge population of *Carex podogyna* Franch. et Sav. (Cyperaceae) maintained under interspecific hybridization

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(Received 14 September 2021, accepted 22 December 2021; J-STAGE Advance published date: 12 May 2022)

Interspecific hybridization is a critical issue in conservation biology because it may drive small populations to extinction through direct or indirect processes. In this study, to develop a conservation strategy for an endangered rear-edge population of *Carex podogyna* in Ashiu, Kyoto, Japan, we performed a molecular genetic analysis of the wild population and an *ex-situ* population established from wild seeds. Microsatellite genotypic data revealed a complete loss of genetic diversity in the wild population, suggesting that it has long been prone to genetic drift due to isolation as a small population. In contrast, microsatellite analysis of 13 *ex-situ* individuals detected multiple alleles that are not harbored in the wild *C. podogyna* population. Sequence analysis revealed that these individuals are likely natural hybrids between *C. podogyna* and a co-occurring species, *C. curvicollis*, although established hybrids have never been found in the natural habitat. Based on our observation of variegated leaves in hybrid individuals, we propose that hybrids have been excluded by natural selection and/or interspecific competition caused by insufficient productivity of photosynthesis, although other genetic and ecological factors may also be influential. Overall, this study indicates that natural mechanisms selectively removing the hybrids have maintained the genetic purity of this rear-edge population of *C. podogyna*, and also emphasizes the importance of genetic assessment in *ex-situ* conservation programs.

Key words: *Carex podogyna*, *Carex* ×*hosoii*, *ex-situ* conservation, interspecific hybridization, rear-edge population

Hybridization between species or lineages within species has attracted much attention from evolutionary biologists because it helps us to understand the process of speciation (Soltis and Soltis, 2009; Abbott et al., 2013). In contrast, the phenomenon poses serious problems for conservation biologists (Allendorf et al., 2001). Harmful effects of hybridization may lead to the decline or extinction of local populations through two main potential mechanisms (Rhymer and Simberloff, 1996; Todesco et al., 2016). First, if hybrids are sterile or have low fitness, population growth rates may decline below the replacement rates due to wasted reproductive effort; this process is termed demographic swamping (Wolf et al., 2001). Second, if introgression via fertile or semifertile hybrids occurs, one or both parental lineages may be replaced by hybrid progeny with superior fitness; this process is defined as genetic assimilation (Wolf et al., 2001). Because these two processes have an especially large impact on small populations that require an urgent conservation program (Price and Waser, 1979), it is important to grasp the whole picture of ongoing hybridization to develop an efficient conservation strategy.

Edited by Yoshihiko Tsumura
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DOI: https://doi.org/10.1266/ggs.21-00087

Rear-edge populations, defined as populations at the low latitudinal margin of species' distribution (Hampe and Petit, 2005), often deserve high conservation value because they have long-term stores of genetic variation (Provan and Maggs, 2012; Rehm et al., 2015; Evans et al., 2016). Evidence from phylogeographic surveys suggests that rear-edge populations often harbor high genetic diversity and/or uniqueness because they are likely to have persisted under the climate oscillations caused by recent glacial–interglacial cycles during the late Quaternary (Hewitt, 2000, 2004). In addition, they are also thought to acquire local adaptations to the warm and dry conditions with which they have to cope (Granda et al., 2018); these adaptations may be a key for species survival in ongoing global warming. Therefore, rear-edge populations are considered to be important for species conservation from long-term perspectives (Hampe and Petit, 2005).

Ashiu Forest Research Station (hereafter Ashiu), located in central Honshu, Japan (Fig. 1A), is a university field site that comprises a wide range of natural forest. The forest is renowned for its rich temperate flora, with approximately 800 seed plants within the area of ca. 4,200 ha (Yasuda and Nagamasu, 1995), which has attracted the attention of many biologists (Nakai, 1941; Kato and Okuyama, 2004; Sakaguchi et al., 2008; Watanabe, 2016). In particular, Ashiu has been recognized as a phytogeographically important area, because south-westernmost populations of several plant species on Honshu, such as *Hemerocallis middendorffii* Trautv. et C.A.Mey and *Viola brevistipulata* (Franch. et Sav.) W.Becker subsp. *brevistipulata* var. *brevistipulata*, are located in this forest (Science Museum Net: http://science-net.kahaku.go.jp/?ln=en). Recently, however, these rear-edge populations are at risk of extinction due to deer over-grazing on vegetation, which has become increasingly intense since the early 2000s (Sakaguchi et al., 2008). For these reasons, there is an increasing awareness that Ashiu is an important area with high conservation priority for several species.

Carex podogyna Franch. et Sav. (Cyperaceae) is a sedge plant endemic to Japan, and one of the species which have a rear-edge population in Ashiu (Fig. 1A). In Ashiu, only one small population occurs, in a riparian terrace (ca. 300 m^2), which is separated from the closest neighboring population (Tango population; Fig. 1A) by approximately 50 km. Over the last two decades, due to deer over-grazing and frequent river floods (Fig. 1B and 1C,

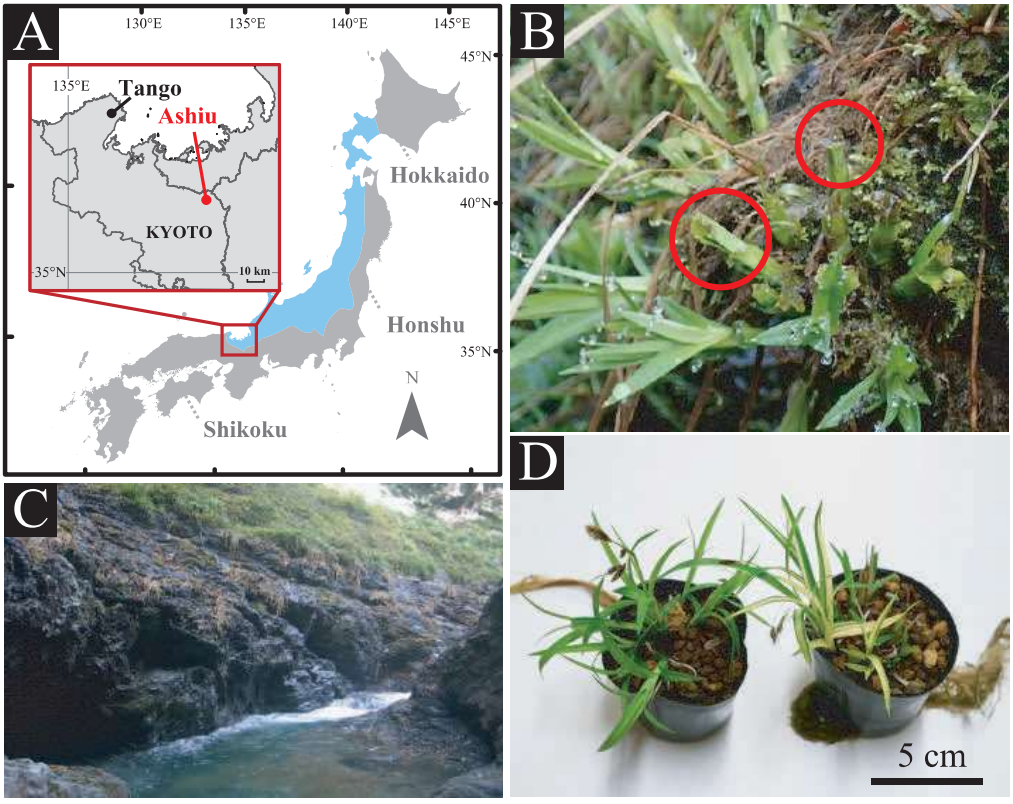

Fig. 1. (A) Map of the study site (Ashiu) with the blue area showing the whole distribution of *C. podogyna*. The black dot in the fine-scale map shows the Tango population. (B) Example of *C. podogyna* in Ashiu, with red circles showing feeding marks by sika deer. (C) View of the habitat of *C. podogyna* in Ashiu, which was heavily eroded by overflowing rivers in 2013. (D) Cultivated individuals of *C. podogyna* (left) and *C.* ×*hosoii* (right).

respectively), the number of *C. podogyna* individuals has rapidly decreased (authors' unpublished data). As a result, *C. podogyna* is designated as a critically endangered species (CR) in Kyoto Prefecture, in which Ashiu is located (Kyoto Prefecture Red list 2015: http://www.pref.kyoto.jp/kankyo/rdb/bio/flower.html). Besides, because this species is distributed preferentially in a Japan Sea-side region with heavy snowfall, the risk of loss of climatically suitable habitat will increase more and more due to the decrease of snowfall caused by global warming. Such a risk was predicted for a dwarf bamboo of the sect. *Sasa* (Tsuyama et al., 2008). For plants in fragile and changing environments, *ex-situ* conservation is considered to be an efficient conservation strategy in the short, medium, and even long term (Mounce et al., 2017); thus, an urgent *ex-situ* conservation program is desirable for the rear-edge population of *C. podogyna* in Ashiu. To establish an *ex-situ* population for *C. podogyna* in Ashiu, the genetic variation of the wild population needs to be evaluated and compared with that of other populations to assess its evolutionary potential. In addition, because *Carex* species have been reported to frequently form interspecific hybrids (Maguilla and Escudero, 2016), it is essential to verify whether hybridization has occurred and, if so, to assess the risk of demographic swamping or genetic assimilation.

The overall goal of this study was to genetically inform the conservation strategy to appropriately establish the *ex-situ* population of *C. podogyna* in Ashiu. To achieve this, we performed molecular genetic analysis on wild and *ex-situ* populations. Specifically, we first evaluated the current genetic diversity of the wild population using microsatellite markers and compared it with that of other populations from other regions. We then tested for the presence of interspecific hybridization and estimated its harmful effect on the survival of the wild population, because there are some closely related species in the vicinity of the wild *C. podogyna* population that can potentially hybridize with *C. podogyna*. Finally, we compared the genetic variation of the *ex-situ* population to that of the wild population in order to determine whether or not genetic variation in the *ex-situ* population fully reflects that in the parental population.

To investigate the genetic status of the *C. podogyna* population in Ashiu, leaf samples were collected from the remaining mature individuals (n = 12). In addition, to compare its genetic diversity with that of other populations, 15 and 16 samples were collected from Yamagata (Tohoku; 38°25′N, 140°27′E) and Gifu (Chubu; 36°26′N, 136°49′E) populations, respectively. Leaves were picked from individuals that were at least 2 meters apart, so that possible clones were not collected (Katsuyama, 2005). The collected samples were immediately dried using silica gel and stored in the dark at room temperature. Genomic DNA was extracted from the dried leaf tissue using a slightly modified cetyltrimethylammonium bromide method (Murray and Thompson, 1980) after removing polysaccharides using HEPES buffer (pH 8.0) (Setoguchi and Ohba, 1995).

For establishing the *ex-situ* population, seeds were collected from these 12 parental individuals of *C. podogyna* in May, 2018. The collected seeds were sown on watered petri dishes separately for each mother, and then 163 seedlings (10 to 18 per parent) were randomly chosen from those germinated. The established 163 seedlings were used for DNA extraction by the same method as described above.

To evaluate genetic variation of *C. podogyna* in Ashiu, microsatellite genotyping was performed using five expressed sequence tag–simple sequence repeat (EST–SSR) markers (Table 1) developed for *C. angustisquama* Franch. (Nagasawa et al., 2018), a related species of *C. podogyna* in sect. Podogynae Holm. Polymerase chain reaction (PCR) and fragment size determination followed Nagasawa et al. (2018). We note that PCR amplification of Cang_7187 failed for *C. shimidzensis* Franch. (Table 2), which was therefore analyzed by DNA sequencing as below. To evaluate the remaining genetic variation of the wild population in Ashiu, genetic diversity parameters in terms of the number of alleles (A) and observed and expected heterozygosity (H_O and H_E, respectively) were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012).

When alleles undetected in the parental population of *C. podogyna* were observed in seedlings, we considered them as alleles that originated from other *Carex* species. *Carex podogyna* is known to naturally hybridize with several related species (Katsuyama, 2005), among which *C. curvicollis* and *C. shimidzensis* are the only two species that have been reported to co-distribute in Ashiu (Yasuda and Nagamasu, 1995); therefore, we hypothesized that *C. podogyna* had hybridized with *C. curvicollis* or *C. shimidzensis*. To test this, DNA sequencing analysis was performed using three individuals of *C. podogyna*

Table 1. Genetic diversity statistics of the five EST-SSR markers used in this study for three populations

Marker	ASH (N=12)			YMG (N=16)			GIF (N=16)		
	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
Cang_4293	1	0.00	0.00	5	0.53	0.62	3	0.63	0.45
Cang_1881	1	0.00	0.00	3	0.20	0.66	2	0.38	0.47
Cang_7187	1	0.00	0.00	2	0.40	0.32	3	0.19	0.61
Cang_18857	1	0.00	0.00	3	0.33	0.52	4	0.50	0.58
Cang_3862	1	0.00	0.00	4	0.33	0.66	4	0.38	0.58
Average	1.00	0.00	0.00	3.40	0.36	0.56	3.20	0.41	0.54

Note: A = number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity.

Table 2. Fragment sizes amplified by five EST–SSR markers developed in Nagasawa et al. (2018)

Species	POP	Genotype	*N*	Cang_4293		Cang_1881		Cang_7187		Cang_18857		Cang_3862	
				Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Carex podogyna	Parental population	A	12	157	157	204	204	234	234	304	304	177	177
	Ex-situ population	A	150	157	157	204	204	234	234	304	304	177	177
		B	8	**149**	157	204	**222**	234	**236**	304	**309**	**169**	177
		C	3	**151**	157	204	**222**	234	**236**	304	**309**	**169**	177
		D	1	**149**	157	204	**222**	234	**236**	304	**311**	**169**	177
		E	1	**149**	157	204	**222**	234	**236**	304	**309**	**169**	177
Carex curvicollis			1	**149**	**149**	220	**222**	**236**	**236**	**309**	**311**	**169**	**169**
			1	147	147	218	218	**236**	**236**	**309**	**309**	**169**	**169**
Carex shimidzensis			1	161	161	**222**	228	–	–	**309**	**309**	175	175

Note: bold numerals mark alleles not detected in the parental *C. podogyna* population; –, no amplification.

from the parental population, two individuals of *C. curvicollis* collected from near the *C. podogyna* population, one individual of *C. shimidzensis* from Ashiu, and 13 putative hybrid individuals from the *ex-situ* population (Table 2). To determine whether individuals with unexpected genotypes were hybrids between *C. podogyna* and *C. curvicollis* or *C. shimidzensis*, the external transcribed spacer (ETS) region was amplified with primers ETS 1f and 18S-R (Starr et al., 2003). PCR was conducted following the protocol of Starr et al. (2003), and the PCR products were sequenced following Takahashi and Setoguchi (2018). The obtained DNA sequences were edited and assembled using BioEdit 7.2.5 (Hall, 1999). Heterozygous sites were called out when the peak height ratio of a secondary peak to a primary peak exceeded 50%. Sequence alignment was performed with ClustalW (Thompson et al., 1994) with default settings. All sequences were deposited in the DNA Data Bank of Japan (DDBJ), and accession numbers of the sequences used in this study are listed in Table 2.

The microsatellite genotyping rate was 100% for the Ashiu, Yamagata and Gifu populations, and then five, 17 and 16 scorable alleles were detected in each population, respectively (Table 1). While no identical genotypes were found within the Yamagata and Gifu populations, all individuals in Ashiu showed the same genotype with all six loci fixed to homozygotes (genotype A; Table 2). In agreement with this result, genetic diversity in terms of H_O and H_E showed a complete loss of genetic diversity in Ashiu, while the Yamagata and Gifu populations harbored substantial genetic variation (Table 1): mean values of H_O and H_E were 0.36 and 0.56 for Yamagata and 0.41 and 0.54 for Gifu, levels which were similar to those of closely related *Carex* species, such as *C. doenitzii* (H_O = 0.45, H_E = 0.59: Nagasawa et al., 2020) and *C. otayae* (H_O = 0.40, H_E = 0.36: Nagasawa et al., 2021). This result indicated that the lack of genetic variation detected in the Ashiu population is unlikely to be due to a lower informativeness of the *C. angustisquama*-derived genetic markers for *C. podogyna*. In addition, given the same sampling strategy for the three populations and the presence of seedlings in the wild Ashiu population (S. F., personal observation), we do not consider that possible clonal reproduction of *C. podogyna* would have resulted in genetic purity in the Ashiu population. Instead, we propose that the rear-edge population in Ashiu lost genetic diversity via strong bottlenecks and/or genetic drift due to isolation, as a small population, from other populations in central distribution areas. Further study is required to reveal whether the observed low genetic diversity in the Ashiu population is a phenomenon specific to rear-edge populations or is also found in other regions, by analyzing more population samples with genetic markers.

For the *ex-situ* population, the microsatellite genotyping rate was 99% and 12 scorable alleles were detected. Although genetic theory predicts that all 163 individuals should share the same genotype as inherited from parental individuals, four new genotypes (genotypes B–E; Table 2), in which the alleles were undetected in parental individuals of *C. podogyna*, were found in 13 individuals (Table 2). Because some of these alleles were not found in other populations of *C. podogyna* (authors' unpublished data from a range-wide phylogeographic study), and consistent with being heterozygotes comprising parental alleles of *C. podogyna* and *C. curvicollis* (Table 2), we hypothesized that these alleles arose via

Table 3. Segregated sites among *C. podogyna*, *C. curvicollis* and *C. shimidzensis*

Species	*N*	Variable site positions in ETS alignment from forward primer (bp)																			
		3	11	13	30	37	83	84	86	103	115	134	150	153	156	166	178	185	203	235	236
Carex podogyna	3	C	T	T	C	C	A	C	C	A	G	T	T	C	T	T	T	T	A	A	A
Carex curvicollis	2	T	–	–	–	–	–	G	–	T	–	C	C	–	–	–	C	C / T	G	G	C
Carex shimidzensis	1	–	C	C	T	G	T	G	C	T	C	–	C	T	G	C	C	C	–	G	T
Putative hybrids	13	**C / T**	–	–	–	–	–	**C / G**	–	**A / T**	–	**T / C**	**T / C**	–	–	–	**T / C**	**C / T**	**A / G**	**A / G**	**A / C**

Species	Variable site positions in ETS alignment from forward primer (bp)										GenBank accession	Voucher specimen accession
	240	247	261	266	317	323	326	370	398	437		
Carex podogyna	A	C	G	C	C	A	T	A	T	C	LC570900–LC570902	KYO 00023452
Carex curvicollis	T	C / T	A / G	G	–	G	C	T	C	A / C	LC570903, LC570904	KYO 00025853
Carex shimidzensis	–	T	–	–	T	–	–	T	–	–		
Putative hybrids	**A / T**	–	–	**C / G**	–	**A / G**	**C / T**	**A / T**	**T / C**	**A / C**	LC570905–LC570917	KYO 00025856

Note: voucher specimens are deposited at the herbarium of Kyoto University; –, the allele is the same as that of *C. podogyna*.

natural hybridization with *C. curvicollis*.

To test this hypothesis, 19 ETS sequences with lengths ranging from 431 bp to 576 bp were obtained. After removing sequence gaps, a sequence alignment of 431 bp long was obtained. The aligned sequences harbored 19 segregating sites between *C. podogyna* and *C. curvicollis*, and 20 segregating sites between *C. podogyna* and *C. shimidzensis* (Table 3). The putative hybrid individuals harbored 17 heterozygous sites, at which each allele matched one or other of the species-specific alleles of *C. podogyna* and *C. curvicollis* (Table 3). Note that the DNA sequences of *C. podogyna*, *C. curvicollis* and putative hybrids were identical within each species/population; that is, they were fixed to three distinct sequences. This pattern of DNA sequence variation indicates that the individuals with unexpected genotypes likely originated from natural hybridization between *C. podogyna* and *C. curvicollis*. Because *C. curvicollis* is a common riparian species in Japan, both species have been found in Ashiu to occur so close to each other that pollen flow can occur. Although a natural hybrid between these two species is known as *C.* ×*hosoii* T. Koyama, this hybrid has never been recorded in Ashiu (Yasuda and Nagamasu, 1995), and actually, we found no hybrid genotype in mature individuals that were sampled from the natural habitat in Ashiu (Table 2). These facts suggest that *C.* ×*hosoii* cannot survive in the natural environment for some reason. Our observation of these hybrid individuals during cultivation provided a possible explanation for the lethality of hybrids in natural environment: most of them formed variegated leaves in the early stage of growth (Fig. 1D). Since variegated leaves are known to inhibit plant growth via impaired photosynthesis (Funayama and Terashima, 1999; Funayama et al., 2001), *C.* ×*hosoii* may be selected against in the natural environment due to its forming variegated leaves, which should be less productive in terms of photosynthesis. Other genetic and ecological factors, including possible hybrid sterility (Koyama, 1956) and extrinsic ecological isolation (as microhabitat segregation is apparent between the two *Carex* species), may be equally influential, and thus need to be examined in future studies.

Our genotypic data revealed that the Ashiu population of *C. podogyna* has experienced a loss of genetic diversity, as well as a severe reduction of effective population size (Aguilar et al., 2008; Cho et al., 2020). Our results also showed that despite some cryptic natural hybridization, the rear-edge *C. podogyna* population still maintained genetic purity, indicating that the *C.* ×*hosoii* in Ashiu does not contribute to genetic introgression, possibly leading to genetic assimilation (Wolf et al., 2001). Nevertheless, the hybridization is likely to reduce the population growth rate due to the wasted reproductive effort, as detected in our seeding experiment in which 8% (13/163) of the seedlings were identified as hybrids. From the above results, together with the increasing risk of loss of suitable habitat due to ongoing global warming, we propose that *ex-situ* conservation is an effective way to safely preserve the remaining genetic diversity of *C. podogyna* in Ashiu as a potential genetic resource for species conservation, although there is little risk of genetic assimilation in the natural environment. Finally, our results emphasize the importance of genetic verification to determine whether an *ex-situ* population consists of genetically pure individuals, even when hybridization is not observed in the natural environment of the source population.

We greatly thank Mr. D. Takahashi for supporting the field survey. We are also grateful to Kyoto Botanical Gardens, especially to Ms. K. Funatsu and Ms. N. Ohki, for cultivation of the *ex-situ* population of *C. podogyna*. Research permission was provided by the Field Science Education and Research Center (Kyoto University). This research was financially supported by the TAKARA Harmonist Fund (the 33rd) to S. S., Pro Natura Foundation Japan (the 28th) for the Ashiu Biological Conservation Project, and the Nissei Fund to M. I.

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