Annals of Botany **98**: 1017–1024, 2006
doi:10.1093/aob/mcl179, available online at www.aob.oxfordjournals.org

Genetic Structure in Aquatic Bladderworts: Clonal Propagation and Hybrid Perpetuation

YOSHIAKI KAMEYAMA* and MASASHI OHARA

Section of Environmental Biology, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido, 060-0810, Japan

Received: 22 February 2006 Returned for revision: 4 April 2006 Accepted: 5 July 2006 Published electronically: 22 August 2006

• *Background and Aims* The free-floating aquatic bladderwort *Utricularia australis f. australis* is a sterile F_1 hybrid of *U. australis* f. *tenuicaulis* and *U. macrorhiza*. However, co-existence of the hybrids and parental species has not been observed. In the present study, the following questions are addressed. (*a*) Does the capacity of the two parental species to reproduce sexually contribute to higher genotypic diversity than that of sterile F_1 hybrid? (*b*) Are there any populations where two parental species and their hybrid co-exist? (*c*) If not, where and how do hybrids originate?
• *Methods* The presence and absence of *Utricularia* was thoroughly investigated in two regions in Japan. An amplified fragment length polymorphism (AFLP) analysis was conducted for 397 individuals collected from all populations (33 in total) where *Utricularia* was observed.
• *Key Results* The mean number of genotypes per population (*G*) and genotypic diversity (*D*) were extremely low irrespective of the capacity to reproduce sexually: *G* was 1·1–1·2 and *D* was 0·02–0·04. The hybrid rarely co-existed with either parental species, and the co-existence of two parental species was not observed. Several AFLP bands observed in the hybrid are absent in both parental genotypes, and parent and hybrid genotypes in the same region do not show greater genetic similarity than those in distant regions.
• *Conclusions* The capacity to reproduce sexually in parental species plays no role in increasing genotypic diversity within populations. The observed genotypes of the hybrid could not have originated from hybridization between the extant parental genotypes within the study regions. Considering the distribution ranges of three investigated taxa, it is clear that the hybrid originated in the past, and hybrid populations have been maintained exclusively by clonal propagation, which may be ensured by both hybrid vigor and long-distance dispersal of clonal offspring.

Key words: AFLP, aquatic bladderwort, free-floating aquatic plant, clonal propagation, genotypic diversity, hybrid perpetuation, natural hybridization, sexual and clonal reproduction, sterile F_1 hybrid, *Utricularia australis* f. *australis*, *Utricularia australis* f. *tenuicaulis*, *Utricularia macrorhiza*.

INTRODUCTION

Of the angiosperms with diverse flowering systems, most have the ability to reproduce clonally, i.e. to produce genetically identical progeny either by vegetative propagation or by forming seeds asexually (Abrahamson, 1980; Richards, 1997). Compared with sexually produced seeds, clonally reproduced offspring usually have a limited dispersal ability (Starfinger and Stöcklin, 1996) but a reduced mortality risk (Jelinski and Cheliak, 1992; Les and Philbrick, 1993), and the propagation of ramets within populations likely to prevent successful seedling recruitment (Abrahamson, 1980). Thus, clonal reproduction strongly influences genetic variation (McLellan *et al*., 1997), effective population size (Barrett *et al*., 1993) and metapopulation dynamics (Olivieri *et al*., 1995; Piquot *et al*., 1998).

Extensive reliance on clonal reproduction with rare to sporadic sexual reproduction is recognized as a striking convergence in aquatic angiosperms, a biological group that shares several attributes of adaptation to the aquatic condition (Les and Philbrick, 1993). For example, some forms of clonal offspring such as turions and shoot fragments are highly effective, economical means of numerical increase, resource acquisition and dispersal under aquatic conditions, reducing the selective value of sexual reproduction (Grace, 1993; Les and Philbrick, 1993). It may be reasonable, therefore, to consider whether aquatic plants have lower genetic variation or a smaller effective population size than terrestrial plants. However, the importance of sexual vs. clonal reproduction and their relationship to genetic variation have rarely been studied in aquatic plants, especially floating-leaved, submerged or free-floating taxa (Barrett *et al*., 1993).

Limited or absent sexual reproduction can result from both ecological and genetic factors (reviewed in Barrett *et al*., 1993; Eckert, 2002). Transient infertility, for example, may arise at the population level because of environmental suppression of seed maturation (Philbrick and Les, 1996) and dominance of a few genotypes in self-incompatible species (Charpentier *et al*., 2000). Permanent genetic sterility may be caused by meiotic irregularities associated with hybridization and changes in ploidy level (Les and Philbrick, 1993) and the accumulation of sterility genes (Klekowski, 1997; Eckert *et al*., 1999; Dorken and Eckert, 2001; Eckert, 2002). Population genetic consequences of reproductive traits, therefore, should be clarified in terms of the causes and levels of sterility and its relationship to clonal reproduction (Eckert *et al*., 2003).

Utricularia australis R. Br (Lentibulariaceae) is a carnivorous, free-floating aquatic bladderwort widely distributed in temperate and tropical regions, except North and

* For correspondence. E-mail kame@ees.hokudai.ac.jp

South America (Taylor, 1989). In spite of its widespread distribution, this taxon displays almost complete sterility (Taylor, 1989). A fertile group has been observed only in Japan, where *U. australis* is classified into two forma: sterile *U. australis* f. *australis* and fertile *U. australis* f. *tenuicaulis* (Komiya and Shibata, 1980; Taylor, 1989; Araki, 2000). While the phylogeny of *Utricularia* has been studied recently based on cpDNA sequences (Jobson *et al*., 2003; Müller and Borsch, 2005), the taxonomic and phylogenetic relationships between *U. australis* and its two forma distributed in Japan, *U. australis* f. *australis* and *U. australis* f. *tenuicaulis*, remain unclear. However, it has been demonstrated that (*a*) *U. australis* f. *australis* is a diploid hybrid that originated by asymmetric hybridization between *U. australis* f. *tenuicaulis* (usually as the female parent) and its close relative *U. macrorhiza* (usually as the male parent); (*b*) the hybrid *U. australis* f. *australis* is almost completely sterile, in which only 0·6 % of the pollen grains can germinate and almost no seeds can be produced by intra- and interspecies crossing; and (*c*) the absence of post-F_1 generation in natural hybrid populations is confirmed by the additive patterns of amplified fragment length polymorphism (AFLP) bands (Kameyama *et al*., 2005). It should be noted that AFLP bands common to the hybrid were invariably derived from either parental species (Kameyama *et al*., 2005), and there are no other parental candidates based on the morphological classification (Taylor, 1989). Interestingly, co-existence of the hybrids and parental species has not been observed (Kameyama *et al*., 2005).

In the present study, several aspects were addressed: first, whether the capacity of the two parental species, *U. australis* f. *tenuicaulis* and *U. macrorhiza*, to reproduce sexually contributes to higher genotypic diversity compared with the sterile F_1 hybrid, *U. australis* f. *australis*. Propagation of the sterile F_1 hybrids depends solely on clonal offspring in the form of many turions and shoot fragments, while the two parental species use both these forms of clonal reproduction as well as sexual reproduction via seed. Thus, it is reasonable to expect that populations with the capacity for sexual reproduction (parental species) will contain more genotypic diversity than those without (sterile hybrid). Secondly, confirmation is needed of the lack of co-existence of three aquatic bladderworts by exhaustive observation and sampling, because, in a previous study (Kameyama *et al*., 2005), sample size was limited (only 2–6 samples from each population). Thirdly, an investigation was conducted to address where and how hybrids originate based on the distribution patterns of three aquatic bladderworts and their genetic relationship. The possible scenarios are (*a*) ongoing hybridization in some populations (three aquatic bladderworts that co-exist in some populations have compatible genotypes); (*b*) ongoing but rare occurrence of hybridization combined with long-distance dispersal of offspring (three aquatic bladderworts that are distributed in distant populations are genetically compatible); and (*c*) past hybridization and long-term clonal perpetuation of the hybrids (extant genotypes of three aquatic bladderworts are mutually incompatible).

MATERIALS AND METHODS

Study site and sample collection

The study was performed in the Tomakomai and Tsugaru regions of northern Japan, which are approx. 250 km apart (Fig. 1). In the Tomakomai region, the presence and absence of three *Utricularia* had been surveyed over 10 years by Masahiro Toyama, Hokkaido Prefecture, Japan (Y. Kameyama, Hokkaido University, Japan, pers. comm.). In the Tsugaru region, the distributions of three *Utricularia* had been thoroughly surveyed in 1999 and 2000, and two reports were available (Toyama and Katsumata, 1999; Uematsu *et al*., 2000). A further field survey was conducted in almost all lakes and ponds in both regions. Plant material was collected from all populations where *Utricularia* was observed: 15 populations in the Tomakomai region and 18 populations in the Tsugaru region (Fig. 1, Table 1). An inflatable boat was used to gain access to the whole surface of each water body. Sample sizes ranged from three to 43 (mean = 12; 397 total), with large populations sampled most intensively (Table 1). Stems of all samples were cleaned with deionized water and frozen at −80 °C for later DNA extraction.

AFLP analysis

Total genomic DNA was isolated from about 50 mg of tissue from each of the 397 frozen stems by the CTAB (cetyl trimethyl ammonium bromide) miniprep procedure (Stewart and Via, 1993).

An AFLP analysis was performed according to the method of Vos *et al*. (1995), with some modifications. Genomic DNA (approx. 0·1 µg per sample) was digested with the restriction enzymes *Eco*RI and *Mse*I at 37 °C for 1·5 h. Double-stranded adaptors were ligated to the ends of the digested DNA fragments at 20 °C, overnight. Preselective amplifications were performed using a primer pair with one additional nucleotide, *Mse*I-C/*Eco*RI-A. Selective amplifications were conducted with three fluorescence-labelled primer combinations, *Mse*I-CC and *Eco*RI-ACT (FAM), *Mse*I-CA and *Eco*RI-ACG (VIC), and *Mse*I-CC and *Eco*RI-AGC (NED). The AFLP Amplification Core Mix (Applied Biosystems, Foster City, CA, USA) and the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) were used for both amplifications. AFLP fragments were detected with an ABI Prism 3100 automated sequencer (Applied Biosystems) and GENESCAN analysis software (Applied Biosystems).

Statistical analysis

The number of genotypes observed (G) and the genotypic diversity (D) within populations were calculated for each taxon. Genotypic diversity was estimated as $D = 1 - \Sigma[n_i(n_i - 1)/n(n - 1)]$, where n is the number of ramets sampled and n_i is the number of ramets with genotype i. The value of D ranges from 0, when all ramets sampled have the same genotype, to 1, when each ramet sampled has a different genotype (Pielou, 1969). Rank abundances of genotypes were calculated for each taxon on the basis of

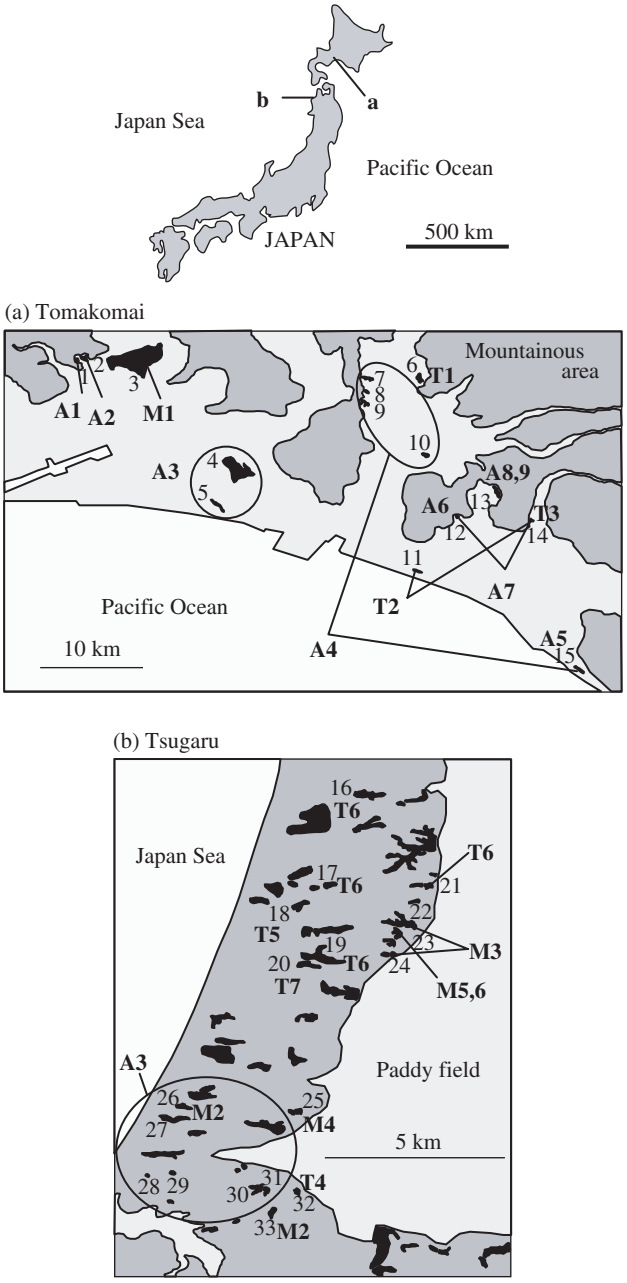

FIG. 1. Population identities (1–33) and observed AFLP phenotypes in the sterile F_1 hybrid *Utricularia australis* f. *australis* (A) and the two parental species, *U. australis* f. *tenuicaulis* (T) and *U. macrorhiza* (M). The two regions, (a) Tomakomai and (b) Tsugaru, are about 250 km apart.

the number of populations in which the genotype was observed.

Principal co-ordinate analysis (PCoA) was conducted to reveal the relationship between genotypes. Genetic similarity was calculated as $S_{ij} = 2N_{ij}/(N_i + N_j)$, where N_{ij} is the number of shared bands between genotypes i and j, and N_i and N_j are the number of bands found in genotypes i and j, respectively (Dice, 1945). Genetic similarity was transformed to dissimilarity using the formula $D_{ij} = 1 - S_{ij}$, and then subjected to PCoA. All calculations were performed with R Package 4·0 software (Casgrain and Legendre, 1999).

Utricularia australis f. *australis* is a sterile F_1 hybrid between *U. australis* f. *tenuicaulis* and *U. macrorhiza*, in which no post-F_1 generations are produced (Kameyama *et al*., 2005). Thus, genotypic similarity between the hybrid and each of the two parental species can be estimated selecting AFLP bands polymorphic in both target taxa, but completely absent in the other parental species. Twenty-two bands were selected for the comparison of the hybrid with *U. australis* f. *tenuicaulis*, and 17 bands for the comparison of the hybrid with *U. macrorhiza*. Genetic similarity (S_{ij}) (Dice, 1945) was calculated as explained above, and an UPGMA (unweighted pair group method with arithmetic mean) cluster analysis was conducted with R Package 4·0 software (Casgrain and Legendre, 1999). Bootstrap values for each branch were determined from 1000 replicates using WinBoot software (Yap and Nelson, 1996). Neighbour-joining trees (Saitou and Nei, 1987) constructed with T-Rex online software (Makarenkov, 2005) confirmed the topologies found with UPGMA except for small differences in the branch length (data not shown).

RESULTS

AFLP polymorphism

In total, 262 bands were observed in the AFLP analysis, with 209 polymorphic bands. Many species-specific bands were observed in both parental species: 51 for *U. australis* f. *tenuicaulis* and 40 for *U. macrorhiza*. Genotypes of the sterile F_1 hybrid, *U. australis* f. *australis* had most of these species-specific bands: 43–46 of the 51 (84–90 %, mean: 88·5 %) *U. australis* f. *tenuicaulis*-specific bands, and 33–34 of the 40 (80–85 %, mean: 83·1 %) *U. macrorhiza*-specific bands. AFLP bands common to the hybrid were invariably derived from either parental species. However, several bands observed in the hybrid were absent in both parental species (7–10 for each genotype, 12 total). The number of polymorphic bands observed within each taxon was 64, 82 and 45 for *U. australis* f. *tenuicaulis*, *U. australis* f. *australi* and *U. macrorhiza*, respectively.

Genotypic diversity

Seven genotypes were observed in ten populations of *U. australis* f. *tenuicaulis* (T1–T7), nine in 18 populations of sterile F_1 hybrid *U. australis* f. *australis* (A1–A9) and six in seven populations of *U. macrorhiza* (M1–M6) (Table 1). Most populations consisted of a single genotype regardless of the potential fertility of the three taxa: the mean number of genotypes within populations was 1·1 (s.e. = 0·10), 1·2 (s.e. = 0·09) and 1·1 (s.e. = 0·14) for *U. australis* f. *tenuicaulis*, *U. australis* f. *australis* and *U. macrorhiza*, respectively. Mean genotypic diversity (D), including monoclonal populations, was 0·05 (s.e. = 0·05), 0·04 (s.e. = 0·03) and 0·02 (s.e. = 0·02) for *U. australis*

TABLE 1. *Sampling locations, population identities and observed AFLP genotypes in sterile F_1 hybrid* Utricularia australis *f.* australis *(A) and two parental species,* U. australis *f.* tenuicaulis *(T) and* U. macrorhiza *(M)*

Region	Population ID	Common name*	Locality	No. of samples in total	ID of AFLP genotype (no. of samples)		
					U. a. f. *tenuicaulis*	*U. a.* f. *australis*	*U. macrorhiza*
Tomakomai	1	(Higashi-interchange)	42°42′N, 141°40′E	4	-	A1 (4)	-
	2	(Higashi-interchange-east)	42°42′N, 141°40′E	8	-	A2 (8)	-
	3	Lake Utonai	42°42′N, 141°43′E	15	-	-	M1 (15)
	4	Benntennuma Pond	42°38′N, 141°45′E	43	-	A3 (43)	-
	5	(Kaigannuma Pond)	42°37′N, 141°45′E	12	-	A3 (12)	-
	6	Matsunonuma Pond	42°41′N, 141°51′E	12	T1 (12)	-	-
	7	Tsurunonuma Pond	42°41′N, 141°50′E	12	-	A4 (12)	-
	8	Okuinuma Pond	42°41′N, 141°50′E	12	-	A4 (12)	-
	9	Tairakinuma Pond	42°40′N, 141°50′E	11	-	A4 (11)	-
	10	Ryuzinnuma Pond	42°39′N, 141°52′E	12	-	A4 (12)	-
	11	(Hamaatsuma)	42°35′N, 141°51′E	16	T2 (16)	-	-
	12	(Ikeda Pond)	42°37′N, 141°53′E	11	-	A6 (1), A7 (10)	-
	13	Naganuma Pond	42°38′N, 141°54′E	12	-	A8 (9), A9 (3)	-
	14	Irishikabetsu Pond	42°37′N, 141°55′E	22	T2 (9), T3 (4)	A7 (9)	-
	15	(Shiomi Pond)	42°33′N, 141°56′E	12	-	A4 (11), A5 (1)	-
Tsugaru	16	Kamisawabenuma Pond	40°53′N, 140°19′E	12	T6 (12)	-	-
	17	(Tsugaru D)	40°51′N, 140°18′E	8	T6 (8)	-	-
	18	(Tsugaru E)	40°51′N, 140°18′E	12	T5 (12)	-	-
	19	Otsutsumi Pond	40°50′N, 140°18′E	12	T6 (12)	-	-
	20	(Otsutsumi-south Pond)	40°50′N, 140°18′E	12	T7 (12)	-	-
	21	(Tsugaru 6)	40°51′N, 140°20′E	3	T6 (3)	-	-
	22	(Tsugaru 7)	40°51′N, 140°19′E	12	-	-	M3 (12)
	23	(Tsugaru 8)	40°51′N, 140°19′E	12	-	-	M5 (11), M6 (1)
	24	(Tsugaru H)	40°50′N, 140°19′E	12	-	-	M3 (12)
	25	Fuzinosawa Pond	40°48′N, 140°18′E	12	-	-	M4 (12)
	26	(Tsugaru 16)	40°48′N, 140°16′E	12	-	A3 (3)	M2 (9)
	27	Sakunuma Pond	40°48′N, 140°16′E	12	-	A3 (12)	-
	28	(Tsugaru 23)	40°47′N, 140°15′E	12	-	A3 (12)	-
	29	(Tsugaru 22)	40°47′N, 140°16′E	12	-	A3 (12)	-
	30	(Tsugaru 24)	40°47′N, 140°17′E	8	-	A3 (8)	-
	31	(Tsugaru 25)	40°47′N, 140°17′E	5	-	A3 (5)	-
	32	(Tsugaru 26)	40°47′N, 140°18′E	12	T4 (12)	-	-
	33	(Tsugaru 29)	40°47′N, 140°17′E	3	-	-	M2 (3)

* Names in parentheses are arbitrarily given by the authors.

f. *tenuicaulis*, *U. australis* f. *australis* and *U. macrorhiza*, respectively.

Genetic structure

The distributions of the three aquatic bladderworts are mutually exclusive (Table 1). Only two co-existing populations of the F_1 hybrid, *U. australis* f. *australis*, and either parental species were observed: population 14 (with *U. australis* f. *tenuicaulis*) and population 26 (with *U. macrorhiza*) (Table 1). It is noteworthy that the two parental species never co-existed (Table 1).

An identical genotype was occasionally observed in multiple populations, even populations distant from one another. In the extreme case, for example, a single genotype (A3) was observed in about half (8/18 = 44 %) of the hybrid populations across the two regions (Table 1, Fig. 1). The number of genotypes observed in multiple populations, however, was relatively small: two of seven genotypes for *U. australis* f. *tenuicaulis*, three of nine genotypes for *U. australis* f. *australis* and two of six genotypes for *U. macrorhiza*, with many other genotypes found in only a single population (Fig. 2).

Genetic similarity

Twenty-two genotypes were analysed by PCoA using 209 polymorphic AFLP bands (Fig. 3). Along the first axis, genotypes of the sterile F_1 hybrid *U. australis* f. *australis* (A) were distributed midway between those of the two parental species, *U. australis* f. *tenuicaulis* (T) and *U. macrorhiza* (M), consistent with the results of Kameyama *et al*. (2005). Within each taxon, severa genetic groups were found along the second and third axes: T1–T5 and T6–T7 from *U. australis* f. *tenuicaulis*; A1–A2, A3–A5 and A6–A9 from *U. australis* f. *australis*; and M1–M2, M3–M4 and M5–M6 from *U. macrorhiza* (Fig. 3). Genetic similarities within each taxon, however, did not correspond to regions. For example, in *U. australis* f. *tenuicaulis*, the genotype group T4–T5 in the Tsugaru region was very different from T6–T7 in the same region (Fig. 3).

Although only a small number of AFLP bands were available with which to construct the UPGMA dendrogram (Fig. 4) (see Materials and Methods), the genetic groups observed were highly consistent with the results of PCoA (Fig. 3). One genetic group of the hybrid (A1–A2) was

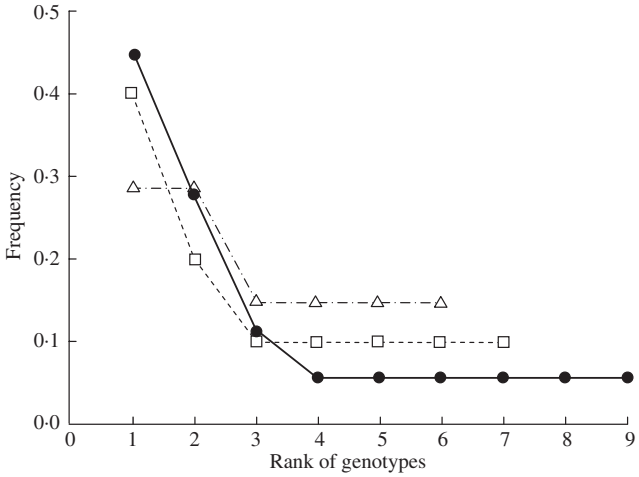

FIG. 2. Rank abundance diagram of clones of the sterile F_1 hybrid *Utricularia australis* f. *australis* (filled circles) and the two parental species, *U. australis* f. *tenuicaulis* (open squares) and *U. macrorhiza* (open triangles). Rankings of the genotypes were estimated for each taxon on the basis of the number of populations in which the genotype was observed.

extremely different from all genotypes of both parental species (Fig. 4). Genetic group A3–A5 had no similar genotypes in *U. australis* f. *tenuicaulis*, but one genotype of *U. macrorhiza* (M4) was relatively similar (Fig. 4). Although genetic group A6–A9 was relatively similar to genotypes in both *U. australis* f. *tenuicaulis* (T6–T7) and *U. macrorhiza* (M1–M2), most of these genotypes were distributed in different regions: for example, T6–T7 in the Tsugaru region and A6–A9 in the Tomakomai region (Fig. 4). Moreover, even when the hybrid and a parental species were found in the same locality, genotypic similarities were extremely low: T2–T3 and A7 in population 14, and M2 and A3 in population 26 (Table 1, Fig. 4).

DISCUSSION

Low genotypic diversity within populations

The sterile F_1 hybrid, *U. australis* f. *australis*, showed extremely low genotypic diversity within populations compared with other clonal plants studied to date: the mean number of genotypes per population (G) was 1·2 and the mean genotypic diversity (D), including monoclonal populations, was 0·04. Even in the parental species, with the capacity to reproduce sexually, the mean number of genotypes per population (G) was 1·1 for both species, and the mean genotypic diversity (D), including monoclonal populations, was 0·05 and 0·02, for *U. australis* f. *tenuicaulis* and *U. macrorhiza*, respectively. Thus, it is concluded that the capacity to reproduce sexually in aquatic bladderworts plays no role in increasing genotypic diversity within populations.

It is generally expected that populations with the capacity for sexual reproduction will contain more genotypic diversity than those without. However, the relative importance of sexual vs. clonal reproduction may vary among clonal plants (Eckert, 2002), and the extensive reliance on clonal reproduction with rare to sporadic sexual reproduction is recognized as a striking convergence in aquatic angiosperms (Les and Philbrick, 1993). In addition, even if many seeds are produced, the seedling establishment largely depends on the existence of safe sites along with the competition with clonal offspring during recruitment (Abrahamson, 1980). Population genetic consequences of reproductive traits, therefore, should be clarified in terms of not only the capacity for sexual reproduction but also its relationship to clonal reproduction, while only few studies have examined these questions (Eckert, 1999; Eckert *et al*., 2003).

The emergent aquatic plant, *Butomus umbellatus*, has marked variation in sexual fertility related to ploidy: diploids produce abundant viable seeds combined with hundreds of tiny clonal bulbils, whereas triploids are sexually sterile and exhibit only limited clonal multiplication through rhizome fragmentation (Eckert *et al*., 2003). Random amplified polymorphic DNA (RAPDs) analysis detected one extremely common and widespread genotype for both fertile and sterile populations (Eckert *et al*., 2003). More importantly, sexually fertile populations did not exhibit higher genotypic diversity than sterile populations, possibly because seeds are outcompeted by bulbils for safe sites during establishment (Eckert *et al*., 2003).

Clonal offspring of free-floating aquatic plants, such as turions and shoot fragments, are highly effective for both numerical increase and expansion within populations (Grace, 1993). In addition, carnivorous, free-floating aquatic bladdderworts can acquire resources solely by shoot fragments, which probably ensure the predominance of clonal reproduction. The rapid expansion of a few founder genotypes via extensive clonal reproduction prevents the subsequent establishment of seedlings and clonal offspring. Competition between clones and stochastic loss of genets may further reduce the genotypic diversity within populations. Widespread expansion of a few clones also inhibits sexual reproduction in self-incompatible species, because it enhances self-pollination or geitonogamy among ramets of the same clone (Eckert, 2000). In aquatic bladderworts, fruits from self-pollination produce fewer seeds than those from cross-pollination: the mean number of seeds per fruit in outcrossed and selfed fruit is 48 and 16 in *U. australis* f. *tenuicaulis*, and 28 and five in *U. macrorhiza*, respectively (Kameyama *et al*., 2005). Thus, these data suggest that rapid and widespread expansion of a few founders excludes the subsequent establishment of seedlings and clonal offspring, and, furthermore, inhibits sexual reproduction because of reduced seed production caused by increased selfing.

Distribution patterns of three aquatic bladderworts

Kameyama *et al*. (2005) clarified the fact that *U. australis* f. *australis* is a sterile F_1 hybrid derived from hybridization between two parental species, *U. australis* f. *tenuicaulis* and *U. macrorhiza*. In the present study, the additive patterns of the AFLP bands and the genetic intermediacy of *U. australis* f. *australis* genotypes (Fig. 3) confirm the hybrid origins and F_1 dominance of this taxon. In addition,

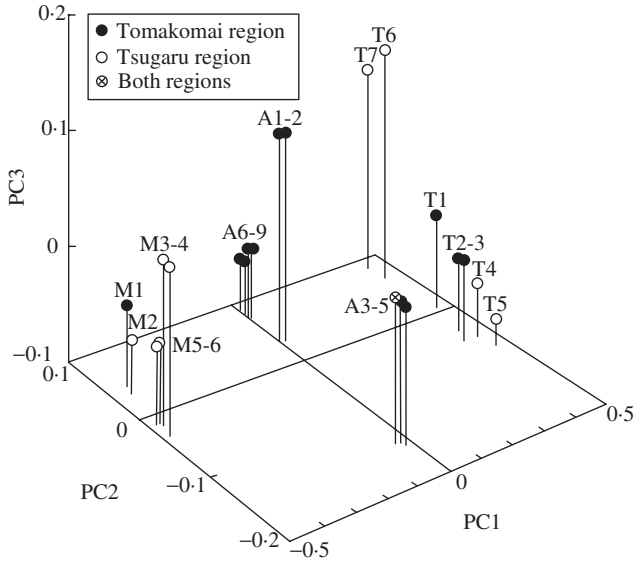

FIG. 3. Principal co-ordinates analysis of 22 genotypes estimated by using 209 AFLP bands from the sterile F_1 hybrid *Utricularia australis* f. *australis* (A) and the two parental species, *U. australis* f. *tenuicaulis* (T) and *U. macrorhiza* (M). The proportion of total variance explained by the first, second and third axes was 38·2, 4·6 and 3·9 %, respectively (46·7 % in total).

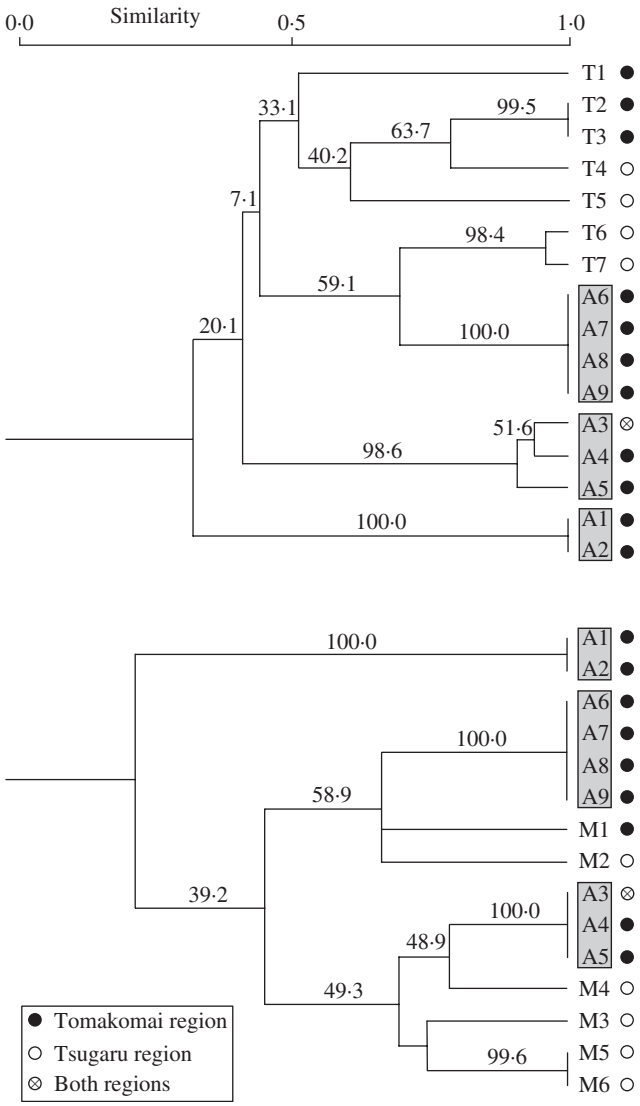

FIG. 4. UPGMA dendrogram constructed for the sterile F_1 hybrid *U. australis* f. *australis* (A) and each of the two parental species: (top figure) *U. australis* f. *tenuicaulis* (T) and (bottom figure) *U. macrorhiza* (M). AFLP bands polymorphic in both target taxa, but absent in the third species, were used for the analyses (see Materials and Methods). Bootstrap values, determined from 1000 replicates, are shown on each branch.

an exhaustive sampling from almost all lakes and ponds in two regions confirmed that the hybrid rarely co-exists with either parental species, and the co-existence of two parental species has not been observed (Table 1).

Interspecific hybrids are highly variable in fertility and vigor, but, in general, F_1 hybrids of closely related species tend to exceed their parents in vegetative vigor and robustness (Grant, 1975; Rieseberg and Carney, 1998; Rieseberg *et al*., 2000). This phenomenon, referred to as hybrid vigor or heterosis, has major implications for evolutionary biology and at least partly explains the success of allopolyploid species and many clonal hybrid lineages (Huskins, 1931; Grootjans *et al*., 1987). Extreme vegetative vigor has been reported in several hydrophyte hybrids, where hybrids compete with or even displace parental species (Les and Philbrick, 1993). The establishment of *U. australis* f. *australis*, however, may be ensured not only by clonal perpetuation but also by long-distance dispersal of clonal offspring: turions and shoot fragments. Long-distance dispersal by waterbirds is a well known characteristic of aquatic organisms (Figuerola and Green, 2002; Green *et al*., 2002), and the existence of several identical genotypes in multiple and distant populations of sterile F_1 hybrid (Figs 1 and 2) supports this observation.

Origin of Utricularia australis *f.* australis

The observed genotypes of the sterile F_1 hybrid could not have originated from hybridization between the extant parental genotypes within the study regions. This is because (*a*) several AFLP bands observed in the hybrid (7–10 for each genotype) are absent in both parental genotypes; and (*b*) parent and hybrid genotypes in the same region do not show greater genetic similarity than those in distant regions (Fig. 4). Thus, possible explanations for the origin of the hybrid genotypes are (*a*) long-distance dispersal from outside the study regions; and (*b*) clonal perpetuation of genotypes that originated from past hybridization event(s) (see Introduction).

First, it is considered whether long-distance dispersal from outside the study regions is a possible explanation. In Japan, *U. australis* f. *tenuicaulis* is distributed widely, found almost everywhere except northernmost Japan, but *U. macrorhiza* is found only in northern Japan (Komiya *et al*., 1997; Y. Kameyama, Hokkaido University, Japan, unpubl. res.). The sterile F_1 hybrid is distributed mainly in northern Japan, in the region where the distribution ranges of the two parental species are broadly overlapping, and only a small number of populations are found in

southern Japan (Kadono, 1994; Y. Kameyama, Hokkaido University, Japan, unpubl. res.). Thus, candidate sites for the hybridization between the two parental species are limited to certain regions in Japan near the study regions. Even though clonal offspring of aquatic bladderworts have high dispersal ability via waterbirds (as was discussed in the previous section), it is unlikely that every population of the hybrid was derived from colonizing propagules outside the study regions.

Thus, the most plausible explanation for the origin of the sterile F_1 hybrid is that extant populations of this taxon stem from the past hybridization event(s). Long-term perpetuation of clones is possible in clonal plants, even if they are sexually sterile (Les and Philbrick, 1993; Hollingsworth *et al*., 1996). This scenario can explain why a small number of hybrid populations are found in southern Japan, where one of the two parental species, *U. macrorhiza*, is not found. In addition, hybrids in southern Japan occasionally co-exist with species known as relicts of the last glacial period (Y. Kameyama, Hokkaido University, Japan, unpubl. res.), and both the Tsugaru and Tomakomai regions were greatly influenced by marine transgressions and regressions during that time (Japan Association for Quaternary Research, 1987). Such past climate change could have led to hybridization between parental species that do not co-exist at the present time. However, more thorough research is required to estimate the time and number of hybridization event(s), because climate change is not the exclusive factor to cause the natural hybridization.

The accurate time and number of hybridization event(s) remain unclear. However, it is clear from the present studies that (*a*) *U. australis* f. *australis* originated from past hybridization event(s); and (*b*) hybrid populations have been maintained exclusively by clonal propagation, which may be ensured by both hybrid vigor and long-distance dispersal of clonal offspring.

ACKNOWLEDGEMENTS

We acknowledge the considerable support of Masahiro Toyama and Satoru Araki. The AFLP analysis was conducted with the advice of Yoshihisa Suyama. This study was supported by the Foundation for Earth Environment, a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology for the 21st Century Center of Excellence Program (E-01), and from the Japan Society for the Promotion of Science (JSPS) for Scientific Research (15370006, 16370007) and Research Fellowships for Young Scientists.

LITERATURE CITED

Abrahamson WG. 1980. Demography and vegetative reproduction. In: Solbrig OT, ed. *Demography and evolution in plant populations*. Oxford: Blackwell Scientific, 89–106.

Araki S. 2000. Isozyme differentiation between two infraspecies taxa of *Utricularia australis* R. Br. (Lentibulariaceae) in Japan. *Acta Phytotaxonomica et Geobotanica* **51**: 31–36.

Barrett SCH, Eckert CG, Husband BC. 1993. Evolutionary processes in aquatic plant populations. *Aquatic Botany* **44**: 105–145.

Casgrain P, Legendre P. 1999. *The R package for multivariate and spatial analysis, version 4·0. User's manual*. Montreal, Canada: Department of Biological Sciences, University of Montreal. http://www.bio.umontreal.ca/Casgrain/en/labo/R/v4/index.html (15 February 2006).

Charpentier A, Grillas P, Thompson JD. 2000. The effects of population size limitation on fecundity in mosaic populations of the clonal macrophyte *Scirpus maritimus* (Cyperaceae). *American Journal of Botany* **87**: 502–507.

Dice LR. 1945. Measures of the amount of ecological association between species. *Ecology* **26**: 297–302.

Dorken ME, Eckert CG. 2001. Severely reduced sexual reproduction in northern populations of a clonal plant, *Decodon verticillatus* (Lythraceae). *Journal of Ecology* **89**: 339–350.

Eckert CG. 1999. Clonal plant research: proliferation, integration, but not much evolution. *American Journal of Botany* **86**: 1649–1654.

Eckert CG. 2000. Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology* **81**: 532–542.

Eckert CG. 2002. The loss of sex in clonal plants. *Evolutionary Ecology* **15**: 501–520.

Eckert CG, Dorken ME, Mitchell SA. 1999. Loss of sex in clonal populations of a flowering plant, *Decodon verticillatus* (Lythraceae). *Evolution* **53**: 1079–1092.

Eckert CG, Lui K, Bronson K, Corradini P, Bruneau A. 2003. Population genetic consequences of extreme variation in sexual and clonal reproduction in an aquatic plant. *Molecular Ecology* **12**: 331–344.

Figuerola J, Green AJ. 2002. Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. *Freshwater Biology* **47**: 483–494.

Grace JB. 1993. The adaptive significance of clonal reproduction in angiosperms: an aquatic perspective. *Aquatic Botany* **44**: 159–180.

Grant V. 1975. *Genetics of flowering plants*. New York: Columbia University Press.

Green AJ, Figuerola J, Sánchez MI. 2002. Implications of waterbird ecology for the dispersal of aquatic organisms. *Acta Oecologia* **23**: 177–189.

Grootjans AP, Allersma RJ, Kik C. 1987. Hybridization of the habitat in disturbed hay meadows. In: van Andel J, ed. *Disturbance in grasslands*, Dordrecht: Dr W. Junk Publishers, 67–77.

Hollingsworth PM, Preston CD, Gornall RJ. 1996. Isozyme evidence for the parentage and multiple origins of *Potamogeton* × *suecicus* (*P. pectinatus* × *P. filiformis*, Potamogetonaceae). *Plant Systematics and Evolution* **202**: 219–232.

Huskins CL. 1931. The origin of *Spartina townsendii*. *Nature* **127**: 781.

Japan Association for Quaternary Research. 1987. *Explanatory text for quaternary maps of Japan*. Tokyo: University of Tokyo Press.

Jelinski DE, Cheliak WM. 1992. Genetic diversity and spatial subdivision of *Populus tremuloides* (Salicaceae) in a heterogeneous landscape. *American Journal of Botany* **79**: 728–736.

Jobson RW, Playford J, Cameron KM, Albert VA. 2003. Molecular phylogenetics of Lentibulariaceae inferred from plastid *rps*16 intron and *trn*L-F DNA sequences: implications for character evolution and biogeography. *Systematic Botany* **28**: 157–171.

Kadono Y. 1994. *Aquatic plants of Japan*. Tokyo: Bun-ichi Sogo-Shuppan [in Japanese].

Kameyama Y, Toyama M, Ohara M. 2005. Hybrid origins and F_1 dominance in the free-floating, sterile bladderwort, *Utricularia australis* f. *australis* (Lentibulariaceae). *American Journal of Botany* **92**: 469–476.

Klekowski EJ Jr. 1997. Somatic mutation theory of clonality. In: de Kroon H, van Groenendael J, eds. *The ecology and evolution of clonal plants*. Leiden: Backhuys Publishers, 227–241

Komiya S, Shibata C. 1980. Distribution of the Lentibulariaceae in Japan. *Bulletin of the Nippon Dental University* **9**: 163–212.

Komiya S, Toyama M, Shibata C, Katsumata K. 1997. Carnivorous plants in Hokkaido, northern Japan. *Bulletin of the Nippon Dental University* **26**: 153–188 [in Japanese].

Les DH, Philbrick CT. 1993. Studies of hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. *Aquatic Botany* **44**: 181–228.

Makarenkov V. 2005. *Program T-Rex: tree and reticulogram reconstruction*. Canada: Université du Québec à Montréal. http://www.trex.uqam.ca/ (12 April 2006).

McLellan AJ, Prati D, Kaltz O, Schmid B. 1997. Structure and analysis of phenotypic and genetic variation in clonal plants. In: de Kroon H, van Groenendael J, eds. *The ecology and evolution of clonal plants*. Leiden: Backhuys Publishers, 185–210.

Müller K, Borsch T. 2005. Phylogenetics of *Utricularia* (Lentibulariaceae) and molecular evolution of the *trn*K intron in a lineage with high substitutional rates. *Plant Systematics and Evolution* **250**: 39–67.

Olivieri I, Michalakis Y, Gouyon PH. 1995. Metapopulation genetics and the evolution of dispersal. *American Naturalist* **146**: 202–228.

Philbrick CT, Les DH. 1996. Evolution of aquatic angiosperm reproductive systems. *Bioscience* **46**: 813–826.

Pielou EC. 1969. *An introduction to mathematical ecology*. New York: Wiley-Interscience.

Piquot Y, Petit D, Valero M, Cuguen J, de Laguerie P, Vernet P. 1998. Variation in sexual and asexual reproduction among young and old populations of the perennial macrophyte *Sparganium erectum*. *Oikos* **82**: 139–148.

Richards AJ. 1997. *Plant breeding systems*, 2nd edn. Cheltenham, UK: Stanley Thorne.

Rieseberg LH, Carney SE. 1998. Plant hybridization. *New Phytologist* **140**: 599–624.

Rieseberg LH, Baird SJE, Gardner KA. 2000. Hybridization, introgression, and linkage evolution. *Plant Molecular Biology* **42**: 205–224.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

Starfinger U, Stöcklin J. 1996. Seed, pollen, and clonal dispersal and their role in structuring plants populations. *Progress in Botany* **57**: 336–355.

Stewart CN Jr, Via LE. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *BioTechniques* **14**: 748–750.

Taylor P. 1989. *The genus* Utricularia – *a taxonomic monograph*. Kew, UK: Royal Botanical Gardens.

Toyama M, Katsumata K. 1999. Insectivorous plants of the western region of Tsugaru Peninsula. *Journal of Insectivorous Plant Society* **50**: 56–61 [in Japanese].

Uematsu S, Katsumata K, Toyama M. 2000. Insectivorous plants of the western region of Tsugaru Peninsula. II. *Journal of Insectivorous Plant Society* **51**: 45–50 [in Japanese].

Vos P, Hogers R., Bleeker M, Reijans M, van de Lee T, Hornes M, *et al*. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Yap IV, Nelson RJ. 1996. WinBoot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. *IRRI Discussion Paper, Series 14*. International Rice Research Institute, Manila, Philippines. http://www.irri.org/science/software/winboot.asp (15 February 2006).