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ORIGINAL PAPER

Conservation unit status inferred for plants by combining interspecific crosses and AFLP

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Abstract Hybridization and introgression are common in plants and lead to morphological similarity between species and taxonomic confusion. This gene flow with closely related species can complicate efforts to determine whether an endangered taxon is evolutionarily distinctive and should be identified as a separate conservation unit. *Potentilla delphinensis* is a rare and threatened endemic species of the Southern French Alps. Two common related taxa (*P. grandiflora* and *P. thuringiaca*) are morphologically similar and occur in the same geographical locations. Thus, whether *P. delphinensis* represents a reliable conservation unit remained unclear. Our evaluation procedure based on a combination of molecular biology and interspecific crosses was used to define taxa within these plants. Plants were sampled from a total of 23 single and mixed localities for the three supposed taxa and were genotyped with 68 polymorphic Amplified Fragment Length Polymorphism (AFLP) loci. Fourty-one seedlings from interspecific crosses were obtained and genotyped. Amplified Fragment Length Polymorphism markers identified four genetically distinct units (*P. delphinensis*, *P. grandiflora* and two distinct groups of *P. thuringiaca*). All individuals of *P. delphinensis* formed a homogeneous and distinct taxon. This taxon was most probably an old allopolyploid from *P. grandiflora* and the related group of *P. thuringiaca*. Interspecific crosses gave low seed set and low germination rate. Furthermore, assignment test indicated that seedlings obtained from interspecific crosses were essentially apomictic rather than hybrids. These results suggest that a reproductive barrier exists between the different taxa. In conclusion, all results supported *P. delphinensis* as a true biological species and justified its conservation unit status. A surprising outcome of this work was the evidence of a potential new cryptic species. This study demonstrated the need to combine a molecular marker-based approach and pollination experiments for an accurate evaluation of plant taxa.

Keywords AFLP · Assignment test · Cryptic species · Interspecific crosses · Plant conservation unit

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Introduction

Identifying reliable conservation units is of particular importance for conservation biology, representing the first step to set priorities for conservation measures. The concept of conservation unit can be applied to taxonomic units at or below the species level and takes into account the high extinction risk of the unit. Morphological traits and the biological species concept are commonly used to distinguish taxonomic units at the species level. The biological species concept involves reproductive isolation between species (''a species is a reproductive community of populations, reproductively isolated from others, that occupies a specific niche in nature'', Mayr 1982, p. 273). However, distinct plant species may not be morphologically distinguishable and the biological species concept may be inappropriate for defining plant taxonomic units when reproductive isolation is unclear. Indeed, hybridization and introgression are common in plants. Hybridization is an important mechanism for plant evolution and is

responsible for the origin of many true species (Grant 1981; Barton 2001). Introgression results in the incorporation of genes from one species' pool into another; however, generally the two species continue to exist side by side as is the case for oaks (Petit et al. 2004), indicating that reproductive isolation is preserved. Species are often difficult to distinguish when using morphological characters, and are genetically close to or intermediate between the two parents (Aldrich et al. 2003). Other cases of morphological similarity are the occurrences of cryptic species. Cryptic species are morphologically indistinguishable and yet reproductively isolated (Grant 1981). To date, very few cryptic species have been identified in plants, and the recent reports of cryptic species are based on strong neutral marker divergence between groups of individuals that are morphologically difficult or impossible to distinguish (Shaw 2000; Rajakaruna et al. 2003; Whittall et al. 2004). In taxonomic groups where confusion is rampant, such as in aquatic plant species, the roles of hybridization, introgression and cryptic species remain unsolved (Whittall et al. 2004).

The evolutionarily significant unit (ESU), formulated by Waples (1991, 1995) provides a concept to prioritize conservation units below the species level (Fraser and Bernatchez 2001). The ESU is a population or a group of populations that is substantially reproductively isolated from other conspecific population units, and represents an important component in the evolutionary legacy of the species (''the evolutionary legacy of a species is the genetic variability that is a product of past evolutionary events and that represents the reservoir upon which future evolutionary potential depends'', Waples 1995, p. 9). As for the biological species concept, the ESU must present effective reproductive isolating mechanisms, such as geographical isolation, ecological specificities or genetic incompatibility. The accumulation of genetic differences through reproductive isolating mechanisms is usually measured by molecular markers to define a potentially evolutionary independent unit.

Whether defined at or below the species level, the conservation unit should be substantially isolated by reproductive barriers. This fact raises the question as to whether hybrids merit conservation status. Hybridization is usually mentioned as a negative force for the preservation of plant species (Levin et al. 1996). Although in some studies, gene flow from an abundant congener to a rare species is viewed as a genetic assimilation and a reduction of the ''purity'' of the rare species (Soltis and Gitzendanner 1999, and citations therein), contemporary hybridization does not seem to cause taxonomic problems in plants (Rieseberg et al. 2006). Allendorf et al. (2001) pointed out that taxa that have arisen by natural hybridization should be eligible for protection, as opposed to taxa originating from anthropogenic hybridization. Hybrid taxa should be eligible for conservation if historical hybridization events were natural, if the hybrid taxa had become species or independent evolutionary units and that their persistence no longer depends on hybridization.

A suitable method to study introgression and hybridization in plants and which helps in defining plant conservation units is based on the use of neutral molecular markers. Molecular markers have proved their efficacy to help resolve the taxonomic status of plants as well as various plant conservation problems (Morrell and Rieseberg 1998; Koontz et al. 2001; Aldrich et al. 2003). In plants, the genetic diversity of very closely related species or interspecific hybrids has successfully been studied using Amplified Fragment Length Polymorphism (AFLP; Beismann et al. 1997; O'Hanlon et al. 1999; Han et al. 2000). The AFLP technique is simple and robust, and overcomes problems associated with development time, cost and reproducibility that can plague plant studies using other marker systems. It is a DNA-fingerprinting method, developed by Vos et al. (1995), that provides information for many loci randomly distributed throughout the genome in a single assay. The large number of these markers, associated with a scrupulous scoring of bands, compensates for the fact that they are dominant (Evanno et al. 2005). Recently, statistical software for population genetic tests and assignment tests have been developed for or extended to AFLP markers (Peakall and Smouse 2001; Duchesne and Bernatchez 2002). However, the ploidy level may affect the AFLP profiles and thus, confuse the genetic relationships between individuals. Indeed, in their investigation on *Solanum*, Kardolus at al. (1998) found an increase in the mean number of fragments along with the increase in ploidy level, but differences were not statistically tested.

The *Potentilla* genus of the Rosaceae family is well known for its difficulties in identifying species and the frequent synonymies (Hansen et al. 2000; Eriksson et al. 2003). Our study focused on an endangered *Potentilla* of the French Alps: *Potentilla delphinensis* Gren. & Godron (PD). The species usually shares localities with two taxonomic relatives, *P. grandiflora* L. (PG) and *P. thuringiaca* Bernh. ex Link (PT).

The phenology of the three taxa differs somewhat but usually overlaps for part of the flowering period (lasting from June to August). Identification of the three taxa on morphological traits can be very difficult. Commonly used morphological characters (number of

leaflets, size of the flower, hair on radical leaf, leaf size) are highly variable within species. One characteristic difference is plant height and volume: *P. delphinensis* is usually larger than the two other species (Grenier and Godron 1848). However, here again, morphological variability is important and intermediate individuals exist. The gigantism of PD may be linked to its higher number of chromosomes. In the genus *Potentilla*, the basal number of chromosomes is $x = 7$, PG is a tetraploid ($2n = 4x = 28$) and PT is a hexaploid ($2n = 6x = 42$) (Lauber and Wagner 1998). In PD, the number of chromosomes has been estimated on only four individuals and the first results indicated that PD might be an octoploid ($2n = 8x = 56$) (R. Verlaque, pers. com.). All this suggests that *P. delphinensis* might be either a recent polyploid hybrid or the result of current introgression between PG and PT, and may thus not be a true species.

Our aims are to resolve the taxonomic confusion in the *P. delphinensis* group. We specifically want to know whether *P. delphinensis* is a reliable conservation unit. To address these issues, we analyzed the genetic structure of the three supposed taxa using AFLP markers. However, because the sole use of AFLP markers may be insufficient to identify recent hybridization events, we performed interspecific artificial crosses. Resulting seedlings were genotyped and we used assignment tests to confirm their origin. According to Fraser and Bernatchez (2001), the ideal to state a conservation unit implies to show evidence of distinctiveness/uniqueness using different types of data, i.e. genetic, karyotypic, ecological, spatial and life history data. However, in practice, funding is usually limited and all data may not be necessary on a case-by-case basis (Fraser and Bernatchez 2001). This paper shows that the combination of molecular biology and cross-species experiments are powerful in the decision process for defining plant taxa.

Materials and methods

Potentilla delphinensis

P. delphinensis Gren. & Godron (PD) is an emblematic Rosaceae of the French Alps that is extremely rare and endangered. It is therefore a priority in conservation in the South East of France. The species is listed as vulnerable in regional and national Red Lists. It is protected by the European Habitats Directive (Natura 2000 network), listed in the Berne and Washington conventions, and substantial funding supports its conservation. In 2001, the Conservatoire Botanique National Alpin (CBNA, Gap, France) started a study to improve the knowledge of the species. The first action was to update the presence/absence data by revisiting previously recorded populations. Next, the current localities were mapped and completely described. Both ex situ and in situ conservation programs were initiated, with germination and pollination experiments and demographic census in two natural populations (Caille 2001).

P. delphinensis occurs between 1500 and 2000 m altitude, usually in sunny and rich meadows. Several locations where it was historically recorded have a doubtful validity (due to confusion with other *Potentilla* species, Fig. 1); in other historic localities it was not found in recent surveys. As a result, it is impossible to determine long-term population trends. Only 10 populations have been recently confirmed, all limited in space and usually including a small number of widely dispersed plants. The current localities are unconnected, which suggests they may face a high extinction risk. Potential threats to this species indicated in the Natura 2000 list are heavy grazing, or conversely the lack of it (leading to competition and canopy closure), and genetic introgression/hybridization with *Potentilla* relatives.

The current project was initiated on request of the CBNA in 2001 (L. Vinciguerra) in order to clarify the taxonomic status of *P. delphinensis*.

Natural populations

Sampling

Mixed or single species populations were sampled during spring and summer 2002 covering the geographical range of *P. delphinensis* (Fig. 2). As our aim was to describe the overall extent of genetic variation of the three species rather than to have a fine description of within-population diversity, only a few specimens from many locations were sampled (5 individuals on average from 23 locations). Species identification on each location was essentially based on morphological and phenological characters and considered previous observations and repeated observations during the last years. Species identification and sampling were performed by taxonomic experts from the CBNA. When a majority of individuals were characteristic of one taxon, the population was considered to belong to this taxon and five plants on average were sampled randomly. In locations where individuals were characteristic of two taxa, populations were considered as mixed and four plants on average were collected for each taxon. Four, five and eight single-species populations were sampled respectively

Fig. 1 *Potentilla delphinensis* (center) and the two close species *Potentilla grandiflora* (left) and *Potentilla thuringiaca* Bernh. (=*Potentilla parviflora* Gaudin) (Right). (From Flora der Schweiz, Band 2. 1970. H.E. Heß, E. Landolt, R. Hirzel. Birkhauser Ed.)

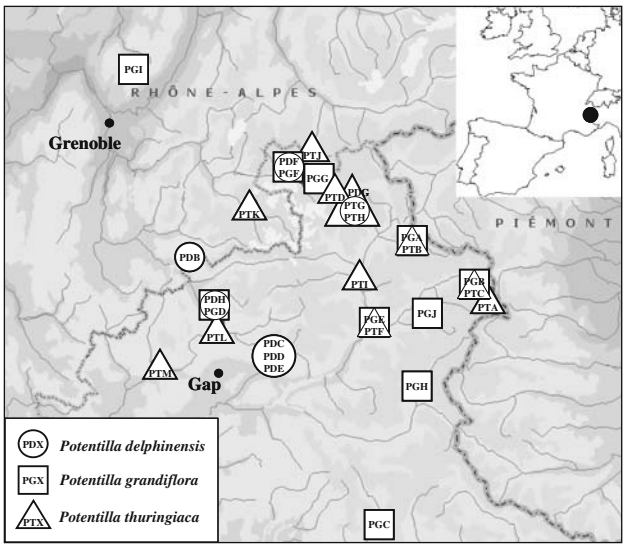

Fig. 2 Sites sampled over the French Alps for the three *Potentilla* species. Identification was based upon morphology and phenology

for *P. delphinensis* (PD), *P. grandiflora* (PG) and *P. thuringiaca* (PT). Two mixed populations of PD and PG, one mixed population of PD and PT and three mixed populations of PG and PT completed the sampling. Tissues were stored in silica gel in the field for fast desiccation.

AFLP fingerprinting

Total genomic DNA was extracted with the ''DNeasy 96 plant kit'' (QIAGEN) according to the manufacturer's protocol using 10 mg of dried leaf material. The approximate amount of DNA and its quality were checked on a 2% agarose gel. AFLP analysis was performed following the protocol described by Vos et al. (1995) modified by Gaudeul et al. (2000). Extracted genomic DNA (~200 ng) was digested by *Mse*I and *Eco*RI (New England BioLabs, Beverly, MA, USA). Selective amplifications were purified and added to 10 μl formamide and 0.3 μl size standard (GeneScan 500 Rox, Perkin Elmer). After 15 min evaporation, samples were analyzed on an ABI PRISM 3100 DNA sequencer (Applied Biosystems). AFLP patterns were visualized using GeneScan Analysis v3.7 (Applied Biosystems).

A total of 12 primer pairs were tested on 4 individuals originating from the 3 taxa. Three primer pairs were selected for the clarity of their restriction fragments profiles and the presence of clear polymorphic peaks. Reproducibility of the chosen primer pairs was tested performing the whole AFLP protocol on 4 individuals with 3 initial concentrations of DNA each (undiluted DNA extract ranging from 47 to 142 ng/μl, 50 times dilution and 200 times dilution). Differences in mean intensity and in mean number of

fragments per genotype were tested between taxa with Kruskal and Wallis non-parametric tests (MINITAB 1998) to examine the influence of ploidy level on the AFLP profiles. This analysis included all fragments from 50 to 450 bp for the three chosen primer pairs. The GeneScan files were imported into Genographer (v 1.6.0, J. Benham, Montana State University, 1998, available at http://hordeum.oscs.montana.edu/genographer/) in order to score the presence or absence of polymorphic fragments over all samples. Only reproducible and unambiguous polymorphic fragments were considered.

Genetic data analysis

An unrooted neighbour-joining phylogram (NJ) based on the similarity coefficient of Nei and Li (1979) was generated and bootstrapped (Felsenstein 1985) using 1000 replicates with PAUP* 4.0b10 (Swofford 2003). Trees were drawn with the program TreeView (Page 1998).

We carried out a hierarchical analysis of molecular variance (AMOVA) for binary data using GENALEX (Peakall and Smouse 2001). The total genetic diversity was partitioned among taxa, among populations within taxa and within populations. Correlations between geographic and genetic distances were tested using a Mantel test.

Two different assignment softwares were used: AFLPOP (Duchesne and Bernatchez 2002) and STRUCTURE (Pritchard et al. 2000). Both programs gave similar results and only the results obtained with STRUCTURE will be presented. STRUCTURE uses a Bayesian clustering method for inferring the genetic structure from multilocus genotypes and simultaneously assigning individuals to clusters. The program has been used successfully with AFLP (Evanno et al. 2005). The dominant AFLP data was recoded as bi-allelic loci by adding missing values for each dominant phenotype (encoded 1 and –9) whereas recessive phenotypes were coded as recessive homozygote (0 and 0). STRUCTURE was used to estimate the most likely number of clusters among all individuals sampled in nature, without taxa and population information. We ran simulations at cluster numbers K ranking from 1 (no structure) to 10 (10 genetic clusters) with burn-in periods of 10000 and 10000 simulations. For each value of K, the software calculated the estimated posterior probability $L(K)$ of the data that is used as the model choice criterion (the maximal value indicates the optimal K). However, because $L(K)$ usually increases slightly with larger values of K, we considered that the true K was not the one with the highest $L(K)$ but the one corresponding to a break in slope of $L(K) = f(K)$. According to Evanno et al. (2005), we selected the true K for the modal value of the second order rate of change of the likelihood function with respect to K (ΔK).

To address whether *P. delphinensis* is an allo- or an autopolyploid, and identify which species it might be derived from, we assigned the AFLP fragments present in the octoploid genomes of PD to the four different groups revealed by NJ and assignment analyses. PD-specific markers were determined as those only present in *P. delphinensis*. Specific fragments for one parental group were defined as being shared just by PD and that particular group, but absent from the two other groups. Common markers were shared by PD and two groups, but absent from the fourth group.

Artificial crosses

Interspecific crosses

From July to September 2002, individuals from natural populations were transplanted into pots in the common-garden of the CBNA. Of these, seven plants of PT originating from three natural populations, eight plants of PD from two populations and nine plants of PG from three populations survived to spring 2003. Because of the drought in spring and summer 2003, we had to synchronize flowering periods of the three species and plants of PT were placed in shade-house to delay flowering. However, very few flowers of PT were still alive when the two other taxa where at peak of flowering. The whole plants were bagged before bud burst. A total of 96 cross-species pollinations were performed on emasculated buds (61 with PD mothers, 24 with PG mothers and 11 with PT mothers). All six possible cross-species pollinations between pollen donors and mother plants were performed using a pool of pollen from different plants. We used a pool of pollen to obtain enough viable pollen, in particular from plants of PT. After pollen deposition, the whole plants were bagged again until fruiting. Drupelets were collected in July.

Intra-specific pollinations and auto-pollinations were also performed, but the very dry season in 2003 led to a high mortality of flowers and the heterogeneous number of treated flowers prevented us from any statistical comparisons with interspecific pollinations. However, high seed set from self-pollinated flowers indicated that the pollen use for all crosses was viable.

The drupelets issued from natural pollination for each taxon were used as control. After 2 weeks of drying, the number of achenes produced by each druplet was counted. We could clearly separate small

achenes that were not fertile from normal-looking, potentially fertile achenes. Normal-looking achenes were germinated on humid Whatman paper in Petri dishes in a culture room at 21°C with 16 h light. Seedlings from interspecific crosses had with their first two leaves were planted in pots filled with a mixture of 60% sand, 20% compost and 20% soil. When there was sufficient material, leaves were sampled for AFLP fingerprinting analysis following the same procedure as for plants from natural populations.

Data analysis

To identify potential hybrids within the seedlings, the procedure with prior group information from STRUCTURE (Pritchard et al. 2000) was used. We assigned the seedlings to the true *K* clusters identified previously with all individuals from natural populations and estimated, for each seedling, the fraction of alleles that are derived from each cluster (assignment probability to each parental group). Because AFLPs are dominant markers and the species have different ploidy values, we did not expect a hybrid to be equally assigned to its two parents. The combination of dominance and number of copy can lead to any kind of assignment proportions. To consider a cluster as a parental group, two minimal thresholds for the fraction of alleles shared between the cluster and the seedling were fixed (10 and 25%). As a consequence, the number of potential hybrid varied, depending on the fixed threshold. According to this variation in the number of hybrids, we estimated a range for the rate of hybridization from hand-pollination experiments. The minimal value (MIN) was calculated as the lowest number of hybrids over the total number of ovules per type of cross and the maximum value (MAX) was calculated as the maximal number of hybrids over the total number of seeds per type of cross. Germination rate was compared between the two treatments (interspecific pollination versus natural pollination) using a binomial test.

Results

Genetic structure of natural populations

The three primer pairs were highly reproducible (proportion of reproducible peaks between 95 and 99%; Table 1) and a total of 68 polymorphic and repeatable markers was used to genotype each individual. Neither the peak intensity, nor total number of fragments per genotype reflected the ploidy level. Summed over the three primer pairs, the peak intensity was significantly higher for the taxa with the lowest ploidy level (PG). Even if the number of fragments was higher for the octoploid PD (189, 168 for the hexaploid and 170 for the tetraploid), the difference was not significant.

The NJ tree (Fig. 3) clearly separated four distinct groups. In particular, one group of PT individuals (subsequently called PT2) was highly differentiated from the rest of the samples (bootstrap value = 100%), including all individuals from the other two species and another group of PT individuals (PT1). The group PT2 was composed of six different populations (populations PTD, PTG, PTH, PTI, PTL and PTM, see Fig. 2) and PT1 was composed of the six other populations sampled as *P. thuringiaca*. The third group clustered all PD individuals (bootstrap value = 85%), showing little genetic variation. The fourth group was composed of most PG individuals, with five PG individuals closer to PT1 cluster (PGC3, PGJ1, PGJ4, PGE2, and PGE5). The presence of these PG individuals at the root of

Table 1 Characteristics of the chosen selective primer pairs for the AFLP procedure and characteristics of the resulting AFLP profiles for the three taxa

Characteristics of the three chosen selective primer pairs			
Sequence primer *Eco*RI	Sequence primer *Mse*I	% Reproducible polymorphic peaks	Number of analyzed markers
5′-GACTGCGTACCAATTC**AGA**-3′	5′-GATGAGTCCTGAGTAA**CAT**-3′	99.2 ± 0.02	33
5′-GACTGCGTACCAATTC**AGT**-3′	5′-GATGAGTCCTGAGTAA**CAT**-3′	98.7 ± 0.03	22
5′-GACTGCGTACCAATTC**ATC**-3′	5′-GATGAGTCCTGAGTAA**CAC**-3′	95.6 ± 0.04	13
Characteristics of the AFLP profiles summed over the three primer pairs			
Taxa (ploidy level)	Mean peak intensity ± S.D	Mean total # fragments/genotype ± S.D.	
PD (8×)	293.1 ± 494.9	188.7 ± 47.3	
PT (6×)	285.1 ± 424.7	167.7 ± 37.1	
PG (4×)	334.9 ± 592.7	170.7 ± 34.2	
Kruskal–Wallis test	$H = 41.1$ df = 2 $P < 0.001$	$H = 4.01$ df = 2 $P = 0.135$	

Selective bases are in bold letters

Kruskal–Wallis non-parametric tests (*H* statistics) allowed to test for difference between taxa/ploidy level

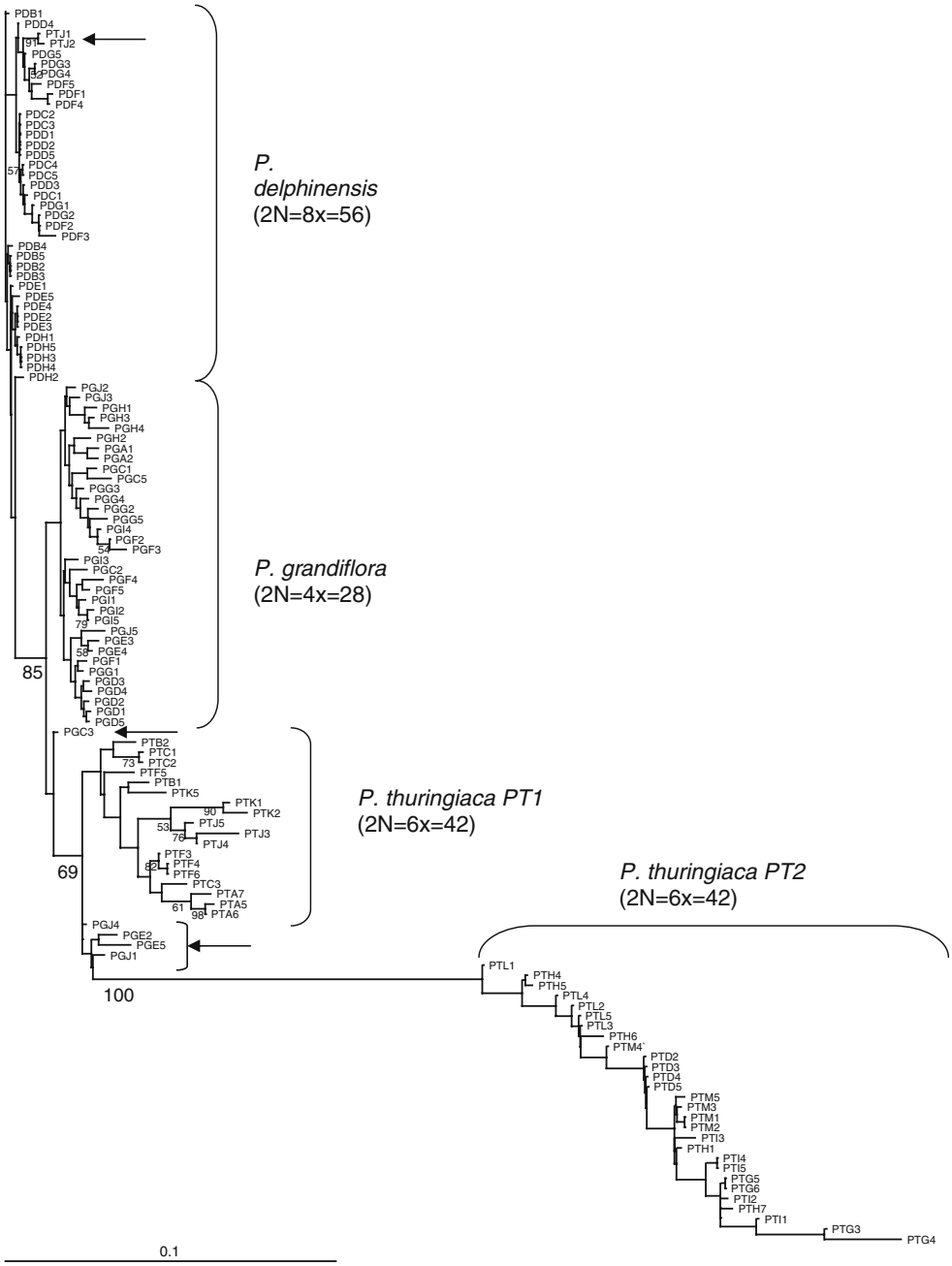

Fig. 3 Unrooted Neighbor Joining phylogram (Nei–Li distance) for 123 individuals and 3 taxa of *Potentilla*. The tree is constructed from 68 AFLP bands. Bootstrap values (>50% only) are shown at the nodes of the branches. Arrows indicated individuals with peculiar or unexpected taxonomic position

PT2 cluster might explain the low bootstrap value (69%) between PG and PT1. No specific populations of PG and PT1 appear closer to PD cluster. Only two individuals completely failed to appear close to the expected species: PTJ1 and PTJ2 were morphologically identified as *P. thuringiaca* but fall with the *P. delphinensis* group. The three other individuals from the same population fall into the PT1 cluster.

The analyses of molecular variance (Table 2) confirmed a strong genetic divergence between the four taxa. In the first analysis performed on the whole sample (PD, PG, PT1 and PT2), 73% of the variation was among taxa, whereas in the second analysis excluding PT2, 52% of the variation was among taxa. The difference between the two AMOVAs pointed out that the divergence between PT2 and the other taxa was important. Figure 4 shows that the genetic difference between PT1 and PT2 was large but did not change as a function of geographic distance. On the contrary, there was a significant positive relation between genetic and geographic distances within PT1 populations and, independently, within PT2 populations. Whatever the geographic distance, the genetic distance between PT1 and PT2 individuals was always

Table 2 Results of the analyses of molecular variance

Source	Estimated variance	Percentage of variance	df	Statistical value	*P*-value
AMOVA on the whole sample (four taxa: PD, PG, PT1 and PT2)					
Among taxa	11.10	73	3	0.73	0.001
Among populations within taxa	2.06	14	24	0.50	0.001
Among individuals within populations	2.05	13	94	0.87	0.001
AMOVA excluding PT2 (3 taxa: PD, PG and PT1)					
Among taxa	4.54	52	2	0.52	0.001
Among populations within taxa	2.02	23	19	0.49	0.001
Among individuals within populations	2.14	25	72	0.75	0.001

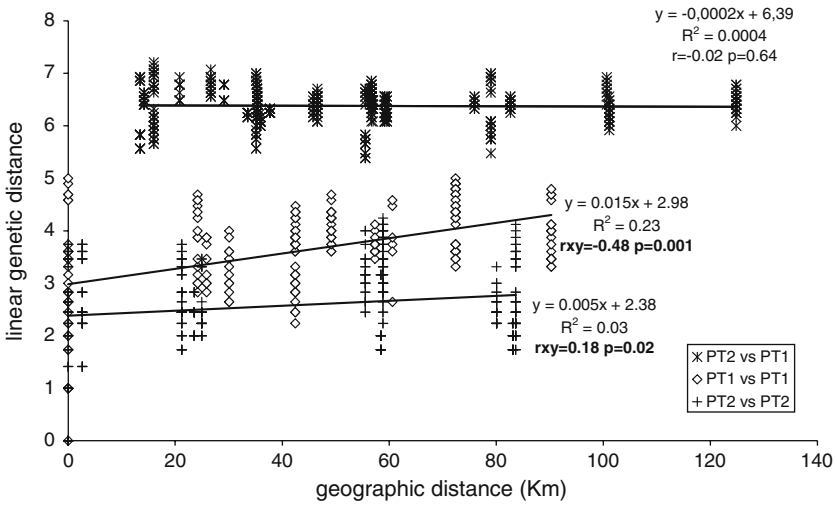

Fig. 4 Genetic distance as a function of linear geographic distance for pairs of PT individuals. For the two lower lines (PT1 and PT2 independently), the coefficient of correlation between geographic and genetic distance (*rxy*) was calculated and tested using a Mantel test (GENALEX, Peakall and Smouse 2001). In the case where one individual is from a PT1 population and the other is from a PT2 population, we calculated a Pearson coefficient of correlation (*r*). Significant correlations are in bold

larger than genetic distances among PT1 or PT2 individuals.

None of the PT plants used in the crosses came from PT2 populations, and no seedling fell close to the PT2 group in preliminary analyses. We therefore performed the assignment test using STRUCTURE on PD, PG and PT1 only. The modal value of the second order rate of change of the likelihood function (ΔK) was clearly identified for $K = 3$ (Fig. 5). The three clusters corresponded essentially to PD, PG and PT1. All individuals were clearly assigned to their expected group, except for the same two individuals PTJ1 and PTJ2 that were assigned for 17.1% to PT1 and 82.6% to PD, and for 21.1% to PT1 and 78.6% to PD respectively. Three other plants (PGE5, PTB2, PDE5) were slightly apart from their expected taxa (Fig. 6).

Among the AFLP fragments identified in the octoploid genome of PD, 30% was common to all four taxa. The distribution of all other bands among the different taxa was represented in Fig. 7. The assignment of the AFLP fragments present in PD to the different groups (PG, PT1 and PT2) suggested an allopolyploid origin.

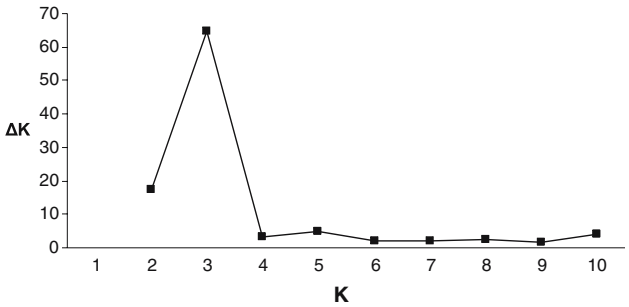

Fig. 5 Identification of the number of clusters within all individuals of PD, PG and PT1. The ''true'' number of clusters K is indicated by the highest modal value. The modal value ΔK for each K was calculated as $\Delta K = |L''(K)|/s[L(K)]$, with $L''(K)$ the second order rate of change of the mean likelihood for K obtained with STRUCTURE ($L(K)$) and $s[L(K)]$, the standard deviation of $L(K)$

Indeed, the majority of the fragments present in PD were common markers shared with PG and PT1 (55%). Furthermore, the presence of PD-specific markers (6%), together with characteristic markers to PG (19%) and PT1 (8%) indicated that PD might be an allopolyploid of ancient origin. Globally, few markers

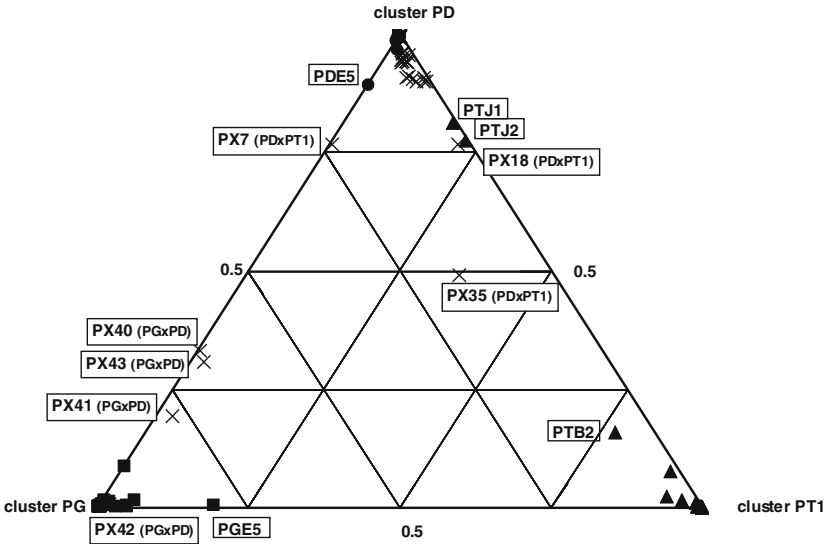

Fig. 6 Triangular plot presenting the assignment probability of samples from natural populations and of seedlings from artificial crosses (×) to the three clusters PD (•), PG (■) and PT1 (▲) identified on the basis of the natural populations. Between brackets, type of cross at the origin of the seedling (mother taxa X father taxa)

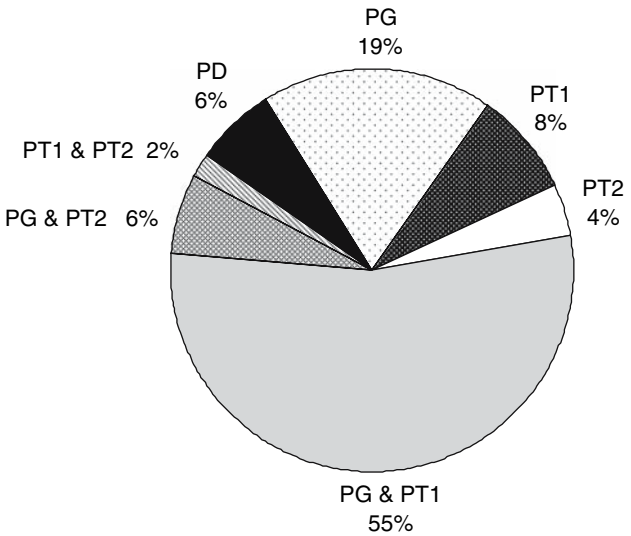

Fig. 7 Pie chart presenting the frequency of the different categories of AFLP fragments present in the octoploid PD and assigned to the three potential parental groups PG, PT1 and PT2. Assignment was based on 68 AFLP markers. PD-specific markers (PD) were those only present in *P. delphinensis*. Taxon-specific markers (PG, PT1 or PT2) were those only shared by one parental taxon and PD. Common markers were shared by PD and two groups (PZ & PW), but absent from the fourth group

were shared with PT2. These PT2-specific markers might also exist in PT1 but were not detected because only five individuals per population were genotyped.

Artificial crosses

Because of the very dry season in summer 2003 in France, the number of successfully treated flowers was very variable (5–32 depending on the type of interspecific cross) and led to heterogeneous and globally low number of seeds per type of cross (Table 3). The important difference in sample size between crosses prevented us from comparing the quantitative results of our different interspecific crosses (the seed set and the direction of the crosses). The absence of surviving seedling from mother PT did not allow comparing the rate of germination between seeds from interspecific crosses and seeds from natural pollination. For PD and PG, the percentage of successful germinations after interspecific pollination was weak (13.3 ± 14.2%) and was significantly lower than for seeds from natural pollination (42.2% for PD, $Z = -4.12$, $P < 0.001$, and 47.8% for PG, $Z = -3.10$, $P = 0.001$). A total of 41 seedlings originating from interspecific crosses could finally be genotyped.

Within the 36 seedlings originating from a cross between PT1 pollen and PD mothers, 33 fell into the PD cluster and 3 were intermediate. PX35 was assigned equally to the PD and PT1 clusters whereas PX18 was assigned for 75% to PD and for 21% to PT and fall close to the two misidentified individuals from the natural populations (PTJ1 and PTJ2). Surprisingly PX7 was assigned to PD (76%) and PG (22%) despite originating from a cross between PD and PT. Within the four seedlings originating from a cross between PD pollen and PG mothers, one fell into the PG cluster and three were intermediate (PX40, PX41 and PX43). They were assigned to PG for approximately 70% and to PD for 30%. The status of hybrids depends on the threshold considered for the minimal percentage of alleles derived from a cluster to consider it as a parental cluster. If the minimal percentage is fixed to 25%, only

Table 3 Results of interspecific crosses and natural pollinations. For the number of potential hybrids, the two values correspond to 25% and 10% minimal thresholds for the fraction of alleles shared between the cluster and the seedling

	Number of crosses	Number of surviving treated flowers	Number of potentially viable achenes	Number of surviving seedlings	Successful germination	Number of potential hybrids	MIN–MAX percentage of hybrids
Cross (♀ × ♂)							
PD × PG	32	31	28	3	10.70%	0	0%
PD × PT	29	26	143	36	25.20%	1–3	0.04–2.1%
PG × PD	8	7	13	4	30.80%	2–3	0.7–23.1%
PG × PT	16	10	17	0	0	–	–
PT × PD	6	5	1	0	0	–	–
PT × PG	5	5	0	–	–	–	–
TOTAL	96	85	202	43	13.3 ± 14.2	3–6	
Natural pollination							
PD		13	233	99	42.80%	$P_{cross}(39/171) < P_{nat}(99/233)$ $Z = -4.12$; $P < 0.001$	
PG		10	46	22	47.80%	$P_{cross}(4/30) < P_{nat}(22/46)$ $Z = -3.10$; $P = 0.001$	

Comparison of the proportion of germination between interspecific crosses (P_{cross}) and natural pollinations (P_{nat}) was tested with a unilateral binomial test (Z statistics)

three seedlings may result from hybridization; if it is fixed to 10%, six seedlings may result from hybridization.

Discussion

Conservation status of *P. delphinensis*

P. delphinensis is a species with high conservation priority in France, based on its rarity and supposed population decline. However, no biological processes were taken into account for this classification, and the morphological similarity with two other species caste some doubt about the validity of its conservation unit status. In this study, we focused on biological and evolutionary processes to identify *P. delphinensis*'s taxonomic status.

Genetic structure analysis from the three morphologically close species of *Potentilla* showed that they were actually composed of four clearly distinct genetic units: *P. delphinensis* populations, *P. grandiflora* populations and two groups of *P. thuringiaca*. All individuals of *P. delphinensis*, from single or mixed populations, clustered together and constituted one distinct group. The NJ tree did not indicate a specific PG or PT1 population as the origin of the PD taxon.

The assignment of the AFLP fragments present in PD to the other taxa (PG, PT1 and PT2) suggested a probably ancient allopolyploid origin. This result tended to disprove the possibility that PD was only an alternate cytotype or chromosomal race of one of the more common *Potentilla* species. However, historical events (such as severe bottlenecks in PD populations) might confuse the interpretation of banding pattern in the genome of PD. Further investigations by cloning nuclear markers would be required to confirm an old allopolyploid origin.

Interspecific crosses between PD, PG and PT1 gave low seed set and few hybrid offspring. Holm and Ghatnekar (1996) obtained similar results after interspecific crosses between diploid *Potentilla argentea* and hexaploïd *P. argentea* and *P. collina* (4.7% mean germination rate on 10 different interspecific crosses). Acharya Goswami and Matfield (1975) obtained only four hybrids of 628 crosses with three different interspecific crosses between *Potentilla* species (including *P. grandiflora*). The high number of pure *P. delphinensis* plants (36) resulting from crosses in which PD was the mother strongly suggested facultative apomixis in that species, similarly to several *Potentilla* that are known to be facultative apomictic (Acharya Goswami and Matfield 1975; Holm and Ghatnekar 1996; Nylehn et al. 2003; Richards 2003). Selfing resulting from error of manipulation or unwanted insect pollination could not be totally excluded.

Germination success was significantly lower for achenes originating from interspecific crosses compared to achenes from natural pollination. This result might indicate that hybrids have a lower survivorship due to chromosomal rearrangements (Rieseberg 2001), that apomictic seeds were less viable than outcrossing seeds and/or that seeds experienced inbreeding depression (Keller and Waller 2002). In that study, the different ploidy levels might be the most important mechanism of reproductive isolation.

Finally, the low survival of interspecific offspring suggests that a reproductive barrier exists between PD, PG and PT1, and strengthened the conclusion that *P. delphinensis* is a distinct biological species *sensu* Mayr (1982).

The combination of molecular markers and cross-species pollination allowed defining *P. delphinensis* as a reliable operational conservation unit. PD seemed to be substantially reproductively isolated from other conspecific units. The accumulation of genetic differences though reproductive isolating mechanisms was sufficient to observe a strong genetic differentiation of PD populations from all other populations. The strong genetic differentiation and the probably ancient origin of PD suggested that *P. delphinensis* became an independent evolutionarily significant unit, and is worthy of conservation (Allendorf et al. 2001).

Cryptic species

Our genetic data indicate that *P. thuringiaca* was composed of two completely isolated taxa, despite morphological similarity. The PT2 taxon was different from the three other taxa (PT1, *P. delphinensis* and *P. grandiflora*), suggesting the absence of gene flow between them. This reproductive barrier was independent of geographic distances as the two PT taxa shared the same distribution range and also shared some localities with the other taxa studied here. Moreover, to our knowledge, the differentiation was not ecological, nor altitudinal or phenological.

The genus *Potentilla* is a group that presents taxonomic challenges. In the Southern Alps, *P. thuringiaca* was recorded under various names and was usually confused with *P. heptaphylla*, a species that no longer exists in this area. The webpage of the French botany network (http://www.tela-botanica.org/) lists numerous synonyms of *P. thuringiaca* including *P. heptaphylla* (*sensu* 1901). One hypothesis is that the cryptic PT2 species is a remnant of *P. heptaphylla*. Only further investigations (morphometric and ecological analyses, molecular marker analysis, chromosome counts and artificial crosses) following further sampling will allow to identify correctly and name the two taxa.

Even though it is difficult to identify *P. grandiflora*, *P. thuringiaca* and *P. delphinensis* on the sole basis of morphology, the identification by specialists on historical and repeated morphological observations proved to be correct for all *P. delphinensis* and most *P. grandiflora* populations. Only two individuals of *P. thuringiaca* (PTJ1 and PTJ2) clearly failed to group with the other individuals of their expected species and were assigned to *P. delphinensis*. Given the proximity of the population PTJ with a *P. delphinensis* population (PDF, Fig. 2), the population they were collected in might actually be a mixed population. However, if these two samples were sampling errors, we would have expected them to have a higher percentage of assignations to PD. Thus, a valid explanation is that these individuals result from natural hybridization. Other individuals that were not exclusively assigned to their expected taxon (PGE5 and PTB2) could be also later generation hybrids because they were located in mixed population (PGE mixed with PTF, PTB mixed with PGA, Fig. 2). Thus, the genotyping of individuals showed that the criteria used by the specialists failed to assign the taxon for very few individuals but that they were completely useless to distinguish the two genetically different groups of *P. thuringiaca*.

Conclusion

This study confirmed the efficiency of the AFLP fingerprinting method to provide information on the status of conservation unit of rare and threatened plants. Contrary to *Solanum* species (Kardolus et al. 1998), we found no evidence of a significant increase in the mean number of fragments along with the increase in ploidy level. An increase in the number of bands would have resulted in the polyploids clustering separately, even if they were not highly genetically differentiated from the parental species. However, in our study, genetic variation of the three chosen primer pairs did not seem to be influenced by the ploidy level and genetic relationships between individuals should thus be preserved.

When hybridization is suspected, the decision process for defining plant conservation unit should automatically include cross-species pollination experiments and seedlings genotyping. This work provides evidence that plants with different ploidy levels can hybridize and that seeds from interspecific crosses can be apomictic rather than hybrids. The combination of molecular marker-based approach and pollination experiments gives the procedure to define plant taxa a real added value.

A surprising outcome of this work was the discovery of a potential new (cryptic) species previously mistaken with *P. thuringiaca*. Cryptic species may be more frequent than what was previously thought and the extensive use of molecular markers may lead to the recognition of a far greater number of species.

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