Western Ukrainian *Cochlearia* (Brassicaceae)—the identity of an isolated edge population

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A strongly isolated population of *Cochlearia* in North-Western Ukraine was analysed using highly discriminating AFLP markers, to clarify the confusing taxonomy of the genus at the eastern edge of its distribution in Europe. The analysis revealed a high level of similarity between the Ukrainian population and *C. pyrenaica,* supporting its inclusion within this species and not within *C. polonica*. *Cochlearia polonica* remains a narrowly distributed endemic of the Polish lowlands. Most genetic variability of the Central European populations of the diploid *C. pyrenaica* (Ukraine, Carpathians, Alps) was found among populations. In addition, the populations significantly differentiated into two distinct groups (Alps *versus* Carpathians and Ukraine). The strong genetic divergence of the Ukrainian population together with its low within population variability (similarity indices between individuals exceeding 0.9) highlights the need for conservation efforts to prevent the irreversible loss of this distinct gene pool of *C. pyrenaica*.

KEYWORDS: AFLP, *Cochlearia polonica*, *Cochlearia pyrenaica*, *Cochlearia tatrae*, conservation, systematics, taxonomy, Ukraine

INTRODUCTION

Most species of the genus *Cochlearia* L. in Europe belong to two sections, namely *Cochlearia* (= *Eucochlearia* Prantl) and *Glaucocochlearia* O.E. Schulz (e.g., Schulz, 1936). These two sections have recently been revised (Koch & al., 1999). Extensive studies of relationships within the genus using morphological, cytological, and molecular data (e.g., Pobedimova, 1971; Vogt, 1985; Nordal & Stabbetorp, 1990; Koch & al., 1996, 1998, 1999, 2003; Koch & Al-Shehbaz, 2000; Koch, 2002) have revealed the general phylogenetic and biogeographical framework. However, disjunct and isolated distributions of most of the species, in addition to the wide and poorly defined morphological variability, require additional fine-scale studies to understand local patterns of diversity. This is especially true for the eastern part of the European range.

Compared to Western Europe, in the East there are numerous inland taxa belonging to the section *Cochlearia*, forming a particular morphologically, cytologically and ecologically differentiated complex, including *Cochlearia pyrenaica* DC. ($2n = 12$), *C. polonica* Fröhl. ($2n = 36$), *C. tatrae* Borb. ($2n = 42$), *C. borzaeana* (Com. & Nyár.) Pobed. ($2n = 48$) (Koch & al., 2003). A recent cytological study by Valachovič & Kochjarová (2000) identified isolated Western Carpathian populations (outside the Tatra Mountains) of *Cochlearia* as highly disjunct diploid ($2n = 12$) *C. pyrenaica,* and not *C. tatrae*, which was often believed to be the only representative of the genus in the Western Carpathians.

There is considerable taxonomic confusion regarding an isolated population in North-Western Ukraine found at the eastern-most edge of the distribution area of *Cochlearia* sect. *Cochlearia* (Tymrakiewicz, 1930). Herbarium samples collected by *Mądalski* in 1934 (*no. 227,* KRAM-Mądalski) were described by him as *C. pyrenaica*. This identification was considered as incorrect by Pobedimova (1970), who claimed that morphological characters (very variable in the case of that population) demonstrated a close relationship with *C. polonica,* an extremely rare Central-European lowland endemic. Additionally, Pobedimova (1970) suggested that there were ecological similarities between these lowland populations compared with the mountain-restricted *C. pyrenaica*. On the other hand, the distribution of *C. pyrenaica* presented in the *Flora Europaea* (Chater & Heywood, 1964) includes mention of the "Ukrainian Carpathians" (in fact, the population is located north of the Carpathian foothills). Vogt (1985) mentioned, based again on morphological characters, that the Ukrainian *Cochlearia* should most probably represent *C. pyrenaica*. In the contemporary Ukrainian literature concerning this population it is treated as *C. polonica*, and is counted among the

most endangered plant populations in the country (e.g., Kotov, 1979; Prokudin, 1987; Shelyag-Sosonka, 1996).

The aim of this study is to elucidate the phylogenetic affinities of this isolated Ukranian population by using molecular markers and chromosome counts, which should provide a more definitive result than those based on morphological comparisons. The study may help clarifying the taxonomic status of *Cochlearia* in Ukraine and estimating the genetic divergence and level of diversity of the Ukrainian population as a means to assess conservation priorities of *Cochlearia* in Central Europe.

MATERIAL AND METHODS

Plant material. — Plant material was collected from the Ukrainian population of putative *C. pyrenaica* ("*C. polonica*"), from the only existing unambiguous population of *C. polonica* (being a result of conservation efforts and restitution activities) and from selected populations of *C. pyrenaica* and *C. tatrae* (Table 1). Forty-seven plants in total were used in the study. Voucher specimens were deposited in KRAM and HEID. Leaf material was collected in the field into plastic airtight bags filled with silica gel. In the laboratory all samples were stored frozen at –80°C prior to DNA extraction. Seeds were also collected in the Ukrainian population to ensure material for chromosome counts.

DNA extraction and AFLP procedure. — Total DNA was extracted from approximately 20–30 mg of dried leaf tissue using the DNeasy Plant Mini Kit system (Qiagen), according to the protocol recommended by the manufacturer. The quality of genomic DNA was checked on agarose gel and DNA concentrations were determined by spectrophotometry using a GeneQuant spectrometer (Amersham Biosciences).

AFLP analysis generally followed the procedure described by Vos & al. (1995). The genomic DNA was digested with *Eco*RI and *Mse*I restriction enzymes (New England Biolabs Inc.). In the following step, double-strand adapters were ligated to *Eco*RI and *Mse*I specific ends by T4 DNA Ligase (Roche Diagnostics). Products of digestion/ligation were checked by electrophoresis in 1.5% agarose gels and subsequently diluted 1 : 10 with sterile de-ionised H_2O. The preselective amplification was performed using primers with single selective nucleotides: *Eco*RI+A and *Mse*I+C. Products were diluted 1 : 20 with sterile de-ionised H_2O. Selective amplifications were performed using *Eco*RI and *Mse*I primers with three selective nucleotides: *Eco*RI-AAG/*Mse*I-CTA, *Eco*RI-ACT/*Mse*I-CAC, *Eco*RI-AGC/*Mse*I-CTA. The *Eco*RI primers were 5'-fluorescent-labelled (6-FAM). The fluorescent-labelled selective amplification products were diluted 1 : 20 with sterile de-ionised H_2O and separated in the POP 4 polymer with an internal size standard GeneScan-500 (ROX), on automated sequencer ABI Prism 3100-Avant (Applied Biosystems). To test the quality and repeatability of the results, one to five samples per population were replicated and carried through all reaction steps. The degree of repeatability was then assessed and a repeatability threshold of 98% was set as standard.

Data analysis. — The raw AFLP data were aligned with the size standard using GeneScan Analysis Software (ver. 3.7, Applied Biosystems) and imported to the Genographer software (ver. 1.6.0; J. Benham, Montana State University, 1998–2001, http://hordeum.oscs.montana.edu/genographer). AFLP fragments were scored in the size range of 50–500 bp and assembled as a binary matrix of presence (1)/absence (0) for further data analysis. The numbers of distinguishing markers were quantified for both taxa and populations using the following criteria: "discriminating" fragments—present in all analysed samples of a respective population/taxon and absent elsewhere, and "private" fragments—unique for respective population/taxon but not common for all of its samples. Intra- and inter-populational relationships were estimated based on a similarity matrix. UPGMA clustering method was applied using Nei and Li's similarity coefficients. For a more holistic picture of molecular variation among taxa and all individuals a principal coordinates analysis (PCO) was performed. Data were analysed using the MVSP software, version 3.10b (Kovach, 1999). Standard genetic diversity, according to Nei (1987), and structure of genetic variation (by the molecular variance analysis, AMOVA) were calculated using ARLEQUIN 2.0 software (Schneider & al., 2000).

Table 1. Localities of studied populations of *Cochlearia* spp. A, Austria; PL, Poland; SK, Slovakia; UA, Ukraine. Representative vouchers are deposited at HEID and KRAM.

No.	Taxon	Site	Coordinates	Samples
1	*Cochlearia polonica*	Centuria river near Olkusz (PL); leg. E. Cieślak	N 50°25′ E 19°29′	7
2	*C. pyrenaica* ("*C. polonica*")	Verchobuzh (UA); leg. E. Cieślak	N 49°51′ E 25°06′	8
3	*C. pyrenaica*	Türnitz, Lower Austria (A); leg. M. Koch	N 47°53′ E 15°28′	5
4	*C. pyrenaica*	Niederalpl, Styria (A); leg. M. Koch	N 47°40′ E 15°24′	5
5	*C. pyrenaica*	Bukovinka, Vel'ka Fatra (SK); leg. M. Ronikier	N 49°00′ E 19°17′	7
6	*C. tatrae*	Čierna Javorova Dolina, Tatry (SK); leg. M. Ronikier	N 49°12′ E 20°11′	8
7	*C. tatrae*	Mięguszowiecki Szczyt (Bandzioch), Tatry (PL); leg. E. Cieślak	N 49°10′ E 20°04′	9

RESULTS

All samples examined exhibited different banding patterns, and consequently 47 AFLP phenotypes were used in our analyses. In total, 181 high quality bands were obtained using the three selective primer combinations, out of which 179 (98.8%) were polymorphic in the whole dataset (Table 2). The level of genetic polymorphism differed among taxa, but, as stated in previous works (Koch & al., 2003), was not correlated with ploidy levels. The highest degree of variability was found in populations of *C. tatrae* and those of *C. pyrenaica* from the Alps; amounting to 78–96% and 82–94% variability respectively. The lowest degree of polymorphism was found in the restituted population of *C. polonica*. No strong differences in the average gene diversity over loci values (Nei, 1987) was found within a species (Table 3); the lowest values characterised populations 3 and 5, while the highest values occurred in both populations of *C. tatrae* (pop. nos. 6 and 7). The number of distinguishing markers was consistent with this. *Cochlearia polonica* (pop. no. 1) had 5 discriminating markers (present across all individuals), and *C. tatrae* had only one, even though *C. tatrae* had 19 private markers (also taxon-restricted, but not present in all individuals) (cf. Tables 2 and 3 for distribution of distinguishing markers).

In the UPGMA analysis all individuals were arranged in population-specific clusters. The cluster analysis grouped individuals into three distinct clusters: (1) *Cochlearia tatrae*, (2) *C. polonica* and (3) *C. pyrenaica* (Fig. 1). These major groups were also seen in the PCO analysis. Almost half of the total variation is explained by the first and the second components (29.78% and 19.51% respectively). These three clusters generally correspond well with taxonomical units (Fig. 2). *Cochlearia polonica* and *C. tatrae* form two very coherent groups, while a further diversification is observed in *C. pyrenaica*, where two alpine populations (pop. nos. 3 and 4) form two close subgroups, and two Eastern populations—from Slovakia (5) and Ukraine (2)—form a common cluster clearly separated from the Alpine group. The third PCO axis provides little additional information, explaining only 8.52% of the total variation (data not shown).

In the AMOVA analysis 40.25% ($p < 0{,}001$) of the total variance was found between taxa (Table 4A), 36.87% of the variance was found between populations within taxa, and 22.88% of the variance was found within populations. In an AMOVA that included only populations of *C. pyrenaica*, 70.73% of total variance was found among populations and only 29.27% within populations (Table 4B). Between the western (pop. nos. 3 and 4) and eastern (pop. nos. 2 and 5) groups of *C. pyrenaica* six discriminating fragments were identified (present in all individuals of one group and absent in the other). The F_{ST} values were high (see Tab. 5 for pairwise F_{ST} values) and are similar to those reported by Koch & al. (2003).

Table 2. Distribution of polymorphic bands among primer pairs used and taxa studied.

	AAG/CTA	ACT/CAC	AGC/CTA	Total polymorphic bands	Discriminating bands[1]	Private bands[2]
Total bands per primer pair	55	57	67	179	–	–
C. polonica [pop. 1]	14	3	10	27	5	2
C. pyrenaica [pop. 2–5]	24	30	31	85	1	14 (11)
C. tatrae [pop. 6–7]	31	17	27	75	1	19 (9)

[1] Discriminating bands—present in all samples from the taxon and totally absent elsewhere.
[2] Private bands—present only in one taxon, but not in its all individuals. Number of bands present in single populations of a taxon or across several populations is given as the first number; no. of those present across all populations of a taxon is given in brackets.

Table 3. Average gene diversity over loci according to Nei (1987) and number of distinguishing bands in populations.

No.	Taxon	Average gene diversity over loci	Discriminating bands[1]	Private bands[2]
1	*C. polonica*	0.053670 (± 0.032109)	5	2
2	*C. pyrenaica*	0.052684 (± 0.030835)	2	1
3	*C. pyrenaica*	0.044199 (± 0.028955)	2	1
4	*C. pyrenaica*	0.077348 (± 0.049065)	0	0
5	*C. pyrenaica*	0.041989 (± 0.023164)	1	1
6	*C. tatrae*	0.088990 (± 0.050672)	0	3
7	*C. tatrae*	0.115408 (± 0.063943)	0	7

[1] Discriminating bands—present in all samples from the population and totally absent elsewhere.
[2] Private bands—present only in one population, but not in its all individuals.

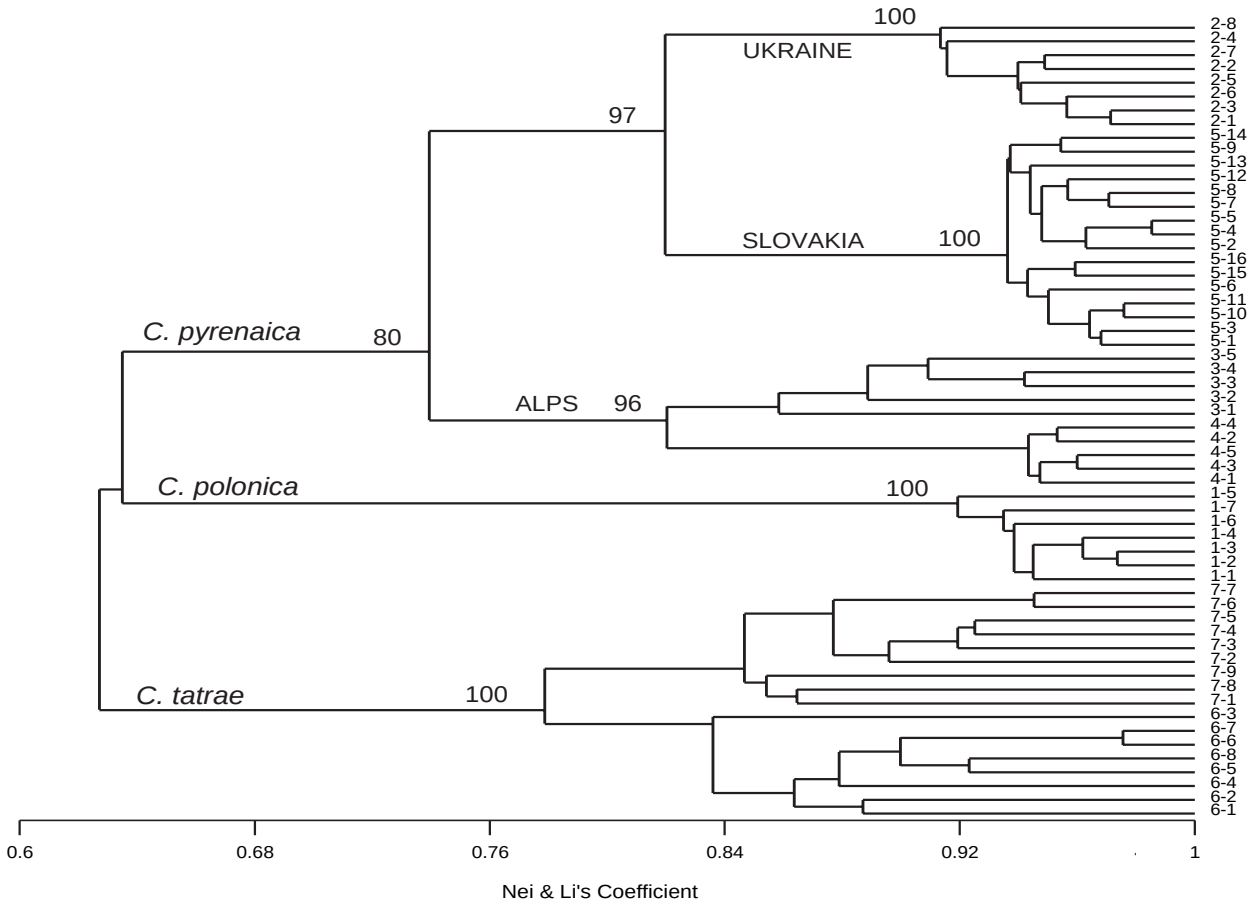

Fig. 1. UPGMA tree based on Nei and Li's coefficient, presenting relationships of all individuals studied (numbers of populations correspond with those in Table 1). Bootstrap values from 1,000 replicates for main nodes are indicated above branches.

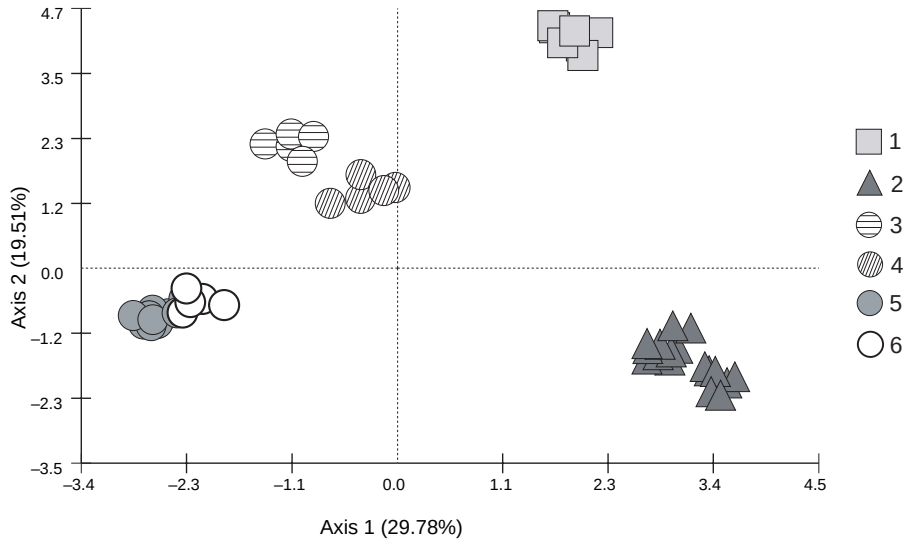

Fig. 2. PCO plot of all individuals studied. Explanation of symbols: 1, *C. polonica* (pop. 1); 2, *C. tatrae* (pop. 6, 7); 3, *C. pyrenaica* (pop. 4); 4, *C. pyrenaica* (pop. 3); 5, *C. pyrenaica* (pop. 5); 6, *C. pyrenaica* (pop. 2).

Table 4. A. Results of the analysis of molecular variance (AMOVA) in *C. polonica*, *C. pyrenaica* and *C. tatrae*, employing 181 AFLP bands. Levels of significance tests are based on 1,023 permutations. Fixation indices: F_{SC} 0.61704, F_{ST} 0.77117, F_{CT} 0.40246. B. Results of the analysis of molecular variance (AMOVA) in 34 individuals (4 populations) of *C. pyrenaica*. Levels of significance tests are based on 1,023 permutations. Fixation index F_{ST}: 0.70727. All p < 0.001.

A	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
	Among groups	2	509.278	10.46463	40.25
	Among populations within groups	4	326.636	9.58698	36.87
	Within populations	51	303.448	5.94997	22.88
	Total	57	1139.362	26.00158	
B	**Source of variation**	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
	Among populations	3	263.625	10.82236	70.73
	Within populations	30	134.375	4.47917	29.27
	Total	33	398.000	15.30153	

DISCUSSION

Relationships between populations inferred from AFLP data: status of the Ukrainian *Cochlearia*. — Our analysis of the genetic variability of *Cochlearia* from an isolated population in Ukraine, in the context of other Central European populations, helps resolve several longstanding taxonomical questions. All of the Ukrainian samples analysed form a population-specific cluster within *Cochlearia pyrenaica*, clearly separated from other studied taxa (*C. polonica* and *C. tatrae*). Some previous studies based on morphological and ecological data had placed the Ukrainian *Cochlearia* with *C. polonica* (Pobedimova, 1970), and has subsequently been followed in current Ukrainian literature. Our results clearly identitify this population as being more closely related to *C. pyrenaica*, and is in accordance with the opinion of Vogt (1985). Our recent chromosome counts for this population (unpubl. data) showed $2n = 12$ chromosomes. Also parallel-conducted cytological investigations of Kochjarová & al. (in press) showed diploid chromosome number $2n = 12$ for this population. Within- and between-population morphological variability does not reflect the strong genetic diversification and does not make foundations for any clear and reliable taxonomical subdivision. Yet, a strong genetic subdivision corresponding with geographical and—presumably—historical traits of lineages, should be included in the future taxonomical considerations of the species. Only a complete phylogeographical study of *C. pyrenaica* incorporating its entire range will allow us to assess the genetic variability of the taxon and help to build its reliable infraspecific taxonomic divisions.

A detailed analysis of the herein analysed selected populations of *C. pyrenaica* brings further information to the structure of intraspecific diversity of this taxon in Central Europe. A very distinct separation of Alpine ("western") and (Sub-)Carpathian ("eastern") populations of *C. pyrenaica* is observed in both UPGMA and PCO analyses. The occurrence of distinct clusters is additionally supported by the presence of several private markers distinguishing these two infraspecific groups. A weak relationship suggested by the 3rd axis (data not shown) additionally shows the divergence between lineages, maybe indicating a link among the Carpathian populations of *C. pyrenaica* and the endemic polyploid *C. tatrae*. This picture supports a hypothesis that the eastern lineage of *C. pyrenaica* or an unknown extinct diploid lineage contributed to the formation of this very interesting allopolyploid taxon (Koch & al., 1996). High diversification of taxa and coherence of populations were supported by the various AMOVAs indicating genetic isolation for the various *C. pyrenaica* populations from the three regions (Eastern Alps, Slovakia, Ukraine). This finding is similar to previous studies showing a separation of diploid *Cochlearia* in Eastern Austria from the Slovakian populations (Koch & al., 2003), a separation of the German and Eastern Austrian *C. pyrenaica* (Koch, 2002) or, on a more regional scale, the genetic differentiation among the Eastern Austrian *C. pyrenaica* populations (Koch, 2002). The Ukrainian population appears to be an eastern edge population of the large European range of *C. pyrenaica* reaching from the Pyrenees to the foothills of the northern Carpathians. Significantly, that all populations of *C. pyrenaica* in Germany, East Austria, Slovakia and Ukraine are located close to the

Table 5. Pairwise F_{ST} values in the studied populations (numbers of populations correspond with those in Table 1). All values are significant on the p < 0.001 level.

	6	7	1	3	4	5	2
6	0						
7	0.40453	0					
1	0.75204	0.72485	0				
3	0.76688	0.71927	0.79171	0			
4	0.66042	0.63444	0.73364	0.55139	0		
5	0.77803	0.75941	0.85245	0.76829	0.72834	0	
2	0.74627	0.72036	0.83349	0.77400	0.65645	0.68476	0

historical border line of the last glaciation maximum from approximately 18,000 years ago, but always outside glaciated areas of that time (Koch, 2002). Ukrainian populations, identified as *C. pyrenaica*, also favour the hypothesis formulated previously (Koch, 2002; Koch & al., 2003), that *C. pyrenaica* survived periglacially maybe even in permafrost habitats. The very clear diversification of two lineages ("eastern" and "western") supports the hypothesis, that fragments of the discontinuous range of *C. pyrenaica* in Europe should be seen as relicts of a formerly wider distribution, separated as a result of extinction processes rather than large-scale postglacial migration or recolonisation. That also explains the constitution of the various inland polyploids closely related to *C. pyrenaica*, with most of them not of a postglacial origin (refer to the discussion for *C. bavarica*, Koch, 2002). Within the regional lineages (Carpathians + pre-Carpathians and Alps), however, isolation of populations could be dated as more recent process, due to clear within-lineage affinities.

Conservation issues. — All taxa analysed here belong to rare plants, in the case of *C. polonica* and *C. tatrae* very narrowly distributed apoendemics. Additionally, due to substantial range discontinuity, all populations of *C. pyrenaica* studied here represent strongly isolated groups with no possible gene flow between populations. Results of our genetic analyses (especially the very high among-population variance as shown by AMOVA) demonstrate a strong divergence of these *C. pyrenaica* populations. This should be an additional reason for conservation efforts to preserve these populations as irreplaceable sources of genetic variability. The pairwise genetic similarity of individuals in the Ukrainian population, estimated based on AFLPs, is very high (in most cases higher than 0.9; data not shown). Even though the population is restricted to a very small area, the PCO diagram shows a correlation between the distribution of the remaining variability and spatial distribution of plants in the population (data not shown), suggesting limited gene flow and possibly reflecting a tendency to self-compatibility, reported to characterise the diploid taxa of *Cochlearia* (Koch & al., 1998; Koch & Bernhardt, 2004). Similar results (with all values above 0.9) were found for the genetically closest Slovak population of *C. pyrenaica* (data not shown). The area of natural occurrence of *C. pyrenaica* in Ukraine has been subjected to draining activities strongly disturbing local water relations (Y. Kobiv, pers. comm.) and most probably ecological changes will put this population on the way of extinction. Our data show the genetic distinctiveness of this population, likely which deserves to be distinguished at infraspecific taxonomic level. Therefore, preservation of genetic material *ex situ* (seed bank) seems to be of utmost importance as a quick step. Field work is also necessary, in order to search for potentially spontaneous dispersal of the species and find optimal areas for restitution (transplantation) in nature. Such restitution helped to save *C. polonica* when its natural populations in South Poland almost disappeared, also due to water level disturbance; this action proved efficient in both demographical (Kwiatkowska, 2001) and genetic (E. Cieślak, M. Ronikier, unpubl. data) respects. Also, experiences from the extremely rare Austrian endemic *C. macrorrhiza* should be taken into account here (Koch & Bernhardt, 2004).

The confirmation of the identity of the Ukrainian population as *C. pyrenaica* and its exclusion from *C. polonica*, has also important implications for the latter taxon. The status of *C. polonica* as a strictly endemic species of Southern Poland remains unaltered. The species is restricted to a single, well established but reintroduced population (and a few vanishing reintroductions descending from it) and should be considered as extremely endangered. The Ukrainian population, however, cannot be considered as a potentially natural genetic resource of this taxon.

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