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Historical vs. present populations of the sedge *Carex repens*: a comparison on the basis of molecular data

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Abstract

Polish historical and present populations of *Carex repens* (= *C. posnaniensis*) were compared with southern European populations of this plant on the basis of their nuclear ITS and chloroplast IGS DNA sequences. It follows from the analysis of the available data that (1) there are no differences in ITS1 and ITS2 sequences between the present populations occurring nearby the Jajtawy population (Poland), where in 1896 Spribille described *C. posnaniensis*, (2) the taxonomic distance between all the populations is relatively small (the greatest was reported between the populations from Poland and Italy), (3) the similarity between the populations decreases with an increasing geographical distance between them. Two populations from Italy exhibit the highest taxonomic distance from the Polish ones. We attribute this finding to the difference in age between these populations, as the Polish populations are much younger relative to the Italian ones and they could have appeared only after the Baltic glaciation ice sheet had receded.

Key words: *Carex* sect. *Ammoglochin*, central population, *Cyperaceae*, marginal population, phenetic analysis

Introduction

Carex repens Bellardi (1793: 42) is one of seven species of sedge of the *Ammoglochin* Dummert section, subgenus *Vignea* (*Cyperaceae*), see Chater (1980). This is a species whose range of occurrence covers the European countries of France, Italy, Germany, Austria, the Czech Republic, Hungary, Romania, and Poland. Spribille described the new species *C. posnaniensis* Sprib. in Kneucker (1896: 184), which was for some time considered to be a Polish endemic (Szulczewski 1932). The specimen described by Spribille was collected in Jajtawy, situated between Toruń and Bydgoszcz. In 1909 Kükenthal proposed this taxon to be synonymous to *C. repens*, and this was commonly accepted by subsequent authors.

In Poland *C. repens* is not considered to be highly threatened, but it occurs only in seven localities at the lower Wisła River between the town of Nieszawa and Chełmno, the latter being the marginal and most northern locality of this species. *Carex repens* is mostly found in sandy proglacial stream valleys, and the valley of the Wisła River, it is particularly found in dry and warm sites at the edges of the upland (Ceynowa-Giełdon 1969, 2001). The aim of our study is to answer the following queries: (1) whether and how the Polish current populations of *C. repens* are similar to the populations of historical localities, (2) how similar are the Polish populations of *C. repens* to its central populations, and (3) to what extent does the geographical distance affect the similarity of the populations.

Population variation was established using nuclear molecular markers ITS (Internal Transcribed Spacer) rDNA. The internal transcribed spacers between genes 18S and 5.8S (ITS 1) and between 5.8S and 26S (ITS

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2) are among the relatively fast changing DNA sequences in comparison with the "conservative" sequence of gene 5.8S (Lee *et al*. 2002). All phylogenetic analyses were also performed using IGS markers which separately code for regions of $tRNA^{Leu}$ and $tRNA^{Phe}$ genes in the chloroplast genome.

These molecular and chloroplast markers are very suitable in the analysis of the relationship between closely related populations and species. Lately, these markers have been successfully applied in the study of the *Cyperaceae*, especially to differentiate between taxa at the level of sections and subspecies, as well as to reconstruct the phylogenetic dependencies among species (Starr *et al*. 1999, Roalson & Friar 2000, 2004, Roalson *et al*. 2001, Hendrichs *et al*. 2004a, b).

Material and methods

DNA isolation, PCR amplification and sequencing of ITS and IGS regions

Total genomic DNA was extracted from live or dried herbarium leaf tissue of single individuals following DNAeasy Plant Mini Kit (Qiagen) and the manufacturer's protocol. A list of specimen whose tissue was taken for study is compiled in Table 1 (the oldest herbarium specimen dated from 1891). The obtained DNAs were used in the PCR reactions, for which ITS or IGS sequences were amplified. From two to five individuals from single populations of *C. repens* were used for PCR amplification and sequenced to determine whether any variation of ITS or IGS regions might exist at the population level.

TABLE 1. Materials of *Carex repens* used in the study. Herbarium vouchers are stored in Adam Mickiewicz University (POZ), University of Warsaw (WA), University of Wrocław (WRSL). The unnumbered herbarium vouchers are supplemented with information that a specimen in question was subjected to molecular analysis.

Material	***Population localization***	***Voucher***	***Date***
Herbarium	Turin, Italy	*Ferrari & Perrino s.n.* (WA)	1898
	Turin, Italy	*Ariangeli 22263* (WA)	1891
	Jajtawy, Poland	*Żukowski s.n.* (POZ)	1976
	Jajtawy, Poland	*Spribille s.n.* (WRSL)	1896
Fresh	Chełmno (Klamry), Poland	*Żukowski s.n.* (POZ)	2002
	Toruń-Bielawy, Poland	*Żukowski s.n.* (POZ)	2002
	Czerniewice, Poland	*Żukowski s.n.* (POZ)	2002
	Ustronie (Przyłubie), Poland	*Żukowski s.n.* (POZ)	2002

DNA for the complete ITS (Internal Transcribed Spacer) region (3' 18S to 5' 26S) was PCR amplified from total genomic DNA using the ITS4i forward and ITS5i revers primers described by Roalson *et al*. (2001). DNA for the complete IGS (Intergenic Spacer) region (3' $tRNA^{Leu}$ to 5' $tRNA^{Phe}$) was PCR amplified from total genomic DNA using the *trnL*e forward and *trnL*f revers primers (Taberlet *et al*. 1991).

The PCR reaction mix for ITS amplification consisted of 10 mM Tris-HCl, pH 8.8, 50 mM KCL, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 0.5 units of Taq polymerase (*Fermentas*), 0.01 mM of each primer, and 20–40 ng total

DNA template in 10 µl reaction volume**.** Double-stranded PCR products were produced on MJ Research thermocycler via initial denaturation at 94°C for 2 min, 36 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 52°C for 1min and DNA strand extension by Taq DNA polymerase at 72°C for 1 min. The PCR was terminated at the end of 36 cycles by final extension at 72°C for 5 min. PCR products were electrophoresed in 2% agarose gel in 1X TBE (pH 8.3) buffer stained with ethidium bromide to confirm a single product and purified using the Gel–Out kit (A&A Biotechnology).

PCR reaction mix for IGS consisted of 10 mM Tris-HCl, pH 8.8, 50 mM KCL, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 0.5 units of Taq polymerase (Fermentas), 0.01 mM of each primer, and 20–40 ng DNA template in 15 µl total reaction volume**.** Double-stranded PCR products were produced on MJ Research thermocycler via 34 cycles of DNA denaturation at 94°C for 30 sec, 94°C for 30 sec, primer annealing at 56°C for 1 min and DNA strand extencion by Taq DNA polymerase at 72°C for 1 min. The PCR was terminated at the end of 34 cycles by final extencion at 72°C for 5 min. PCR products were electrophoresed in 2% agarose gel in 1X TBE (pH 8.3) buffer stained with ethidium bromide to confirm a single product and purified using the Gel-Out kit (A&A Biotechnology).

The dsDNA obtained was sequenced directly on both strands using Applied Biosystems 3130*xl* Genetic Analyzer. The sequences of both strands were combined and proof read with Chromas v1.45 and GeneDoc v2.4. Representative species consensus sequences for ITS and IGS marker have been deposited in GeneBank (accessions: AY620233 and DQ486862).

Phenetic analysis

Coding regions (18S RNA, 5.8S RNA, 26S RNA), ($tRNA^{Leu}$, $tRNA^{Phe}$) and spacer regions (ITS 1, ITS 2), (IGS) were determined by comparison to the published sequences for other *Carex* species (Roalson *et al*. 2001). Complete sequences for the entire ITS region (ITS 1-5.8 sRNA-ITS 2) were obtained for all individuals and included in the analysis. Ambiguous regions for IGS sequences alignment were excluded from all distance calculations and phylogenetic analysies in order to reduce systematic error.

DNA sequences were aligned using Clustal W v1.4 (Thompson *et al*. 1994) and Muscle (Edgar 2004) then adjusted manually to minimize gap number using GeneDoc v2.4. Phylogenetic Neighbor-Joining (NJ) analysis was performed using PAUP * 4.0beta for Macintosh (Poe & Swofford 1999). For dendrogram visualization, the TreeView v. 1.6.6 program was used.

Principal Component Analysis (PCA) test was used to explain genetic variations between all studied populations. All phylogenetic and statistical analysies were performed in two different methods: (1) with ITS sequences data alone, (2) combined ITS and IGS sequences data. For (PCA) test Tanagra 1.4.13 program was used.

Results

The ITS spacer lengths for all individuals *C. repens* examined was determined to be 270 bp for ITS 1, and from 221 bp to 225 bp for ITS 2, which is consistent with the range of values determined for these species by Starr *et al*. (1999). GC mean content for ITS 1 is about 61.06% and 63.54% for ITS 2. The 5.8S RNA subunit in *C. repens* was 166 bp in length. The IGS spacer lengths separating $tRNA^{Leu}$ and $tRNA^{Phe}$ chloroplast genes for analyzed individuals was determined to be from 362 bp to 363 bp and GC mean content for IGS is about 26.76%. Summary statistics for ITS 1, ITS 2, combined ITS 1/ITS 2 and IGS sequences for all individuals sequenced in this study are given in Table 2. The phylogenetic NJ trees showing the relations between studied populations for ITS and combined ITS/IGS data set are shown in Fig 1.

TABLE 2. General DNA sequences statistics for ITS 1, ITS 2, combined ITS 1/ITS 2 and IGS.

	ITS1	***ITS2***	***Combined ITS1+ITS2***	***IGS***
Length range (bp)	217	221–225	438–442	362–363
GC content average (%)	60,83–61,29	62,67–64,41	61,76–62,87	26,45–27,07
Number of variable sites	1	7	8	4
Number of constant sites	216	220	437	359
Number of parsimony informative	1	2	4	1
Number of autopomorphy sites	1	4	3	3
Aligned length (bp)	217	229	446	364

In the first example, where only ITS data were used, the PCA method components 1 and 2 describe 94.96% of the genetic variation between all studied populations and 93.1% when combined ITS/IGS data were used. Relative position of the objects in two-dimensional space described by principal components 1 and 2 of ITS and combined ITS/IGS data set in PCA analyses are presented by plots in Figure 1. The population group, for which we observe no differences in ITS 1 and ITS 2 sequences was reported to include: Jajtawy-1896, Jajtawy-1976, Toruń-Bielawy-2002 and Przylubie-2002. This group contains current *C. repens* populations and populations from historical sites found in Poland. The reported distance between all these populations is small: the greatest (0.0168) was reported between the Polish and Italian populations.

Discussion

So far no viable data has been provided to confirm the hypothesis that *Carex posnaniensis* is the same species as *C. repens* (Fig. 2). In their descriptions of the two species both Spribille (Kneucker 1896) and Kükenthal (1909) used only a few morphological features. Hence the deviations observed for *C. posnaniensis* by Spribille from the features characterizing *C. repens* may be ascribed to the fact that the Polish population occur at the margin of the geographical range of this species. In such marginal populations it is common to find that some species exhibit different features than found in the more typical forms of this species. This is probably the effect of the already well-described genetic phenomena such as inbreeding and bottleneck (Lynch & Gabriel 1990, Barret & Kohn 1991).

The results of our studies, where ITS and IGS molecular markers have been studied, a great similarity between the present Polish population of *C. repens* and the populations occurring in the center of the geographical range of this taxon is indicated. In spite of the relatively small taxonomic distance between all the populations, it is possible to note that this distance increases with a growing geographical distance between the populations. This explains why two populations from Italy, which are geographically in the center of the natural range of *C. repens*, are in the phenograms the most distanced from each other. Moreover, the taxonomic distance between these two populations with respect to the distance between other populations has been noted to be the largest. This is probably the result of the difference in age between these populations, and differences in their level of variability. In comparison with the Italian populations, the Polish are significantly younger, as they were able to appear only after the Baltic glaciation. Besides age, the distance between these populations, and in particular between the Italian ones, is also affected by inbreeding - which generates variation - and a certain geographical isolation, hindering pollen dispersal.

It was also observed that there are no differences in ITS 1 and ITS 2 sequences between populations occurring in the area of the Jajtawy population, from where Spribille described *C. posnaniensis*. The population at the locality from where *C. posnaniensis* was described still existed until the late 1970s, but it no longer there and is now an historical locality. Similar populations 6–40 km from Jajtawy, may be ascribed to this taxon, because it can be argued that this population originates from the diaspora of the original type locality.

The results of our study, revealing small genetic distances between the examined populations, indicate that populations described by Spribille as *C. posnaniensis* are not a separate species from *C. repens*. It is likely that in Poland, *C. posnaniensis* displays slightly different morphological features than *C. repens*, resulting form its occurrence at the limit of the species range, in different environmental conditions.

Plants of *C. repens* produce generative shoots with ripe seeds, which exhibit germinating disability, which has not been reported before. Moreover, it has been found that the sequence differentiation level is lower in IGS regions than in ITS marker sequences. The low number of variable sites indicates that IGS sequences are more conservative in character and confirm to a slower rate of mutations in chloroplast genomic DNA during evolution. We also observed no significant difference between two marker sequences within *C. repens* populations that are 100 years apart. This finding seems to suggest that these markers are effective tools to describe relations, not only between species in one section (Starr *et al*. 1999, Hendrichs *et al*. 2004a, b), but also between populations of the same species. Although we used two distinct molecular markers from different cell compartments and cell genomes, our sampling of populations across the species is far from complete. Therefore our study should be treated as a preliminary examination in which we aim to reconstruct past processes and relationships within the species during the last thousands of years. In the future we plan to expand our hypothetically reconstructed trees of dependencies between populations of *C. repens*, verifying them using other molecular markers or techniques, and include a wider spectrum of populations of this species.

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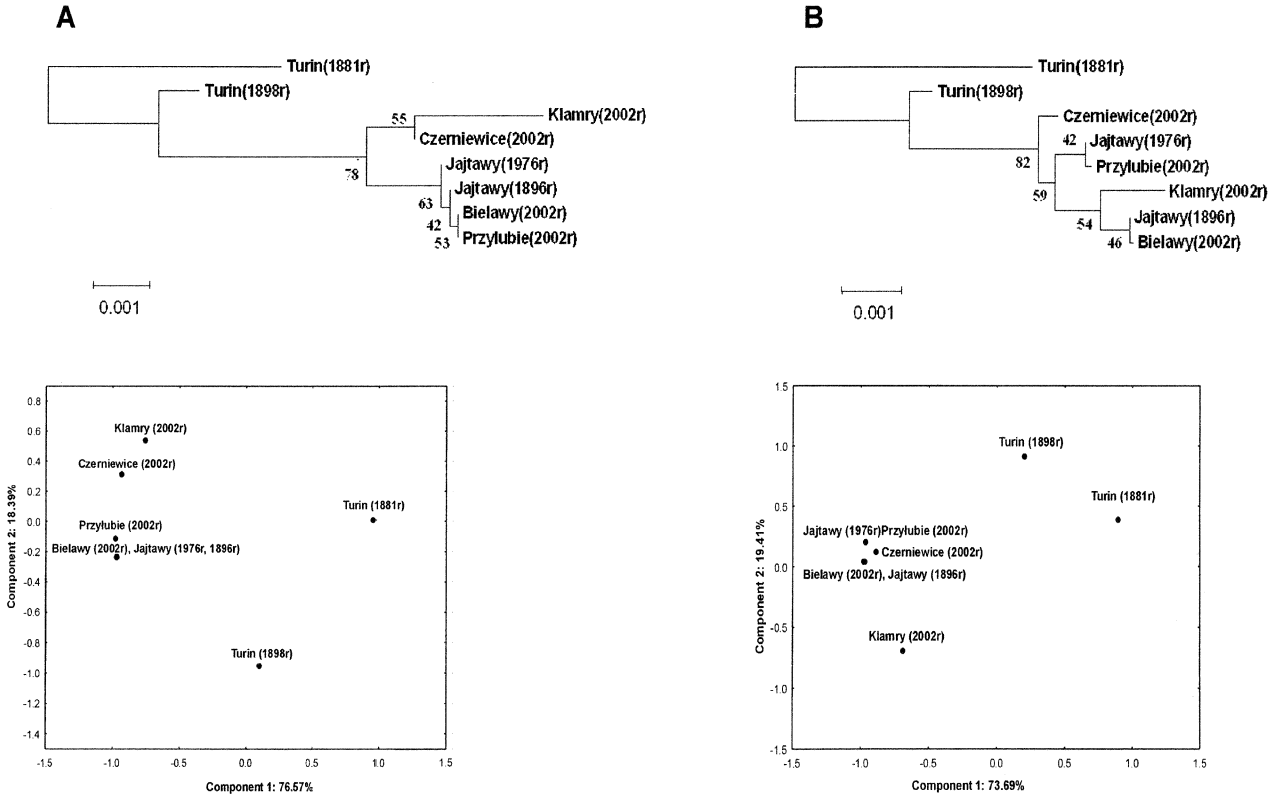

Fig. 1. Phylogenetic Neighbour –Joining (NJ) trees and Principal Components Analysis (PCA) in two- dimensional plots showing relationships between Polish and historical populations of *Carex repens*. A – for ITS sequences data set only, B - for combined ITS/IGS data.

Fig. 2. Herbarium voucher of *Carex posnaniensis* from 1898. After morphological verification by W. Żukowski and T. Egorova and molecular analysis this taxon is currently recognized as *Carex repens*.