

Low allozyme variation in *Carex loliacea* (Cyperaceae), a declining woodland sedge

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The genetic diversity within and among 17 populations of *Carex loliacea* from Estonia, Finland, Sweden, Poland and southcentral Alaska was evaluated by isozyme analysis. An extremely low allozyme variation was found. Fourteen populations were monomorphic at all 18 isozymes. Only three Estonian populations studied showed limited variation of one isozyme, malate dehydrogenase MDH-A, with two allozymes. Almost all allozyme variation was observed as homozygous variants. Of 400 individuals analyzed, only six revealed heterozygous morphs. The high level of inbreeding ($F = 0.91$) clearly shows that *C. loliacea* is a predominantly selfing species. The variation of MDH-A showed some geographic distribution. Finnish, Swedish and Alaskan populations form one group, and Polish and Estonian populations form another. The pattern of intraspecific geographic differentiation may reflect the postglacial recolonization routes.

Key words: allozymes, *Carex loliacea*, decreasing distribution, geographical variation

Introduction

Carex loliacea is a declining species in Estonia and declining, rare or extinct in many other regions in Europe (Hegi 1939, Schweitzer & Polakowski 1994, Garve & Kiffe 1997, Pawlikowski 2001, Hallanaro *et al.* 2002). However, there is a severe lack of knowledge on a population genetic level. This information would be important since within-population genetic variation is needed to adapt to constantly changing and varying environments. Low levels of genetic variation are therefore mostly associated with

high extinction risks (Lande 1988, Young 1996). The principal reason for the decline of *C. loliacea* is presumed to be intensive land draining causing destruction and loss of habitat. The species is associated with rich wet sites, such as swamp forests, spruce mires and paludified forests. Because *C. loliacea* is sensitive to draining, it prefers relatively pristine stands, especially along forest streams or in depressions. It also favours clear-cut areas exploiting the abundant light and moisture.

Carex loliacea belongs to the subgenus *Vignea* (Egorova 1999). It is a boreal-montane

species, with a circumpolar distribution but evidently having wide gaps in the North Atlantic and Bering Sea areas (Hultén & Fries 1986). It is a morphologically uniform herbaceous perennial with short, slender rhizomes, forming sparse tufts and with gynecandrous spikes (Hegi 1939, Eichwald 1966, Hämet-Ahti *et al.* 1998, Krall *et al.* 1999). The fruit production is relatively high. According to Novikov (1980), an average of 40 generative culms and 300–400 seeds can be found on one square meter. However, seedling recruitment is rare for undisturbed vegetation (personal observations). The seeds are dispersed by water and birds (Novikov 1980).

According to the inflorescence type, *Carex* species are generally assumed to be wind pollinated (Hesse 1980, Tarasevich 1992, Alekseev 1996, Egorova 1999). As wind-pollinated plants, sedges are supposed to be mostly outcrossed. Isoenzyme studies have shown outcrossing for many sedges and selfing for only some species (Ford *et al.* 1991, Whitkus 1992, Hedrén & Prentice 1996). Alekseev (1996) also noticed that both self- and cross-pollination exist within the genus. There are no data on the pollination mode of *C. loliacea*, and no studies of its breeding system have been carried out.

In Estonia, *C. loliacea* is frequent in the northeastern and southeastern parts; in other regions it occurs sporadically at most (Kuusk *et al.* 2003). In Finland, the species is common throughout the country (Hämet-Ahti *et al.* 1998). In Sweden, it is mostly confined to the northern and central parts (Mossberg *et al.* 1992). In Poland, the species is quite rare, restricted to the northern part of the country, which represents the southern margin of its distribution range in Europe (Pawlikowski 2001). In Alaska it grows sporadically in the southeastern three-quarters of the mainland (Tande & Lipkin 2003).

Isoenzyme data had been used successfully to study the genetic diversity in different *Carex* species (Ford *et al.* 1991, Whitkus 1992, Hedrén 1996, Hedrén & Prentice 1996, Ford *et al.* 1998, Jonsson 1998, Stenström *et al.* 2001, Tyler 2002a, 2002b, Tyler *et al.* 2002, Hedrén 2003, Tyler 2003), but there the genetic diversity and geographical structure of *C. loliacea* were not studied. The aims of this work were (1) to investigate the extent of allozyme variation in

C. loliacea, (2) to determine the mating system of the species, (3) to describe the distribution of genetic diversity of *C. loliacea* within and among populations in different regions, and (4) to discuss whether the decline of the species could be associated with its mating system.

Material and methods

Seed collection

Seeds of *C. loliacea* were collected in the summers of 2003–2005 from six populations in Estonia, two in Finland, three in Sweden, three in Poland and three in south-central Alaska (Table 1). The seed collection sites in Estonia were distributed throughout the country. The major habitats of the Estonian populations were swamp forests and paludified forests with *Picea abies* as the dominant tree species, accompanied by *Betula pubescens*, *Alnus glutinosa*, *Pinus sylvestris* and others. The Polish collection sites are situated in the northeastern part of the country. The Polish habitats were *Picea*–*Alnus* (*Picea*–*Betula*–*Alnus*) forests on the margin of raised bogs or fens. In Finland, the collection sites were in the northeastern part of the country, in the Kuusamo commune. The Finnish habitats were usually spruce mires on slopes and along streams. The Swedish collection sites were situated in the Vindeln commune in the central part of the country, and the habitats were similar to those in Finland. In south-central part of Alaska Peninsula *C. loliacea* was found along streams and by wet pathways, accompanied by *Picea mariana* and *P. glauca*.

Most populations of *C. loliacea* studied were small, usually occupying only few square meters. Only the EE1, EE3 and EE4 sites in Estonia and POL3 in Poland were larger, covering each up to 0.5–1.5 km². In Finland and Sweden, the species is rather common but the population size is usually small.

Each seed accession collected consisted of a bulk seed sample from an individual population. Seeds were collected from at least five separate tussocks in each population, except from the Alaskan populations, which consisted of two tussocks each. This study analysed 15–25 seed

progeny of a few mother plants from each population. Seeds collected from mother plants in the wild were formed through pollination from plants comprising a population, and the Wright's statistics allows to quantify the outcrossing rate in each population that is polymorphic for allozymes.

The collected seeds were stored in paper bags in the laboratory at approximately 20 °C for four months. For stratification, seeds were kept wet in darkness in a refrigerator at 4 °C for two months. Germination was carried out in the laboratory at fluctuating day/night temperature of 28/17 °C. Specimens are deposited in the herbarium of the Institute of Agricultural and Environmental Sciences (TAA, Tartu, Estonia).

Isozyme analysis

Eleven enzymes were examined: malate dehydrogenase (MDH, EC 1.1.1.37), shikimate dehydrogenase (SKD, EC 1.1.1.25), aspartate aminotransferase (AAT, EC 2.6.1.1), superoxide dismutase (SOD, EC 1.15.1.1), 6-phosphoglucate dehydrogenase (6PGD, EC 1.1.1.44), phosphoglucoisomerase (PGI, EC 5.3.1.9), peroxidase (PRX, EC 1.11.1.7), phosphoglucomutase (PGM, EC 2.7.5.1), alcohol dehydrogenase (ADH, EC 1.1.1.1), leucine aminopeptidase (LAP, EC 3.4.11.1) and esterase (EST, EC 3.1.1.2).

Enzyme extracts were prepared from seedling leaves (about 1–2 months old) by grinding in 0.05 M Tris(hydroxymethyl)aminomethane (Tris)–0.01 M EDTA buffer containing 5 mM cysteine. After adding 20–50 mg of sucrose–Sephadex G200 mixture (4:1) to increase their viscosity, the extracts were subjected to electrophoresis in vertical polyacrylamide gel slabs (120 × 70 × 2 mm). The following four gel-buffer systems modified from Jaaska (1997) and Oja (1999) were applied for different enzymes to attain better band resolution:

Gel 1: 10% acrylamide, 0.2% N,N'-methylenebis-acrylamide (Bis), 0.25 M Tris, 0.1 M HCl; applied for EST and SOD.

Gel 2: 10% acrylamide, 0.2% Bis, 0.15 M Tris, 0.1 M HCl; applied for LAP, ADH, AAT, SKD, PGI, PGM and 6PGD.

Table 1. Collection sites of seeds of *Carex loliacea* with geographical coordinates. N = number of individuals analyzed, N_M = number of maternal individuals analysed.

Country	Code	Collection site	Lat. N	Long. E	N	N _M
Poland	POL1	Romincka Forest, close to village Czarnowo Srednie	54°20'00"	22°27'29"	17	5
	POL2	Romincka Forest, "Mechacz Wielki" nature reserve	54°20'12"	22°26'14"	25	10
	POL3	Romincka Forest, "Zytkiemska Struga" nature reserve	54°21'05"	22°37'29"	25	10
Finland	FIN1	SSO from Oulanka Biol. Station, near to Ylimmäinen Hiidenlampi	66°21'48"	29°20'10"	25	5
	FIN2	Kuusamo, Juuma	66°20'48"	29°19'79"	25	5
Sweden	SWE1	Vindeln, Stora Sandsjö	64°05'52"	19°05'87"	25	10
	SWE2	Vindeln, Lappkuliden	64°16'57"	19°39'12"	25	10
	SWE3	Vindeln, Selsberg	64°00'76"	19°35'62"	25	5
Estonia	EE1	Tartumaa, Järvelja	58°16'69"	27°19'30"	25	10
	EE2	Pärnumaa, Urissaare	58°00'54"	24°36'48"	18	5
	EE3	Ida-Virumaa, near to the Poruni river	59°09'81"	27°47'98"	25	10
	EE4	Ida-Virumaa, Mustajõe	59°17'08"	27°53'61"	25	5
	EE5	Ida-Virumaa, Aadamäe	59°04'95"	26°48'93"	25	5
	EE6	Lääne-Virumaa, Suigu	59°09'03"	26°49'25"	25	5
Alaska	AL1	Lääne-Virumaa, Suigu	61°25'78"	149°24'61"	15	2
	AL2	Alaska, 20 km N of Anchorage, near Mirror Lake	61°24'90"	149°10'89"	25	2
	AL3	Alaska, northeast of Anchorage, road to Eklutna Lake, Alaska, Wasilla, Big Lake	61°31'60"	149°51'75"	25	2

Gel 3: 7.5% acrylamide, 0.2% Bis, 0.4 M Tris, 0.1 M HCl; applied for MDH and PRX.

N,N,N',N'-Tetramethylethylenediamine (0.05 ml %) and ammonium persulfate (1 mg %) were added to the gel mixtures to initiate and catalyze their photopolymerization between two daylight fluorescence bulbs for 1 h. The upper cathode was 80 mM glycine with 10 mM Tris. The lower anode buffer was always 0.1 M Tris-acetate with the initial pH about 8.9, and it was used repeatedly as long as the pH remained > 7. Ice-refrigerated electrophoresis was carried out by applying a pulsed current at 15 mA and 20–30 V cm⁻¹ for about 2–2.5 h until the bromphenol blue marker dye reached the gel end. After electrophoresis, the gels were stained for isozymes by applying standard histochemical methods (Wendel & Weeden 1989).

The isozyme results are described at the level of isozyme phenotypes that correspond to respective genotypes. Isozymes encoded by separate loci are designated by capital letters followed by a number reflecting allozymes in the order of decreasing mobility. The allozyme numeration is unified for *C. loliacea* and its related species under study. Heterozygous phenotypes are denoted by a slash separating numbered allozymes, e.g. MDH-A1/2. Genetic interpretation of zymograms is based on the available information on the subunit structure and principles described by Wendel and Weeden (1989). In total 400 individuals of *C. loliacea* from 17 populations were used in the analyses.

Data analysis

To characterise the genetic diversity quantitatively, the following parameters were calculated: the number of alleles per locus (*A*), the percentage of polymorphic loci (*P*), Wright's fixation index (*F*), the observed (*H_o*) and expected heterozygosity (*H_e*). The analysis of progeny lines in studies of mating systems in plants is a widely used and accepted approach that is theoretically founded in works of Brown and Allard (1970) and Clegg (1980). We used the Wright's inbreeding coefficient *F* (Wright 1965) computed from polymorphic allozymes in the progeny grown

from seeds collected in the wild to estimate the extent of selfing in natural populations. An isozyme was considered polymorphic when two or more allozymes were detected, regardless of their frequency. The number of polymorphic populations of *C. loliacea* that could be used for calculations was limited. Most populations proved to be totally genetically homogenous.

Results

The isozyme variation observed among and within populations of *C. loliacea* was very low. Eleven enzymes with 18 isozymes and 19 allozymes were interpreted. Only one (MDH-A) of the 18 isozymes was polymorphic. Except for MDH-A all isozymes displayed complete homozygosity. Isozyme MDH-A showed variation with two distinctly separated allozymes A1 and A2. Isoenzymes of SKD, AAT, PGI, PGM and 6PGD were totally monomorphic in all populations, with homozygous SKD-A3, AAT-C1, PGI-A2, PGM-A1 and 6PGD-A1, respectively. Peroxidase showed three homozygous isozymes, PRX-A1, PRX-D1 and PRX-F1, in all populations. LAP revealed two homozygous isozymes, LAP-A3 and LAP-B1. EST showed two interpretable monomorphic isozymes, EST-A2 and EST-B2. SOD displayed four homozygous isozymes. Isozyme SOD-A was much faster than the remaining three isozymes, SOD-B, SOD-C and SOD-D. ADH showed a clear invariant band for isozyme ADH-A. Additional zones of activity were observed for ADH and MDH, but these were not considered because we were unable to measure them adequately for all individuals.

The mean number of alleles per polymorphic locus was 1.05 and percentage of polymorphic loci was 5.56. Mean observed and expected heterozygosity and Wright's fixation index values in polymorphic populations are given in Table 2. In all polymorphic populations the expected heterozygosity based upon Hardy-Weinberg expectations was much higher than the observed heterozygosity (Table 2), demonstrating the deficiency of heterozygotes and indicating dominance of self-fertilization in these populations. All except the three largest Estonian populations (EE1, EE3 and EE4) were monomorphic at all isozymes.

Population EE3 revealed heterozygous MDH-A1/2 in three individuals out of 25 resulting in a fixation index $F = 0.73$. Population EE4 revealed only one individual with heterozygous MDH-A1/2 ($F = 0.92$). Population EE1 was polymorphic for the same two MDH allozymes, but no heterozygotes were detected, indicating no outcrossing in this population. The distribution of the two allozymes showed some geographic pattern. MDH-A1 was the only variant in the Polish populations and a prevalent morph in Estonian populations. The Finnish, Swedish and Alaskan populations were monomorphic for alternate allozyme A2 (Table 3 and Fig. 1). However, the Alaskan population AL3 showed two individuals heterozygous for A1/2, but no homozygous variant A1, indicating a possible outcrossing with gene flow from neighbouring population with homozygous allozyme A1 not sampled. The geographic distribution of the allozymes is shown in Fig. 1.

Discussion

Levels and patterns of genetic variation in populations of plant species depend on the mating system, population size, seed dispersal etc. The great decrease in population sizes of wild plant species due to ongoing habitat fragmentation leads to smaller and more isolated populations, whose reduced genetic variation makes them more vulnerable (Oostermeijer *et al.* 2003, Godt *et al.* 2005). *Carex loliacea* has small and fragmented habitats and the isoenzyme analysis indicates that the species has extremely low genetic diversity within and among populations. Fourteen populations out of the 17 studied, i.e. 82% of the sites, were monomorphic at all 18 isozymes. Only the three largest Estonian



Fig. 1. Sampling areas of *Carex loliacea* and geographical distribution of MDH enzyme phenotypes: MDH-A1 = grey dot, MDH-A2 = black dots, both MDH-A1 and MDH-A2 = black square.

populations (EE1, EE3 and EE4) displayed low polymorphism of one isozyme, MDH-A, with two allozymes.

Table 2. Genetic variability at the MDH-A isozyme in polymorphic populations EE1, EE3, EE4 of *Carex liliacea*: number of individuals analyzed (N), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F).

Population	N	H_o	H_e	F
EE1	25	0	0.40	1
EE3	25	0.12	0.45	0.73
EE4	25	0.04	0.50	0.92

Table 3. Allele frequencies for MDH-A allozymes in the *Carex loliacea* studied. 18 loci coding for SKD, AAT, PGI, PGM, 6PGD, PRX, LAP, ADH, SOD and EST were totally invariable. The site codes are the same as those in Table 1.

[illegible]

Few authors have found similarly very low allozyme variation in *Carex* species. Waterway (1990) reported extremely low polymorphism and no heterozygotes in any of the populations of *C. gynodynamis*, an endemic in the California Floristic province. All individuals sampled of that species were monomorphic for the same allozymes for 16 out of 17 isozymes studied. Only one isozyme, GPI-1, showed any variation. Waterway (1990) supposed that the low level of polymorphism in *C. gynodynamis* might be caused by genetic bottlenecks. A low degree of genetic variation was also found in the *C. pachystachya* complex (Whitkus 1992), in which the mean number of alleles per polymorphic locus was 1.2 and only 20% of all loci were polymorphic. Schell and Waterway (1992) found low levels of isozyme heterozygosity and polymorphism both within and between populations of the rare and endemic sedge *C. misera*. They found an average of 1.5 alleles per polymorphic locus and 30% of loci polymorphic for this species. Still, these parameters of the *C. pachystachya* complex and of *C. misera* show a higher allozyme variation than in *C. loliacea*, which has 1.05 alleles per polymorphic locus and only 5.5% of loci polymorphic, despite the fact that *C. loliacea* is not an endemic but has a wide circumpolar distribution. Hamrick and Godt (1996) showed that the extent and distribution of genetic diversity within and among populations are greatly influenced by the mating system. The three Estonian polymorphic populations of *C. loliacea* possessed *F* values ranging from 0.73 to 1, indicating that selfing is highly prevalent within the populations. The capability of *C. loliacea* populations consisting of a single tussock to produce seeds able to germinate (pers. obs.) argues in favour of self-fertilization. In addition, the close proximity of pistillate and staminate flowers and the loosely caespitose growth habit promote selfing in such sedges and may therefore result in a low level of heterozygosity (Ford *et al.* 1998). Ford *et al.* (1998) classified *Carex* species into two groups. Group 1 consists of species with hermaphroditic spikes and caespitose growth. The likelihood of selfing is increased in this group. The species have low intrapopulation variation but high interpopulation and interspecific variation. Group 2 is characterised by

prevalent rhizomatous growth and widely spaced unisexual spikes. The chance of outcrossing is increased among this group. According to this classification, *C. loliacea* belongs to the group 1.

The principal method of seed dispersal for *C. loliacea*, as for the other wet forest *Carex* species, is by water, e.g. during the spring and autumn overflows or along a stream or a river. The plant may form culms up to 80 cm long and is able to distribute its seeds itself up to this distance. This mode is quite plausible because the populations are often small and composed of compactly spaced, loose tussocks. As *C. loliacea* is a woodland understory species and has during the flowering time very short culms (they elongate later during seed maturation), the potential genetic contact between populations through wind pollination is not likely. According to our observations, *C. loliacea* forms sparse tussocks with very short rhizomes (0.1–6.5 cm), and the possibility for vegetative propagation through the root-cuttings from a parental tussock is very low. Thus, the relatively low genetic diversity in *C. loliacea* could be explained by its fragmented distribution pattern together with predominant selfing.

Bolkovskikh *et al.* (1969) reported the chromosome number for diploid *Carex* species $2n = 16$ or 18 . According to the high chromosome number ($2n = 54$ or 56) published in the literature (Heilborn 1924, Moore & Calder 1964, Novikov 1980), *C. loliacea* is supposed to be a hexaploid. But as the species revealed isozyme electrophoretic patterns typical of a diploid, with no increase in the isozyme number and heterozygosity we may suppose that it is a diploidized polyploid, i. e. functionally diploid. A noteworthy result of our study is that the diploidized polyploid *C. loliacea* revealed an extremely low level of isozyme heterozygosity, which has been found previously in only some sedge species (Waterway 1990, Whitkus 1992), but is characteristic of many highly polyploid homosporous ferns (Haufler & Soltis 1986, Haufler 1987).

The spatial structure of genetic variability has been widely studied on local and geographical scales and was found to be influenced by life form, seed dispersal, geographic range, and breeding system of species (reviewed by Hamrick & Godt 1996). Selfing species show higher allozyme divergence among populations

as opposed to outbreeding species, which have most diversity within populations. Consistent with this general view, the largely selfing *C. loliacea* revealed a distinct geographic differentiation between Polish and Fennoscandian populations with alternate allozymes at the polymorphic MDH-A locus. However, no further differentiation was found between Swedish and Finnish populations.

Evolutionary conservative isozyme loci with limited allelic variation may give plausible evidence for species phylogeographic inferences. For example Cronberg (1998) in his paper about phylogeography of *Sphagnum* spp. used evidence about the geographic distribution of two alleles, *Idh*-1A and *Skd*-1B, for making conclusions about postglacial migration roots of species. The observed pattern of intraspecific geographic differentiation by alternate MDH-A allozymes in *C. loliacea* may also reflect the impact of glaciation in northern Europe. Poland can have been a refugium during the last glaciation from where *C. loliacea* migrated northwards. Northern Poland is today the southernmost area in Europe where climatic conditions are still suitable for the species. Another glacial refugium for northern Europe might have been in the central regions of Russia or Alaska that remained unglaciated during the last Ice Age (Velichko *et al.* 1997). Thus, *C. loliacea* could have migrated to Estonia from two different sources: from the south (Poland) and from the east (Russia), but to Fennoscandia most probably from Russia, because MDH-A1 characteristic of Poland populations was not found in the Fennoscandian populations. Further investigations of local populations in different geographical regions, especially from Russia, would be necessary to elucidate the postglacial phylogeographic history of *C. loliacea*.

Several explanations can be supposed to explain the low genetic diversity of *C. loliacea*. The reduced genetic diversity in small populations may have resulted from inbreeding, genetic drift or bottlenecks (Lande 1988, Young *et al.* 1996). Due to active draining of wet forests, *C. loliacea* suffers from fragmentation of populations and loss of habitat. The populations of *C. loliacea* are often small and widely separated. This results in restricted gene flow among them and may lead to higher probability of self-pollination or cross-pollination between nearby close

genetic relatives within populations, decreasing genetic variation and facing a high risk of extinction. Thus, our data agree with the general view that genetic diversity and population size are often positively correlated, and that small populations frequently have lower genetic diversity than large populations (Brigham 2003, Godt *et al.* 2005). The small fragmented populations in Estonia appeared totally monomorphic at all isozyme loci, whereas larger northeastern and southeastern Estonian populations revealed diallelic polymorphism of MDH-A, indicating the possible importance of the population size. Some other *Carex* species with restricted and fragmented distribution have also found to display low genetic diversity (Hedréen 1997, Ford *et al.* 1998). Hooftman *et al.* (2004) showed that habitat fragmentation not only affects the rare species in an ecosystem, but also reduces the survival probabilities of the common species. As *C. loliacea* is in decline throughout its distribution area, it would be beneficial to pay more attention to preserving its natural populations to avoid it becoming very rare or even extinct.

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