Cryptogamie, Bryologie, 2003, 24 (2): 147-154
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Isozyme variability among Central European species of the aquatic moss *Cinclidotus*

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(Received 10 October 2002, accepted 28 March 2003)

Abstract – An electrophoretic survey of isozyme systems of some populations in 4 Central European *Cinclidotus* species shows that the species are genetically clearly separated. Nine of sixteen tested enzyme systems were scoreable. Qualitative differences between species are detected in five of the scored enzyme systems. The theory of a hybrid origin of *Cinclidotus danubicus* is discussed but dismissed.

Aquatic moss / *Cinclidotus* / variability of species / isozyme / Central Europa

INTRODUCTION

Cinclidotus is a genus endemic to Europe and the Near East. In Central Europe, four (*C. nigricans*, *C. fontinaloides*, *C. aquaticus, C. danubicus*) or five species (if *Dialytrichia mucronata* is included in *Cinclidotus*) are known. Of these species *Cinclidotus danubicus* was not recognized before 1906, when it was described from the river Danube between Pöchlarn and Krems in Lower Austria. As herbarium revisions revealed (Philippi, 1967), the species has apparently not been found in the last century prior to its description and not in the upper parts of the Danube. Next, *Cinclidotus danubicus* was found along the Danube in 1925 in Hungary, 1928 along the Düna in former Yugoslavia. From Yugoslavia it was later described as *C. herzogii* Pavl. (Pavletic, 1955). In Germany, it was found first along the Rhine near Mainz in 1911 and later in 1921 and 1929 along the Upper Rhine, 1933 and 1938 also between Basel and Konstanz (data taken from Philippi, 1967). As Philippi (1967) stated, the northermost locality was Zons near Cologne (Feld, 1958) and the species was at that time not known in the Netherlands. In the meantime, the species has spread further along the Rhine (Frahm & Abts, 1993), probably also enhanced by improved water quality and increased water temperatures (Frahm, 1997). In addition to the previously known localities, Philippi (1967) reported the species from the river systems of the Doubs in Switzerland and France, the Saône, the Seine and the Tiber in Italy. Later, the species was found as new in Slowakia, Belgium and in additional localities in France (Frahm, 1997), which indicates a massive spreading during the past centuries.

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In their description of *C. danubicus*, Schiffner and Baumgartner considered the species as related to *C. fontinaloides* and *C. nigricans*, however, as stated by Amann (1933) and Philippi (1967), it shows relations to *C. aquaticus* and is in many respects intermediate between the latter and *C. fontinaloides* or *C. nigricans* (Table 1):

Tab. 1. Comparison of morphological characters of the species of *Cinclidotus* in Europe.

	C. fontinaloides	*C. danubicus*	*C. nigricans*	*C. aquaticus*
Leaves	Straight when wet Contorted when dry	Slightly hamate Slightly contorted when dry	Straight when wet Contorted when dry	Strongly hamate Straight when dry
	Widest at midleaf	Widest in lower third	Widest in lower third	Widest at leaf base, gradually contracted
Costa	Costa 1/10 of leaf base, excurrent at leaf tip	Costa 1/5 of leaf base, excurrent at leaf tip	Costa 1/10 of leaf base, excurrent at leaf tip	Costa (1/2)-1/3(-1/5) at leaf base, not excurrent at leaf tip
Diameter of laminal cells	8-10 (-12) µm	12-15 (-19) µm	7-13(-15) µm	8-10 (-12) µm
Habitat	Around high water line, flooded only scarcely	Below high water line, periodically flooded	Around high water line	Underwater most of the year

The facts that

a. *C. danubicus* is known only sterile (Frey *et al.*, 1995).
b. it was not known before 1906 (Schiffner & Baumgartner, 1906).
c. its characters are intermediate between *C. aquaticus* and *C. fontinaloides* or *nigricans* (cf. Table 1).
d. it has apparently spread over the last centuries from Austria to Hungary, Yugoslavia, Italy, Slowakia, France, Belgium, Germany and the Netherlands (Boros, 1971; Frahm, 1997; Hörmann, 1965; Korneck, 1960; Lambinon & Empain, 1973; Pavletic, 1965 [as *C. herzogii*]; Philippi, 1967; Touw & Rubers, 1989)

may suggest that *C. danubicus* might be of recent hybrid origin. The reason for the relatively late origin could be that both potential parental species occur in different habitats. *Cinclidotus fontinaloides* and *C. nigricans* are characteristic of borders of large lowland streams such as Danube or Rhine, and its tributaries. In contrast, *C. aquaticus* occurs in springs and streams in the calcareous Alps. Thus the chance for a crossing is quite small. It requires that both species not only grow together but also produce gametangia, which happens rarely in aquatic mosses. *C. danubicus* usually grows together with *C. nigricans* and *C. fontinaloides* but was also found growing together with *C. aquaticus*, each one time in tributaries of the Doubs and Saône (Philippi, 1967), respectively.

To test the hypothesis of a hybrid origin of *C. danubicus*, isozymes of *C. danubicus*, *C. fontinaloides*, *C. nigricans* and *C. aquaticus* were electrophoretically analyzed. Isozyme analysis has already been applied successfully for the detection of hybrid origins of a number of species including within the Bryophyta (e.g., in the Mniaceae Stoneburner *et al.*, 1991).

MATERIALS AND METHODS

The following samples were collected and cultured for at least 4 weeks before they were extracted for analysis:

A) Cinclidotus aquaticus: Germany, North Rhine-Westphalia, brook exit behind quarry « Liet » in Warstein, *ca* 340 msm, 2 samples. France, Dept. Haute-Saône, Grotte de la Solborde near Echenez, *ca* 3 km south of Vesoul, Karst spring, *ca* 300 msm, 2 samples. Austria, Salzburg, Fürstenbrunn, Fürstenquelle, 460 msm, *Gruber 3785.*

B) Cinclidotus fontinaloides: Germany, Rheinland-Pfalz, western Rhine shore at Rolandseck, Kreis Ahrweiler, *ca* 80 msm, 10 samples.

C) Cinclidotus danubicus: Germany, Rheinland-Pfalz, western Rhine shore at Rolandseck, Kreis Ahrweiler, *ca* 80 msm, 10 samples.

D) Cinclidotus nigricans: Germany, Rheinland-Pfalz, western Rhine shore at Rolandseck, Kreis Ahrweiler, *ca* 80 msm, 10 samples.

Ten samples of species B, C, and D (at distances of at least 5 m) were collected to obtain information about variability within the population. The samples were grown submersed in water with aeration at 15 °C and 1 500 Lux. Voucher specimen are located in the bryophyte herbarium of the botanical institute at the University of Bonn (BONN).

The following enzyme systems were included in the analysis: alcohol-dehydrogenase (ADH), *cis*-aconitase (ACO), acid phosphatase (ACP), aldolase (ALD), aspartate-aminotransferase (AAT), esterases (EST), formate-dehydrogenase (FDH), glucose-6-phosphate-isomerase (GPI/PGI), glutamate-dehydrogenase (GDH), hexokinase (HEX), isocitrate-dehydrogenase (IDH), malate-dehydrogenase (MDH), menadione-reductase (MNR), phosphoglucomutase (PGM), peroxidase (PRX), triosephosphate-isomerase (TPI), shikimate-dehydrogenase (SKD).

For extraction, electrophoresis and staining methods summarized by Cronberg (1995) were mainly used. Additional staining recipes were taken from Weeden & Wendel (1989) and Wendel & Weeden (1989). Gels were mainly stained using the agar overlay method with 1% agar. Most of the gels were documented by hand and some by additional photographs. The isozyme loci were named after the enzyme system and numbered from the most anodal to the most cathodal one (e.g. Gpi-1). Alleles were numbered likewise and denoted as superscripts (e.g. Gpi-$2^{1,1}$). Information about enzyme structure, localization and expected number of loci was taken from Kephart (1990).

RESULTS

Interspecific variation was present for GDH, GPI, HEX, IDH, MDH, SKD and TPI. Scorable banding patterns could be achieved for GDH, GPI, HEX, MDH and TPI. No variation between the populations of *C. aquaticus* or within the populations of the other species was observed. No results could be obtained for ACO, ADH and FDH. The enzyme systems ACP, EST and PRX produced only smeared bands. For AAT, ALD and PGM, a single band that was monomorphic within and among the species was observed.

Glucose-6-phosphate-isomerase (GPI)

For the dimeric GPI enzyme system each species revealed two or four different electromorphs (Fig. 1). The anodal banding region that is common to all species represents one locus (Gpi-1) that was only poorly resolved and hence was not used for interpretation. The cathodal banding region represents a second putative locus Gpi-2 with two different alleles.

One electromorph is present in *C. fontinaloides* (band no. 2, homodimer Gpi-$2^{1,1}$) and *C. nigricans* (band no. 4, homodimer Gpi-$2^{2,2}$).

In *C. danubicus* three isozyme electromorphs are observed (band no. 2-4). One of these is unique among the treated species (band no. 3). Its migration behaviour is intermediate between band 2 and 4 so it might be a hybrid heterodimeric isozyme Gpi-$2^{1,2}$. *Cinclidotus danubicus* exhibits an heterozygous banding pattern for the Gpi enzyme system.

Besides Gpi-$2^{1,1}$ in *C. aquaticus* a homodimer for the second allele (Gpi-$2^{2,2}$) seemed to be present in one gel. It produced a very weak and therefore questionable band. If this band actually represents a homodimeric isozyme, the question arises why no heterodimer Gpi-$2^{1,2}$ is present. The banding pattern in *C. danubicus* shows presence of a functional heterodimeric Gpi-$2^{1,2}$ isozyme. This leaves two possibilities for the questionable band in *C. aquaticus*: the weak band is an artifact or represents a third allele of Gpi-2 with a homodimer migrating to the same position as Gpi-$2^{2,2}$.

Band no.	*locialleles*	*C. aquaticus*	*C. fontinaloides*	*C. nigricans*	*C. danubicus*
1	Gpi-$^{?}$	-------	-------	-------	-------
2	Gpi-$2^{1,1}$	-------	-------		-------
3	Gpi-$2^{1,2}$				-------
4	Gpi-$2^{2,2}$	(-------)		-------	-------

Fig. 1. Schematic banding pattern for GPI; bands numbered from the most anodal to the most cathodal electromorph; dubious (extremely weak) band is put in parentheses; else staining intensity was not regarded.

Triosephosphate-Isomerase (TPI)

For the dimeric TPI system three different electromorphs are documented (Fig. 2). The most anodal one represents a probably monomorphic locus Tpi-1. It is found in all four species. For the second locus two different alleles could be detected.

Band no.	*loci^{alleles}*	*C. aquaticus*	*C. fontinaloides*	*C. nigricans*	*C. danubicus*
1	Tpi-^{1}	-------	-------	-------	-------
2	Tpi-$2^{1,1}$	-------		-------	-------
3	Tpi-$2^{2,2}$		-------		

Fig. 2. Schematic banding pattern for TPI.

The homodimer Tpi-$2^{1,1}$ is present in all species except *C. fontinaloides*. Here Tpi-2 is found for a second allele resulting in a more slowly migrating homodimer Tpi-$2^{2,2}$.

Malate-Dehydrogenase (MDH)

The dimeric malate dehydrogenase system is represented by a single locus in all four species (Fig. 3). Again, *C. fontinaloides* seems to possess a slightly different electromorph that migrates more slowly and is thus separated from the other species.

Band no.	*Loci*^{alleles}	*C. aquaticus*	*C. fontinaloides*	*C. nigricans*	*C. danubicus*
1	Mdh-$1^{1,1}$	-------		-------	-------
2	Mdh-$1^{2,2}$		-------		

Fig. 3. Schematic banding pattern for MD.

Isocitrate dehydrogenase (IDH)

The multibanded patterns for the dimeric IDH system were only poorly resolved but hint at a system of at least two alleles (Fig. 4). Interestingly *C. danubicus* shows a unique extra band in the cathodal region. As this electromorph is more extreme in migration behaviour than all others it has possibly evolved independently or at least not by hybridization of some of the visible electromorphs. In one of the first gels a faint band in the banding region 2 of *C. fontinaloides* was visible but since it was not found another time it could not be decided whether it was an artifact or possibly lost activity due to storage. Band 4 in *C. danubicus* was always weak.

Banding region	*Loci*^{alleles}	*C. aquaticus*	*C. fontinaloides*	*C. nigricans*	*C. danubicus*
1	(Idh-$1^{1,1}$)	-------	-------	-------	
2	(Idh-$1^{1,2}$)	-------	(-------)	-------	
3	(Idh-$1^{2,2}$)	-------	-------	-------	-------
4	(Idh-$1^{3,3}$)				-------

Fig. 4. Schematic pattern of banding regions for IDH. Proposed loci and alleles in parentheses.

Hexokinase (HEX)

For monomeric hexokinase three electromorphs were detected in *C. aquaticus* and *C. nigricans* (Fig. 5). Only one (Hex-2) is present in *C. fontinaloides* and *C. danubicus*. Banding patterns are uncommon in this case: *C. aquaticus* and *C. nigricans* possess three bands that represent three different loci or two loci with one being heterozygous. *C. fontinaloides* and *C. danubicus* in contrast show only one locus. This may hint either at the existence of null alleles in the latter species or ghost bands in the others.

Band no	*Loci*^{alleles}	*C. aquaticus*	*C. fontinaloides*	*C. nigricans*	*C. danubicus*
1	Hex-1	-------		-------	
2	Hex–2	-------	-------	-------	-------
3	Hex-3	-------		-------	

Fig. 5. Schematic banding pattern for HEX.

DISCUSSION

General considerations.

The theory that *C. danubicus* might be a hybrid of *C. aquaticus* and either *C. fontinaloides* or *C. nigricans* is based mainly on ecological and morphological characters. Leaf form and costa width at leaf base of *C. danubicus* appear intermediate between the presumed parents. *Cinclidotus aquaticus* prefers habitats that are underwater for most of the year whereas *C. fontinaloides* grows mostly above the high water line where it is flooded only scarcely. Again *C. danubicus* is intermediate: in places where it is found together with *C. fontinaloides* and *C. nigricans*, it grows below both of them but still in regions that dry out for rather long periods.

One possible reason for the late discovery of *C. danubicus* is former confusion with *C. fontinaloides*. The two species are often found at the same sites with *C. danubicus* growing just a little nearer to the water. Many herbarium specimens show that these two species were often mixed (Philippi, 1967). Furthermore *C. danubicus* has been found only sterile until now. Earlier bryologists tended to collect preferably fertile plants and might have ignored *C. danubicus*, taking it for sterile *C. fontinaloides*.

Chromosome counts and ploidy level.

Our first assumption and the impulse for this analysis was that *C. danubicus* might be a „young" hybrid, as it was found only 1906. This theory is undermined by chromosome counts: while the possible parent species all have thirteen chromosomes, *C. danubicus* has only twelve (Fritsch, 1991).

The ploidy level in the analyzed species is not yet clear. Assuming a base number of x = 6-7 (Smith, 1978) all scored species (n=12/13) were regarded as diploid, though only *C. danubicus* obviously showed diploidy by a band resulting from a heterodimer of GPI. The isozyme patterns of the other species better fit haploidy yet they might be homozygote diploids due to self-fertilization. In this case the isozymic structure does not show in obvious patterns and the individuals may appear functionally haploid. Even allopolyploidy does not necessarily result in typical polyploid gene expression as it is sometimes distorted by gene silencing and posttranslational protein modifications (Stoneburner *et al.*, 1991). On the other hand haploid gene expression of an enzyme system can be disguised by additional loci that provide additional isozymes to a system with a known number of isozymes, simulating the presence of a second set of chromosomes. Thus, interpretation of isozyme patterns in general is often ambiguous and often needs to be supplemented by DNA analyses.

Isozymic evidence

Cinclidotus danubicus shows an intermediate state for the GPI enzyme system. In this case the probable parents would not be *C. aquaticus* and *C. fontinaloides* or *nigricans* as we assumed, but the latter two species. Yet, hybridization between *C. fontinaloides* and *nigricans* is not supported by any other isozymic correspondences nor is it congruent with variation in morphological characters. Moreover, the intermediate state of some morphological and ecological characters of both species does not lay in the range of *C. danubicus* characters: *C. danubicus* grows nearer to the water, its cell diameter ranges a little higher and its leaves are slightly hamate.

For IDH system *C. danubicus* shows a unique band that is not intermediate between any other visible bands and thus cannot be of hybrid origin. It might be a mutation characteristic to *C. danubicus*. Unfortunately IDH is an enzyme system that easily produces « ghost bands », i.e. weak bands ascribed to degradation products of primary isozymes – considering the possible hybrid status – an allele that was not included in the analysis due to incomplete sampling. So this system alone is not suitable to prove an independent position.

In four of the five enzyme systems (IDH, HEX, MDH, TPI) that were scored, *C. aquaticus* and *C. nigricans* had identical isozymes. For MDH and TPI the banding pattern is nearly identical for all four species but *C. fontinaloides*. It differs from all other species in one locus each. For HEX it lacks Hex-1 and Hex-3 like *C. danubicus* but beyond that they have no other special isozymes in common.

Hybridization does not necessarily result in additional intermediate bands for multimeric enzymes in the hybrid's isozyme pattern: the maternal progenitor may provide a chloroplast isozyme whereas the paternal progenitor adds a cytosolic isozyme. Since these are strictly cytologically separated in the hybrid's cells no heteromultimers with intermediate migration behaviour are present. Yet the hybrid is expected to show both parents' bands. Apart from GPI this was not observed in any other of the studied enzyme systems.

On the whole the results of isozyme analysis do not encourage the theory that *C. danubicus* is a hybrid of *C. aquaticus* and *C. fontinaloides*.

Infrapopulational variation

An interesting result of this study is the high genetic uniformity of the four species of *Cinclidotus* from the studied sites. There is no genetic diversity within the populations of all the three species (*C. nigricans, danubicus, fontinaloides*) collected along the Rhine. Of the three populations of *C. aquaticus* that were studied one is from the northernmost part of its closed range in the French Jura, another from Austria and the last from a highly disjunct locality. The occurrence in Westphalia was reported for the first time by Wiemeyer (1916) and Loeske (1916), and later confirmed by Töns (1957). This population still exists, although in poor condition. Although it is 500 kilometres north of the closed range and 250 km apart from the next occurrence, it is genetically identical to the southern populations. This disjunct occurrence is therefore likely of recent origin and may have been caused by dispersal by birds or man.

Genetic uniformity is, however, found in several bryophyte species studied so far, including widespread species such as *Funaria hygrometrica, Lunularia cruciata, Sphagnum subnitens, S. lindbergii, Marchantia globosa, Rhynchostegium riparioides,* and *Plagiochila porelloides* (Cronberg, 2000). In the case of predominantly sterile aquatic mosses, the genetic uniformity may be a result of clonal reproduction and downstream dispersal.

Acknowledgements. We thank Johann Gruber for providing material of *Cinclidotus aquaticus* from Austria, Bernard Goffinet for substantially improving the manuscript as well as two anonymous reviewers for helpful comments.

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