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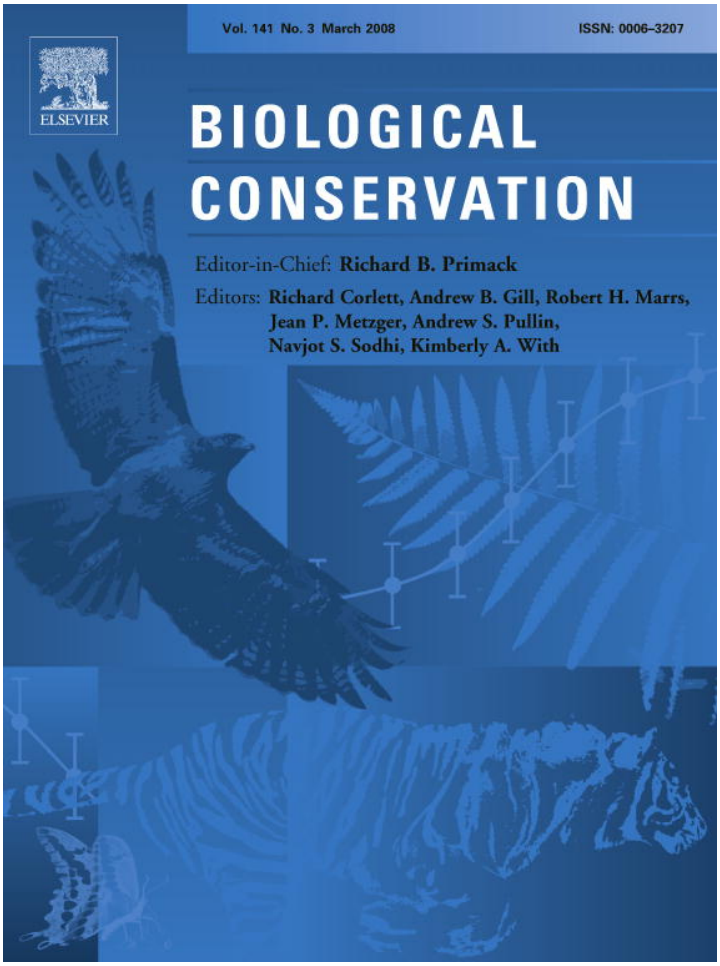

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DNA sequences identify invasive alien *Cardamine* at Lake Constance

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ABSTRACT

The shores of Lake Constance in southwest Germany inhabit an endemic and highly endangered lake-shore community. In spring 2004, a so far unknown *Cardamine* (Brassicaceae) was detected at the lake-shores. In the subsequent years, this taxon has spread rapidly, 95 locations have been recorded until spring 2007. We tested the utility of DNA sequences to distinguish between two alternative hypotheses regarding the appearance of this new invasive taxon: a local formation via hybridization between native species versus an introduction of a non-native taxon. The relative DNA contents was analysed as an additional independent character. DNA sequences provided substantial evidence that the unknown *Cardamine* taxon, recently discovered at Lake Constance in southwest Germany, is a non-native species introduced from abroad. Sequences of the internal transcribed spacer of the large subunits of nuclear ribosomal DNA and two noncoding regions of chloroplast DNA (*trn*L intron, *trn*L/F spacer) were distant from sequences of all native *Cardamine* species providing evidence against a hybridization hypothesis. In contrast, DNA sequences of the unknown *Cardamine* were identical to one accession of *Cardamine flexuosa* auct. non With. (Asian *C. flexuosa*) from Japan. The introduction history of Asian *C. flexuosa* at Lake Constance and the potential threat to the native lake-shore vegetation is discussed. Our study highlights the potential of DNA sequences to identify invasive genotypes and source regions.

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1. Introduction

Lake Constance (southwest Germany) is the largest northern pre-alpine lake with a surface area of 536 km^2 and a shore length of 273 km. It is distinguished from most other pre-alpine lakes by its natural water-level fluctuation with highest water levels during late spring and early summer (Jöhnk et al., 2004). The exposed parts of the lake-shore contain an amphibious plant community phytosociologically described as Deschampsietum rhenanae (Lang, 1990). This community is characterized by narrow endemic species, mainly *Myosotis rehsteineri* Wartm., *Deschampsia littoralis* (Gaudin) Reut., and other rare amphibious plants (*Littorella uniflora* (L.) Asch., *Ranunculus reptans* L.). The small herbaceous and perennial species are weak competitors but are well adapted to the seasonal water-level fluctuations of Lake Constance (Peintinger et al., 2007). The occurrence of the Deschampsietum has been reduced dramatically during the last 100 years (Dienst and Strang, 2002; Dienst et al., 2004; Strang and Dienst, 2004). In spring 2004, a so far unknown *Cardamine* (Brassicaceae) was detected at the shores of Lake Constance. In the subsequent years, this taxon (called C. "Lake Constance" throughout the

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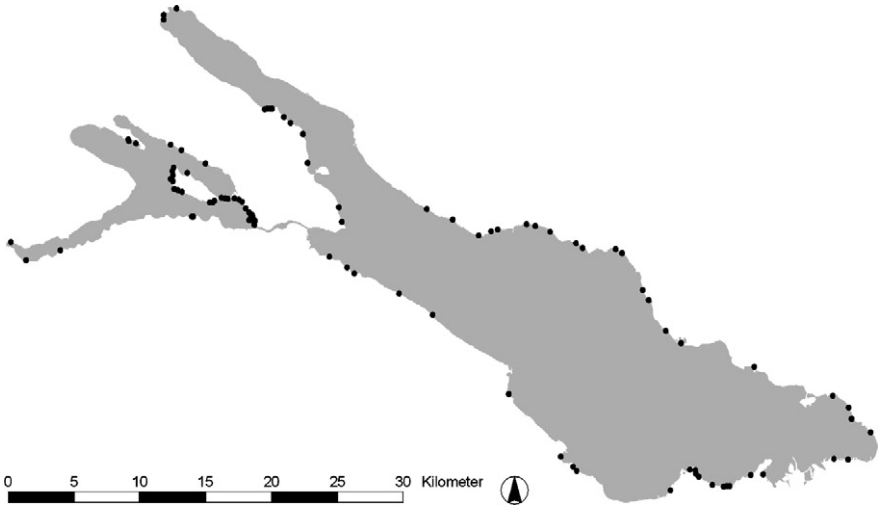

Fig. 1 – Distribution of Asian *Cardamine flexuosa* at Lake Constance (southwest Germany) in spring 2007.

paper) has spread rapidly and invaded the native lake-shore communities, 95 locations have been recorded until spring 2007 (Fig. 1). The unknown *Cardamine* resembled the native species *Cardamine hirsuta* L. and *Cardamine flexuosa* With., both successful colonizers, but had no basal leaf rosette. Photographs and plants from Lake Constance have been examined by a number of specialists but without a clear result. Two hypotheses were formulated regarding the origin of the unknown plants: (1) The plants could be of recent hybrid origin. *Cardamine* species occurring at the shores of Lake Constance are *C. hirsuta* ($2n = 2x = 16$), *C. flexuosa* ($2n = 4x = 32$), and *Cardamine pratensis* L, which occurs in different ploidy levels ($2n = 2x - 6x$). Hybrids were reported between *C. hirsuta* and *C. flexuosa* (*C. ×zahlbruckneriana* O. E. Schulz) and between *C. flexuosa* and *C. pratensis* (*C. ×haussknechtiana* O. E. Schulz). However, none of these hybrids has been reported to reproduce successfully in Central Europe. (2) As a second hypothesis, the plants could have been introduced from abroad. Putative source regions would be North America or Eastern Asia, which both have a high number of native and also endemic *Cardamine* species. *Cardamine* in eastern Asia has only recently been subject to detailed molecular systematic studies (Lihova et al., 2006a).

In order to estimate a potential threat to native biodiversity it is necessary to identify the origin of invasive plant species as early as possible. The ecology of introduced species in their native range may provide important information in an attempt to predict where potential invaders will be successful. DNA sequences provide powerful tools for analysing interspecific hybridization (Franzke and Mummenhoff, 1999; Lihova et al., 2006b) and have recently been suggested as tools for invasive species identification (DNA barcoding, Armstrong and Ball, 2005). It has been shown that DNA sequences commonly used for phylogenetic studies might perform well even though they were not produced to be used as barcodes (Chase et al., 2005). The internal transcribed spacer of the large subunits of nuclear ribosomal DNA and two noncoding regions of chloroplast DNA (*trn*L intron, *trn*L/F spacer) have been used widely for phylogenetic reconstruction in the large genus *Cardamine* (Franzke et al., 1998; Bleeker et al., 2002a; Lihova et al., 2006a). DNA sequences are available for about 150 of approximately 200 *Cardamine* species worldwide. For many species multiple accessions are available. Ploidy analyses are also a useful tool to distinguish between different taxonomic entities in genera which are rich of polyploids like *Cardamine* Lihova et al. (2007) or the closely related genus *Rorippa* (Bleeker and Matthies, 2005).

In this paper we test the utility of DNA sequences to distinguish between two alternative hypotheses regarding the appearance of a new invasive taxon: a local formation via hybridization between native species versus an introduction of a non-native taxon. The relative DNA contents was analysed as an additional independent character.

2. Materials and methods

2.1. Origin of plant material

In spring 2006, we visited six locations of *C.* "Lake Constance" at western parts of the lake. At five of these locations, *C.* "Lake Constance" co-occurred with other *Cardamine* species. Seven morphological characters (height of stem, length of petals, length of sepals, length of middle stem leaf, length of first lateral leaflet of middle stem leaf, presence/absence of stem hairs, presence/absence of basal leaf rosette) were used to evaluate morphological variation of *Cardamine* taxa at the lake shore. *C. pratensis* could easily be distinguished by its large petals. *C.* "Lake Constance" was characterized by lacking a basal leaf rosette, all other quantitative morphological characters analysed were in the range of *C. hirsuta* and *C. flexuosa*. The hairness of the stem, a character used to distinguish between *C. hirsuta* and *C. flexuosa*, varied in *C.* "Lake Constance", but in most plants the stem was less hairy than in *C. flexuosa* (Klausmeyer, 2006). The number of stamens in *C.* "Lake Constance" was six like in *C. flexuosa*.

2.2. Ploidy analysis

Flow cytometry was used for the determination of relative DNA amount. Fresh leaf material was harvested in the field

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and stored at 4 °C for 2–3 days until further analyses. Approximately 0.5 cm^2 leaf material was chopped with a sharp razor blade in a DAPI solution and filtered into a sample tube. Subsequent flow cytometry analysis was performed on a Partec Ploidy Analyser-I (Partec, Münster, Germany) using an UV arc lamp. A diploid *C. hirsuta* collected in the area of Osnabrück (Germany) was used as an internal standard.

2.3. DNA extraction, amplification and sequencing

Total DNA was isolated by the CTAB method of Doyle and Doyle (1987). Amplification of the ITS 1 and ITS 2 regions, *trn*L intron, and *trn*L-F spacer were performed following the protocol of Franzke et al. (1998). The primers were as follows: ITS 4 and ITS 5 (White et al., 1990) for the ITS regions; primers c and d (Taberlet et al., 1991) for the *trn*L intron; and primers e and f (Taberlet et al., 1991) for the *trn*L-F spacer. Amplification products were resolved on 1.5% agarose gels and visualised by UV light after staining with ethidium bromide. Before DNA sequencing, amplification products were purified (Qiaquick PCR Purification Kit). Sequence reactions were performed using the ABI PRISM BigDye Ready Reaction Terminator Cycle Sequencing Kit (Applied Biosystems). The sequence reaction products were separated electrophoretically on an ABI 377 (Applied Biosystems).

2.4. Data analysis

The sequences of the ITS 1, ITS 2, *trn*L intron, and *trn*L/F spacer regions of *C.* "Lake Constance" were used to perform BLAST searches. For each region the five species with highest sequence similarities compared to the query sequence were kept for further analysis. If there were multiple accessions of the same species with high similarities to the query sequence, we kept the accession with highest similarity. DNA sequences of species with high sequence similarities to the query sequences (closest five species for each marker) and the sequences of the native taxa *C. hirsuta*, *C. flexuosa*, and *C. pratensis* were aligned manually. Regions with ambiguous alignment and indels were excluded from analysis. In ITS regions of *C.* "Lake Constance", a few intra-individual single-nucleotide polymorphisms were observed, suggesting presence of divergent ITS copies. These polymorphic sites were also excluded from the alignment. Dissimilarities were calculated as total number of pairwise character differences. A hierarchical cluster analysis (UPGMA) based on a combined data set using all four regions (ITS 1, ITS 2, *trn*L, *trn*L/F) was performed in order to analyse the distance structure among the taxa.

3. Results

3.1. Ploidy analyses

In total, we analysed 32 individuals of *C.* "Lake Constance", 16 individuals of *C. hirsuta*, five individuals of *C. flexuosa*, and five individuals of *C. pratensis*. As expected, no variation was detected within *C. hirsuta*. Accessions from Lake Constance had the same DNA amount as plants collected in the area of Osnabrück (northwest Germany), which were used as internal standard (1 s, Fig. 2). The relative DNA contents of *C.* "Lake Constance" varied between 3.0 s (3 × DNA amount of *C. hirsuta*) and 3.6 s (mean = 3.33, coefficient of variation = 4.4%), *C. flexuosa* had a relative DNA contents of 2.3 s. Obviously, plants with different ploidy levels were analysed in *C. pratensis*, one individuals had a relative DNA contents of 2.4 s, the other four individuals varied between 3.2 s and 3.5 s (Fig. 2).

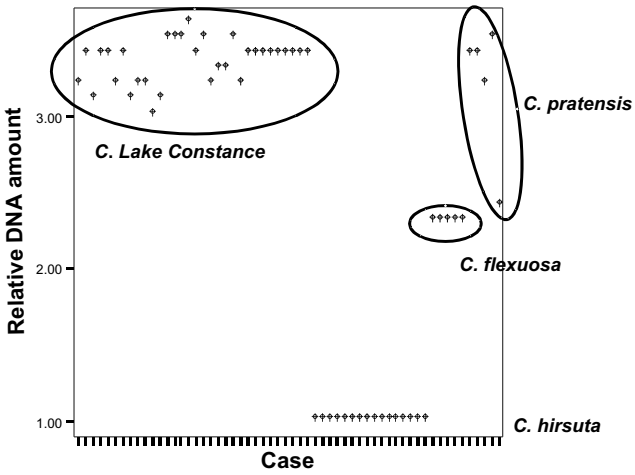

Fig. 2 – Relative DNA amount of 58 *Cardamine* individuals collected at six locations at western Lake Constance. Diploid *C. hirsuta* from north-west Germany was used as an internal standard.

3.2. DNA sequencing

We sequenced four individuals of *C.* "Lake Constance", and one individual each of *C. hirsuta* and *C. flexuosa*. Table 1 includes origin of plant material and GenBank accession numbers for the DNA sequences obtained. Sequences of *C. pratensis* from the area of Lake Constance were available from a former study (Franzke and Hurka, 2000).

All four accessions of *C.* "Lake Constance" had identical ITS 1, ITS 2, *trn*L, and *trn*L/F sequences. The query sequences used in the BLAST searches were 267 bp (ITS 1), 187 bp (ITS 2), 467 bp (*trn*L), and 200 bp (*trn*L/F) in length. Due to multiple *trn*F gene duplications and subsequent pseudogene formation, the *trn*L/F spacer of *Cardamine* and related genera is characterized by considerable length variation between and within species (Bleeker et al., 2002a,b; Koch et al., 2005). Therefore, only 200 bp from the 5' end downstream were used in the BLAST searches and subsequent analysis.

Nine *Cardamine* taxa, among them six Eastern Asian taxa (*C. flexuosa* auct. non With., *C. fallax* (O. E. Schulz) Nakai, *C. scutata* Thunb., *C. niigatensis* H. Hara, *C. dentipetala* Matsum., and *C. longifructus* Ohwi), one Eurasian taxon (*C. parviflora* L.), one North American taxon (*C. pensylvanica* Willd.), and the Australian *C. paucijuga* Turcz., had *trn*L intron sequences identical to the query sequence (Table 2). Four of these species (*C. flexuosa* auct. non With., *C. pensylvanica*, *C. fallax*, *C. paucijuga*) and the Eastern Asian *C. keysseri* O. E. Schulz had sequences identical to the 200 bp query *trn*L/F sequence. Nuclear ITS sequences provided a higher resolution, only one taxon (*C. flexuosa* auct. non With.) had ITS 1 and ITS 2

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Table 1 – Origin of *Cardamine* plants used for DNA sequencing (all shore of Lake Constance)

Taxon	Location	GenBank numbers		
		ITS	*Trn*L	*Trn*L/F
Cardamine "Lake Constance"	Reichenau Niederzell, N 47°42′ E 9°32′	EU342873· EU342874	EU34875	EU34876
	Litzelstetten, N 47°42′ E 9°10′	·	·	·
	Konstanz-Staad, N 47°40′ E 9°12′	·	·	·
	Dingelsdorf, N 47°44′ E 9°09′	·	·	·
Cardamine flexuosa	Reichenau Niederzell, N 47°42′ E 9°32′	EU342877 EU342878	EU342879	EU342880
Cardamine hirsuta	Konstanz-Staad, N 47°40′ E 9°12′	EU342881 EU342882	EU342883	EU342884

Table 2 – Results of BLAST searches using the obtained ITS sequences and chloroplast DNA sequences of *Cardamine* "Lake Constance" as query

ITS 1 (267 nucleotides)	ITS 2 (187 nucleotides)	*Trn*L intron (467 nucleotides)	*Trn*L/F spacer (200 nucleotides)
C. flexuosa auct. (DQ 268430, 0)	*C. flexuosa* auct. (DQ 268430, 0)	*C. flexuosa* auct. (DQ 268093, 0)	*C. flexuosa* auct. (DQ 268260, 0)
C. niigatensis (DQ 268479, 2)	*C. scutata* (DQ 268489, 0)	*C. niigatensis* (DQ 268164, 0)	*C. pensylvanica* (DQ 268303, 0)
C. scutata (DQ 268489, 2)	*C. niigatensis* (DQ 268479, 1)	*C. scutata* (DQ 268156, 0)	*C. paucijuga* (AY047640, 0)
C. pensylvanica (DQ 268467, 3)	*C. pensylvanica* (DQ 268467, 2)	*C. longifructus* (DQ 268155, 0)	*C. keysseri* (AY047651, 0)
C. flexuosa (DQ 268423, 3)	*C. flexuosa* (DQ 268423, 2)	*C. dentipetala* (DQ 268149, 0)	*C. fallax* (DQ 268290, 0)
		C. pensylvanica (DQ 268136, 0)	
		C. paucijuga (AY047640, 0)	
		C. fallax (DQ 268123, 0)	
		C. parviflora (DQ 268406, 0)	

All species with identical sequences (*trn*L, *trn*L/F) or the five closest sequences (ITS) were included. GenBank accession numbers and the number of pairwise character differences are given in parentheses.

sequences identical to *C.* "Lake Constance" (Table 2). *C. niigatensis* and *C. scutata* had two nucleotide substitutions compared to the query ITS 1 sequence, *C. pensylvanica* and the European *C. flexuosa* With. both had three nucleotide substitutions. The ITS 2 region of *C. scutata* was identical to *C.* "Lake Constance", *C. niigatensis* had one nucleotide substitution, *C. pensylvanica* and *C. flexuosa* With. both had two nucleotide substitutions (Table 2).

Fig. 3 shows the distance structure based on an UPGMA cluster analysis using all four sequenced DNA markers. The sequences of *C.* "Lake Constance" were identical to *C. flexuosa* auct. non With. from Japan and are close to a number of other taxa from eastern Asia and one taxon from North America. The sequences of all three co-occurring native species (*C. hirsuta*, *C. flexuosa*, *C. pratensis*) are distant from *C.* "Lake Constance".

4. Discussion

4.1. *Identification of Asian C. flexuosa at Lake Constance*

DNA sequences provide substantial evidence that the unknown *Cardamine* taxon, recently discovered at Lake Constance in southwest Germany, is a non-native species introduced from abroad. Sequences of the internal transcribed spacer of the large subunits of nuclear ribosomal DNA and two noncoding regions of chloroplast DNA (*trn*L intron, *trn*L/F spacer) were distant from sequences of all native *Cardamine* species providing evidence against a hybridization hypothesis. In contrast, DNA sequences of the unknown *Cardamine* were identical to one accession of *C. flexuosa* auct. non With (Asian *C. flexuosa*) collected in Japan. It has been shown recently that Asian weedy populations traditionally assigned to *C. flexuosa* form an independent evolutionary lineage and represent a distinct taxon from European *C. flexuosa* (Lihova et al., 2006a). However, what was called Asian *C. flexuosa* by Lihova et al. (2006a) is not a monophyletic taxon. Lihova et al. (2006a) analysed 29 accessions of Asian *C. flexuosa*, including accessions representing introduced weedy populations in Australia and North America. In the phylogenetic analyses of ITS sequences using Bayesian inference, accession JP 11 of Asian *C. flexuosa* (the one identical to *C.* "Lake Constance") formed a clade with a second accession of Asian *C. flexuosa* from Japan. This clade was close to one accession of *C. scutata* from Japan, one accession of Asian *C. flexuosa* from North America, and several accessions of *C. niigatensis* from Japan. Several other accessions of Asian *C. flexuosa* from various regions were not resolved close to accession JP 11. Thus, in a phylogenetic context, Japan is identified as the most likely source region of *Cardamine* "Lake Constance", too.

Different diploid genome sizes exist in *Cardamine* making it difficult to estimate ploidy levels directly from relative DNA amounts (Bleeker, unpublished data). *C.* "Lake Constance" had a relative DNA contents 3.0–3.6 times diploid *C. hirsuta* and approximately 1.5 times tetraploid *C. flexuosa*. These data provide evidence for hexaploid *C.* "Lake Con-

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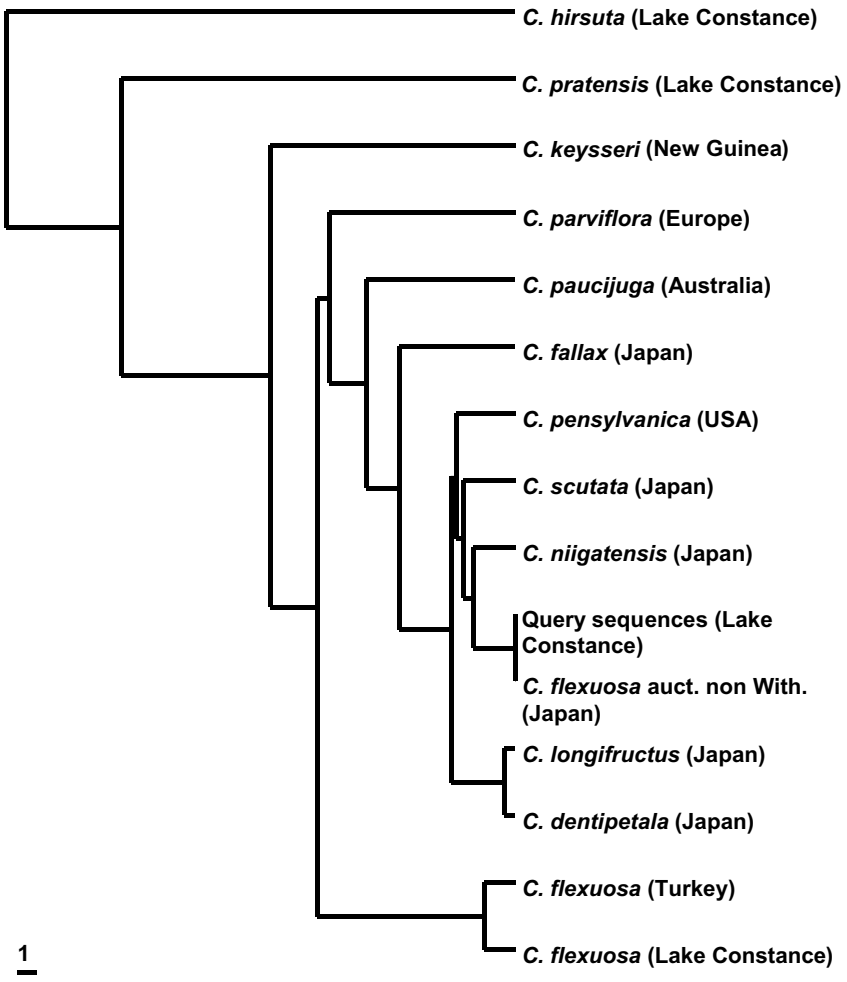

Fig. 3 – Cluster analysis (UPGMA) based on total numbers of pairwise character differences using a combined data set of all four sequenced DNA regions (ITS 1, ITS 2, *trn*L, *trn*L/F).

stance" assuming identical diploid genome sizes in European and Asian *C. flexuosa*. However, Lihova et al. (2006a) listed Asian flexuosa as an octoploid taxon. Presumably, different ploidy levels (6×, 8×) occur in Asian *C. flexuosa*.

4.2. *Conservation implications*

Identifying the introduction pathways of invasive species is a major challenge in order to set up conservation strategies (prevent secondary introductions). However, the introduction pathway of Asian *C. flexuosa* from southeast Asia to Lake Constance is unknown. A direct introduction from southeast Asia via migrating birds seems unlikely since no bird species is regularly migrating from Central European lakes to southeast Asia. Interestingly, plants morphologically close to Asian *C. flexuosa* have recently been detected in a paddy field in northern Italy (leg. T. Götz 2006, private herbarium). Thus it cannot be excluded that Asian *C. flexuosa* has been introduced into other regions of Europe, too. An introduction with seeds from southeast Asia to northern Italy and from Italy to Lake Constance via migrating birds would be possible. An alternative hypothesis would be a direct introduction from Japan to Lake Constance via tourists.

At Lake Constance in southwest Germany Asian *C. flexuosa* has first been observed in spring 2004 after extreme low water levels in 2003. The lake-shore vegetation is subject to recurrent vegetation monitoring so that it is unlikely that the plants have been overlooked. In 2005 and 2006, about 30 locations were detected in the western parts of the lake. After the plant has been identified as an invasive neophyte (Klausmeyer, 2006), more field botanists got interested and the number of records increased rapidly. In winter 2006/2007 Asian *C. flexuosa* was also detected in eastern parts of the lake. Fig. 1 shows the distribution of Asian *C. flexuosa* at Lake Constance in spring 2007.

Asian *C. flexuosa* is a self-compatible taxon producing large numbers of seeds but it is highly plastic in age and size at maturity (Kudoh et al., 1996). In Japan, the species prefers wet habitats and in paddy fields it is a winter-annual (Yatsu et al., 2003). In contrast to *C. hirsuta*, the Asian *C. flexuosa* is well adapted to flooding. Experiments show, that seeds survived submerged condition for four months and germination rate was higher in the flooded treatment than in the control (Yatsu et al., 2003). The recent spread of Asian *C. flexuosa* at Lake Constance has been favoured by mild winters and low water levels of the lake. Flowering plants have been observed

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all over the mild winter 2006/2007. Asian *C. flexuosa* has established itself mainly at the middle water line providing evidence that swimming seeds are a main vector of dispersal. The low water level of Lake Constance (last extreme high water in 1999) provided the space for seed germination and seedling establishment.

At present, it is too early to conclude whether Asian *C. flexuosa* will represent a threat to the Deschampsietum rhenanae, an unique lake-shore community especially protected by the fauna-flora-habitat directive of the European Union. Recently, the Asian *Cardamine* is more frequent on fine-grained and nutrient-rich sediments than on nutrient-poor gravel shores which are characteristic for the Deschampsietum rhenanae. Therefore, it seems unlikely that the species will out compete the endangered amphibious plant species. It is more likely that it will change the community structure of ephemeral vegetation on bare and organic sediments (with *Catabrosa aquatica* (L.) P. Beauv., *Rorippa* spp., *Barbarea vulgaris* R. Br.). High waters, like during the flood in 1999, could also transport seeds of Asian *C. flexuosa* into the upper regions of the lake-shore and into surrounding gardens. It is also likely that seeds of Asian *C. flexuosa* will be transported downwards the River Rhine (which flows through Lake Constance). In Japan, Asian *C. flexuosa* represents a serious weed in paddy fields, moist gardens and orchards (Lihova et al., 2006a). The life history characteristics of Asian *C. flexuosa* are quite similar to *C. hirsuta*, which is a notorious weed in European gardens. An additional question will be whether Asian *C. flexuosa* will hybridize with native *Cardamine* species possibly leading to the formation of new invasive genotypes (Ellstrand and Schierenbeck, 2000; Bleeker et al., 2007).

4.3. *DNA barcodes and invasive species identification*

Our study highlights the potential of DNA sequences to identify invasive genotypes and source regions. In general, nuclear encoded ITS sequences provided a higher resolution compared with cpDNA regions. The *trn*L intron and the *trn*L/F spacer of chloroplast DNA were not variable enough to assign the query sequences to a single taxon. The nuclear ribosomal ITS region, even with its recognized limitations, is a prime candidate as an effective locus for DNA barcoding in angiosperms (Kress et al., 2005). The maintenance of multiple uncorrected ITS copies is seen as one of the major problems in using ITS as a barcoding tool (Cowan et al., 2006). In Asian *C. flexuosa*, the presence of multiple uncorrected ITS copies is likely due to a hybrid origin of the taxon (Lihova et al., 2006a). However, we show that ITS sequences can even be used as an identification tool after eliminating sites showing intra-individual single-nucleotide polymorphisms. DeSalle et al. (2005) stated that although distance based approaches are appropriate to identify the nearest neighbour to the query sequence, the success of a DNA sequence as a species identification tool – the barcode – depends on the existence of unique substitutions that distinguish among closely related species. However, finding molecular characters which are associated with morphological characters that distinguish between closely related species is a difficult task in many plant groups. A taxonomic study focusing on the correct name and the delimitation of Asian *C. flexuosa* is currently performed by K. Marhold and colleagues. Therefore, it is too early to ask for diagnostic molecular characters -a true barcode- of Asian *C. flexuosa*.

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