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Ice age cloning – comparison of the Quaternary evolutionary histories of sexual and clonal forms of spiny loaches (*Cobitis*; Teleostei) using the analysis of mitochondrial DNA variation

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Abstract

Recent advances in population history reconstruction offered a powerful tool for comparisons of the abilities of sexual and clonal forms to respond to Quaternary climatic oscillations, ultimately leading to inferences about the advantages and disadvantages of a given mode of reproduction. We reconstructed the Quaternary historical biogeography of the sexual parental species and clonal hybrid lineages within the Europe-wide hybrid complex of *Cobitis* spiny loaches. *Cobitis elongatoides* and *Cobitis taenia* recolonizing Europe from separated refuges met in central Europe and the Pontic region giving rise to hybrid lineages during the Holocene. *Cobitis elongatoides* due to its long-term reproductive contact with the remaining parental species of the complex — *C. tanaitica* and *C.* spec. — gave rise to two clonal hybrid lineages probably during the last interglacial or even earlier, which survived the Würmian glaciation with *C. elongatoides*. These lineages followed *C. elongatoides* post-glacial expansion and probably decreased its dispersal rate. Our data indicate the frequent origins of asexuality irrespective of the parental populations involved and the comparable dispersal potential of diploid and triploid lineages.

Keywords: asexuality, *Cobitis*, glacial refuges, hybridization, phylogeography

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Introduction

More than a century after the introduction of the 'paradox of sex' (Weissman 1889), there is little disagreement that strict asexuality among higher organisms generally represents an evolutionary dead end due to the inability of clones to escape mutation or parasite loads or to cope with the changing environment (Felsenstein 1974; Hamilton 1980; Howard & Lively 1998). Yet, recent findings contrasting traditional assumptions in some model groups (Vrijenhoek 1993; Judson & Normark 1996; Alves *et al*. 1998) together with a growing amount of discovered asexual complexes (Vrijenhoek *et al*. 1989; for review of later described cases see Alves *et al*. 2001) suggest that we are far from understanding the roles of sex and the asexual reproductive mode in evolution.

Theoretical predictions have been tested on suitable animal, plant and fungi model organisms, among which vertebrates occupy a privileged position, due to relatively easy sampling and rather well-developed methodological approaches. Since the discovery of the first clonal vertebrate, *Poecilia formosa* (Hubbs & Hubbs 1932), significant research effort led to some conclusions about asexuality among metazoans in general and among vertebrates in particular. Generally, asexual lineages are recent offshoots of extant Mendelian taxa, although some lineages have apparently persisted without sex for longer (Quattro *et al*. 1992a; Spolsky *et al*. 1992; Judson & Normark 1996) than predicted by mutation-accumulation models (Lynch *et al*. 1993). Clonal vertebrates studied so far apparently have

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originated by hybridization (Vrijenhoek *et al*. 1989) generally involving females of only one parental species, although reciprocal hybridization has been found in some asexual complexes (Janko *et al*. 2003). Strong genetic, developmental and/or ecological constraints on the origin of asexuality likely exist because most asexual complexes have originated from crosses involving one or few related females, although multiple independent origins have been demonstrated in some systems, especially among sperm-dependent parthenogens (Avise *et al*. 1992; Vrijenhoek 1998; and citations therein). The resulting clonal diversity may help the asexual populations in the minimization of their parasite load or better resource utilization (Vrijenhoek 1979; Hamilton *et al*. 1990). Better dispersal relative to sexual progenitors likely plays a crucial role in the maintenance of true parthenogens, allowing them to escape parasites and avoid competition with parental species. In sperm-dependent parthenogens, however, where asexual females need to receive sperm from a sexual host either to stimulate the oocyte development (gynogenesis) or to replace the excluded parental genome (hybridogenesis) and are therefore bound to their sperm donors, dispersal likely plays a different role (Vrijenhoek 1998), possibly in the maintenance of their metapopulation structure via 'infections' of new host populations.

Empirical evaluation of traits significant in an adaptation to asexuality is largely based on comparisons of related sexual and asexual lineages. Since conclusions made upon laboratory experiments — despite the advantage of the direct comparison of the effects of simulated parameters on both lineages — are simplifying due to an inability to simulate the complex interactions of environmental factors, significant effort has been focused on studies of natural populations (see, e.g. Avise *et al*. 1992; Alves *et al*. 2001; Cunha *et al*. 2004). Such experiments are nonetheless challenged by an uncertain evolutionary history of the compared populations. Recent advances in population genetics and phylogeography has given us an unprecedented tool to reconstruct population history and to estimate contemporary as well as past population parameters (Avise 2000; Knowles & Maddison 2002). Such information overlaid upon inferred environmental events may ameliorate the testing of hypothetical advantages and disadvantages of a given reproductive mode. For example, the inference of the historical biogeography of parental taxa and the geographical origins of related asexual hybrids may be used to address the question of whether the lower genetic diversity of asexuals is due to the constraints on the origins of asexuality, or it is simply due to the lack of long-term reproductive contact of the sexual progenitors.

In the present study, we compare the Quaternary historical biogeography of sexual and clonal forms of the European spined loaches of the genus *Cobitis*. Four species, *C. taenia*, *C. elongatoides*, *C. tanaitica* and a formally yet undescribed *C.* spec., are known to hybridize (Bohlen & Ráb 2001), giving rise to virtually all-female gynogenetic hybrid lineages (Saat 1991). We have shown previously that the *elongatoides–taenia* hybrid lineages arose by multiple reciprocal hybridizations, whereas *elongatoides–tanaitica* hybrids originated exclusively by the mating of *C. elongatoides* females with *C. tanaitica* males (Janko *et al*. 2003). Triploids, the predominant hybrid form, arise via fertilizations of the unreduced gametes of diploid hybrids (Janko *et al*. 2003), which are occasionally found in some populations (mainly in the Odra and Elbe rivers; Bohlen *et al*. 2002). Our data also indicate that *C.* sp. gave rise to a triploid lineage upon hybridization with *C. elongatoides*. Trihybrid lineages were also documented, likely having originated by fertilization of the diploid ovum by a sperm of a species not involved in the original hybridization.

Mitochondrial DNA (mtDNA) is a strong tool for the elucidation of population history and once becoming 'frozen' in a clonal lineage, mtDNA genealogy virtually matches their branching history (Avise *et al*. 1992; reviewed in Avise 2000). Studying the mtDNA variation in three parental taxa of the complex *C. elongatoides*, *C tanaitica* and *C.* sp., we inferred their glacial refuges and postglacial dispersal routes. We integrated this information with the results for *C. taenia* (Culling *et al*. submitted) and assessed the geographical origin and dispersal of the asexual lineages. Comparison of the inferred dispersal potential of the parental species and hybrids led us to hypothesize the effect of sperm parasitism on host populations.

Materials and methods

Samples

Spiny loaches were obtained from 35 localities throughout Europe (Figs 1 and 2; Table 1). Taxonomic identification of specimens, their ploidy and genome composition were determined by allozyme and karyotype analyses as in Janko *et al*. (2003), and in several unclear cases, flow cytometry was used to determine the ploidy of specimens following the protocol of (Flajšhans & Vajcova 2000). In total, 32 specimens of *C. elongatoides*, four *C. tanaitica*, seven *C.* spec. and 83 hybrids of various genomic compositions were analysed (Table 1). Two specimens of *Cobitis albicoloris* were used as outgroups in the phylogenetic analyses, which was justified by the phylogenetic relationships within the genus (Perdices & Doadrio 2001). Given that both *C. elongatoides* and *C. taenia* were maternal ancestors of the *elongatoides–taenia* hybrids (Janko *et al*. 2003), we used data on the geographical partitioning of the genetic variability of the *C. taenia* cytochrome *b* gene from Culling *et al*. (submitted) to place our data into a phylogenetic framework and to determine the geographical origin of the hybrid lineages.

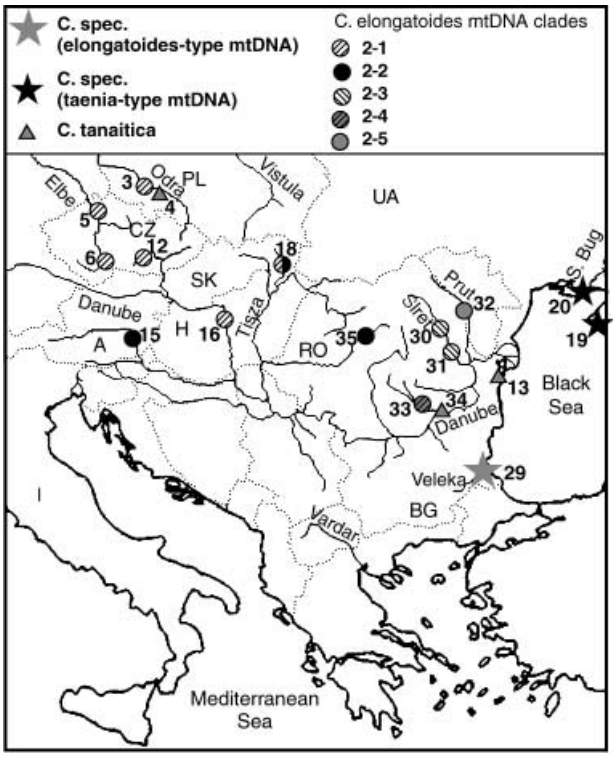


Fig. 1 Sampling sites of parental species and the geographical distribution of *Cobitis elongatoides* nested clades with localities labelled according to Table 1. Countries where fish were sampled are the Czech Republic (CZ), Poland (PL), Ukraine (UA), Slovakia (SK), Romania (RO), Bulgaria (BG), Hungary (H) and Austria (A).

Molecular biological techniques

Total genomic DNA was extracted from muscle or fin tissues using the DNeasy tissue kit (QIAGEN) following the manufacturer's specifications. A 1190-bp fragment of mtDNA encompassing a small part of the Glutamic tRNA gene, complete cytochrome *b* (cyt *b*) gene and a part of the Threonine tRNA gene was amplified by the polymerase chain reaction (PCR) using primers L 15267 (Briolay *et al*. 1998) and ThrR (Doadrio *et al*. 2002). Reactions were performed according to the protocol of Janko *et al*. (2003). The PCR fragments were screened on 1% agarose gel, purified with QIAquick PCR purification kit (QIAGEN), and both strands were directly cycle sequenced by the dideoxy-chain-termination method. Primers used for sequencing were the same as for PCR and for some specimens, we used an internal primer INT1R (5′-TCGCCCGAGGACTATATTAT-3′) from Culling *et al*. (submitted). Sequencing reactions were performed as described in Janko *et al*. (2003) and analysed on an ABI Prism 310 automated sequencer. Sequences were deposited in the GenBank database under the Accession numbers AY706159–AY706203.

Phylogenetic analyses

Sequences of 1088 bp of the cyt *b* gene were aligned using the SEQMAN II program, version 5.05 of the DNASTAR software package, to each other and to the published sequence of the complete cyt *b* of the cyprinid *Barbus barbus* (GenBank Accession no. AF112123; Tsigenopoulos & Berrebi 2000). Sequences of both chains, L and H, were compared to resolve ambiguities. Nucleotide divergences between haplotypes were estimated with the PAUP* software package, version 4.0b10 (Swofford 1999) using the HKY 85 + gamma model of DNA substitution (Hasegawa *et al*. 1985). The HKY 85 + gamma model was selected as an appropriate model for our data set using the hierarchical likelihood-ratio test implemented in the MODELTEST program, version 3.06 (Posada & Crandall 1998).

Phylogenetic relationships among haplotypes were reconstructed by the neighbour-joining algorithm (NJ) using the HKY 85 + gamma corrected distance matrix, and by the maximum-likelihood (ML) criterion using the heuristic search and parameter settings as estimated with the MODELTEST base frequencies: A = 0.2782, C = 0.2462, G = 0.1401, T = 0.3355, transitions/transversion (ti/tv) ratio = 7.0913, gamma shape parameter = 0. 1824. Both analyses were performed with PAUP*. To infer statistical support for the internal branches of the NJ tree, a nonparametric bootstrap test with 1000 replicates was performed. We employed the statistical parsimony of Templeton *et al*. (1992) implemented in the TCS program, version 1.06 (Clement *et al*. 2000) to reconstruct a minimum-spanning haplotype tree (MST). This approach evaluates the maximum number of nucleotide differences between haplotypes for which the use of parsimony is justified, i.e. infers the probability that observed character changes defining the connections of haplotypes are due to no more than single mutations. The age of the clades was estimated either by calculating the mean pairwise within-clade divergences using the mtDNA 'clock' calibration for the *Cobitis* cyt *b* gene by Perdices & Doadrio (2001) assuming the divergence rate of 0.84% per million years (Myr), or using the method of Saillard *et al*. (2000), which infers the average distance from the most recent common ancestor (MRCA) of the clade as expressed in the number of mutation steps ($\rho = \{n_1 l_1 + \ldots + n_k l_1\}/n$, where n is the number of specimens and l is the length of k^{th} branch expressed in mutation steps, and the variance of the estimate $\sigma^2_H = \{ n_1^2 l_1 + \ldots + n_k^2 l_1\}/n^2$). In the latter approach, the absolute timing was assessed by multiplying the observed values by a mutation rate of 0.0042 mutations per site per Myr.

Nested clade phylogeographical analysis

We applied nested clade phylogeographical analysis (NCPA) (Templeton *et al*. 1995; Templeton 1998) to detect and biologically interpret statistically significant phylogeographic

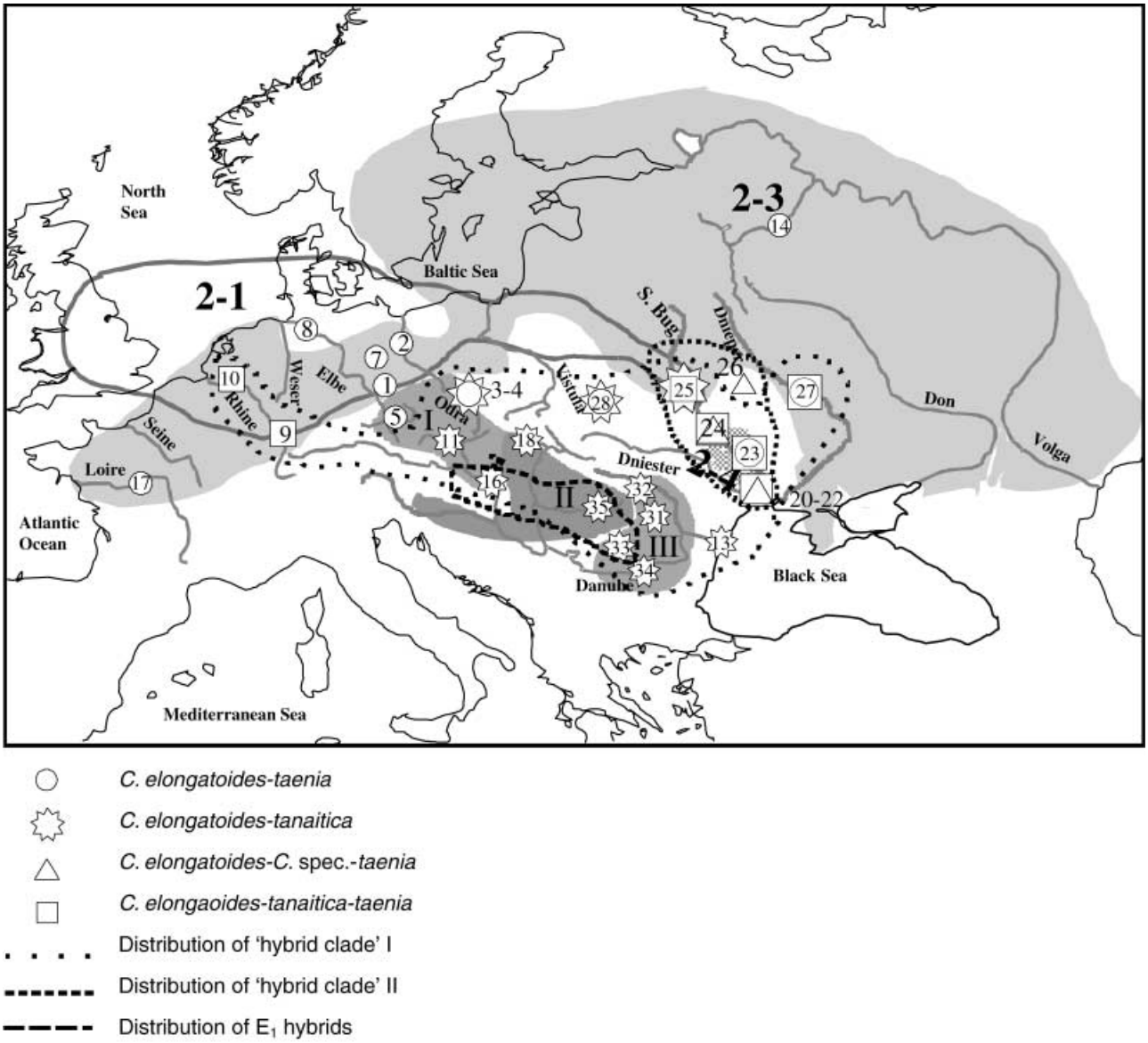


Fig. 2 Sampling sites of hybrid asexuals. Sites are labelled according to Table 1. Areas encircled with a grey line or filled with different tones of grey labelled with arabic numerals indicate approximate geographical distributions of the two-step nested clades of *Cobitis taenia* (Culling *et al*. submitted), 2-1, 2-3, and 2-4, respectively. Roman numerals I, II and III, marking three remaining grayish areas correspond to the *Cobitis elongatoides* three-step nested clades 3-1, 3-2 and 3-3, respectively.

patterns in the loach mtDNA data set. We designed the hierarchical set of nested clades of the MST using the algorithm of Templeton *et al*. (1987) and Templeton & Sing (1993) to identify clades grouped by mutational changes step by step, until the final nesting level comprised the whole haplotype network (Fig. 4). For each clade at each hierarchical level, statistically significant departures from expectation under the null hypothesis of no geographical association were determined for the mean distance of members of a given clade from its geographical centre (D_c), for its mean distance from the entire nested clade (D_n) and for the mean differences between each measure for tip vs. interior clades (I–T). For all calculations, the great circle distances were assumed. Calculations were performed in GEODIS software, version 2.01, with 1000 permutations of the clades against the sampling sites to generate the null distribution (Posada *et al*. 2000). We used the dichotomous inference key provided in Templeton (2004) to identify plausible processes compatible with the observed patterns.

Dispersal rate estimate

In order to compare the dispersal potential of the parental species and hybrid lineages we estimated the average

single-generation migration distances using the method of Neigel *et al*. (1991), which assumes that the geographical distribution of a genealogical lineage results from the multigenerational process of dispersion from a single site of origin. Using the matrix of genetic distances corrected to the best-fit model (HKY 85 + gamma) we reconstructed the phylogenetic tree with the UPGMA algorithm (Sneath & Sokal 1973) implemented in PAUP*. The age of inferred lineages was estimated using the mtDNA 'clock' of 0.0042 mutations per site per Myr (Perdices & Doadrio 2001). We inferred the geographical centre of each such lineage as an abundance-weighted average of latitudinal and longitudinal coordinates of each location, where a given lineage was distributed. To estimate the parameter σ^2_H, defined as the variance of the geographical positions of individuals related by a common ancestor n generations ago, we calculated the variance of distances from the lineage centre for all lineages of a specified age-class. Per generation dispersal distance (δ) was calculated using the formula: $\delta = (\sigma^2_H / n)''$. We analysed north/south and east/west migrations separately since the combination of the two spatial coordinates leads to a loss of information due to the possibility of direction-dependent migration (Neigel *et al*. 1991). Spearman rank correlation coefficients were calculated to test the correlation of estimated parameters with the lineage ages (the use of a one-tail test was justified by the model predictions (Neigel *et al*. 1991) assuming only a positive correlation of σ^2_H and a negative one of δ). The method is based on the expectation that under restricted gene flow in continuously distributed species there should be a positive correlation between the spatial distribution and the age of a lineage. The original model of a nonequilibrium stage assumes an unconstrained random-walk process leading to the linear increase of the parameter σ^2_H and independence of δ on the age of a lineage. Departures from the model-predicted correlation of σ^2_H due to the imposition of an upper limit by range restrictions serve to estimate the state of achievement of the migration–drift equilibrium and are thus informative about the population history of a species. According to Neigel & Avise (1993), intermediate-age species, which have already met some geographical constraints but still continue to disperse, should express a positive but nonlinear correlation of σ^2_H and a negative correlation of δ with the ages of the included lineages and in old species having achieved migration–drift equilibrium, σ^2_H should be independent of time, whereas δ should be negatively correlated with lineage age.

Results

Sequence variation

The sequence of 1088 bp of cyt *b* was determined for 126 individuals. A total of 188 sites were variable (17.3% of total sites), of which 145 were phylogenetically informative (13.3% of total sites). Within the ingroup, 125 sites were variable (11.49%), 78 of which were phylogenetically informative (7.17%). All observed polymorphisms were single nucleotide substitutions. The combination of nucleotide states at variable positions defined 43 distinct haplotypes.

Phylogeny of haplotypes

According to both the ML and NJ tree topologies, the haplotypes clustered in two main clades with 100% bootstrap support in NJ (Fig. 3). One of these clades, corresponding to clade A of Janko *et al*. (2003), comprised all of the *C. elongatoides* haplotypes. The three haplotypes of *C. tanaitica* and the haplotype found in the Bulgarian population of *C.* spec. were also clustered in this clade. The other clade, corresponding to clade B of Janko *et al*. (2003), contained the haplotypes encountered by Culling *et al*. (submitted) in *C. taenia* and three haplotypes found in the Ukrainian populations of *C.* sp. All *elongatoides–taenia* hybrids carried *taenia*-type haplotypes except a single specimen from the Elbe River basin, which had *elongatoides*-type mtDNA. All the other hybrid lineages possessed only *elongatoides*-type mtDNA (Table 1).

The internal topology of the clades differed slightly between ML and NJ analyses and only a few branches obtained bootstrap support higher than 50% in the NJ tree. According to the statistical parsimony of Templeton *et al*. (1992), the maximum number of mutational steps allowing parsimonious connections between the haplotypes with probabilities equal to or higher than 95% was 14. Parsimony cladogram construction using this criterion resulted in two disjoint unrooted trees, corresponding to the two major clades (Fig. 4). Within the *C. elongatoides* tree, haplotypes detected in *C. tanaitica* did not form a monophyletic cluster relative to the haplotypes found in *C. elongatoides*, and they appeared to be most closely related to *C. elongatoides* from the lower Danube. *Cobitis* species haplotypes clustered in both trees (Fig. 4). The topology of the *C. taenia* tree was identical to the topology of the minimum-spanning tree of Culling *et al*. (submitted) with some additional haplotypes of hybrids and *C.* sp. added in this study. The majority of the hybrids with *taenia*-type mtDNA possessed the most frequent *C. taenia* haplotype, hereafter called T1, or haplotypes derived by single mutations from it (T2, T3), and they clustered in clade 1-1 of the nesting design by Culling *et al*. (submitted). Other hybrid lineages with *taenia*-type mtDNA clustered in clades 1-6 and 1-7 also possessed haplotypes either widespread in the maternal ancestor (T4, T9) corresponding to haplotypes 3 and 2, respectively, of Culling *et al*. (submitted) or derived by single mutations from them. In some cases, '*elongatoides*-type' hybrids possessed haplotypes that were sampled in *C. elongatoides* (E13) or most likely existed in its populations (E1), but most of them grouped in two distinct clades, hereafter

Table 1 Geographical origin of the samples of *Cobitis elongatoides* (C. el.), *C. taenia* (C. tae.), *C. tanaitica* (C. tan.), *C.* sp. and their hybrids (marks C. X tae./Y el. or C. Y el./Z tan. refer to the genomic composition of hybrids where X, Y and Z correspond to numerals indicating numbers of chromosome sets each species contributed to the hybrid genotype). Haplotype distribution is shown with absolute sample frequencies in parentheses

No.*	Water body	Country†	Biotype	Haplotype
1	Neisse River (Odra River basin)	D	C. 1 tae./2 el.	T1 (1)
2	Odra River	D	C. 1 tae./1 el.	T1 (1)
			C. 2 tae./1 el.	T2 (2)
3	Storawa River (Odra River basin)	PL	C. el.	E8 (2)
			C. tan.	E21 (1)
			C. 2 el./1 tan.	E31 (2)
4	Polska Woda River (Odra River basin)	PL	C. el.	E8 (2)
			C. 1 tae./2 el.	T1 (1)
			C. 2 el./1 tan.	E31 (1)
5	Pšovka creek (Elbe River basin)	CZ	C. el.	E6 (2), E8 (1)
			C. 1 tae./2 el.	T3 (3)
6	Lužnice River (Elbe River basin)	CZ	C. el.	E2 (1), E4 (1)
7	Lake Muegelsee (Elbe River basin)	D	C. 2 tae./1 el.	T1 (1)
			C. 1 tae./1 el.	E8 (1), T3 (1)
8	Ilav River (Elbe River basin)	D	C. 2 tae./1 el.	T3 (2)
9	Rhine River	D	C. 1 el./1 tan./1 tae.	E30 (2)
10	Rhine River	D	C. 1 el./1 tan./1 tae.	E30 (2)
11	Dyje River (Danube River basin)	CZ	C. 2 el./1 tan.	E13 (2), E31 (1)
12	Dyje River (Danube River basin)	CZ	C. el.	6 (2), 7 (1)
13	Lake Sinoe	RO	C. tan.	E17 (1), E20 (1)
			C. 1 el./2 tan.	E29 (1)
14	Moskva River (Volga River basin)	RU	*C.* 2 el./1 tae.	T1 (2)
15	Mur River (Danube River basin)	A	C. el.	E11 (4), E12 (1)
16	Szodrakosz creek (Danube River basin)	H	C. el.	E3 (2), E8 (1)
			C. 2 el./1 tan.	E1 (2), E29 (1), E30 (1)
			C. 3 el./1 tan.	E30 (1)
17	Loire River	FR	C. 1 el./2 tae.	T1 (4)
18	Čierná Voda River (Danube River basin)	SK	C. el.	E5 (1), E7 (1), E10 (1)
			C. 2 el./1 tan.	E29 (1), E30 (3)
19	Cornaya River	UA	C. spec.	T7 (2), T8 (1)
20	Southern Bug River	UA	C. spec.	T5 (1)
			C. 1 el./1 spec./1 tae .	E28 (1)
21	Southern Bug River	UA	C. 1 el./1 spec./1 tae.	E23 (1), E25 (1), E26(1)
			C. 1 el./1 tan./1 tae.	E29 (2)
22	Kodyma (southern Bug River basin)	UA	C. 1 el./1 spec./1 tae.	E27 (1)
23	Savranka R. (southern Bug River basin)	UA	C. 1 el./2 tae.	T4 (1), T6 (1)
			C. 1 el./1 tan./1 tae.	E29 (1)
24	Southern Bug River	UA	C. 1 el./1 spec./1 tae.	24 (1)
			C. 1 el./1 tan./1 tae.	29 (1)
25	Slutch River (Dnieper River basin)	UA	C. 2 el./1 tan.	E32 (2)
			C. 1 el./1 tan./1 tae.	E32 (3)
26	Slutch River (Dnieper River basin)	UA	C. 1 el./1 spec./1 tae.	E23 (4)
27	Irpien River (Dnieper River basin)	UA	C. 1 el./2 tae	T9 (2), T10 (1)
			C. 1 el./1 tan./1 tae.	E29 (1), E30 (1), E31 (1)
28	Western Bugiver River (Vistula River basin)	UA	C. 1 el./2 tae.	T1 (1)
			C. 1 el./1 tan./1 tae.	E31 (1)
29	Veleka River	BG	C. spec.	E22 (3)
30	Tazlau River (Danube River basin)	RO	C. el.	E13 (1)
31	Birlat River (Danube River basin)	RO	C. el.	E13 (1), E33 (1)
			C. 2 el./1 tan.	E29 (3)
32	Prut River (Danube River basin)	RO	C. el.	E18 (2)
			C. 2 el./1 tan.	E14 (1), E29 (2)
33	Comana River (Danube River basin)	RO	C. el.	E15 (1), E16 (1)
			C. 2 el./1 tan.	E1 (1)
			C. 2 el./1 tan.	E29 (1)
34	Danube River	RO	C. tan.	E19 (1)
			C. 1 el./1 tan.	E30 (1)
			C. 2 el./1 tan.	E29 (1), E30 (1)
35	Mures River (Danube River basin)	RO	C. el.	E9 (3)
			C. 2 el./1 tan.	E1 (1)

*The localities are numbered according to Fig. 1; †D, Germany; PL, Poland; CZ, Czech Republic; RO, Romania; RU, Russia; A, Austria; H, Hungary; FR, France; SK, Slovakia; UA, Ukraine; BG, Bulgaria.

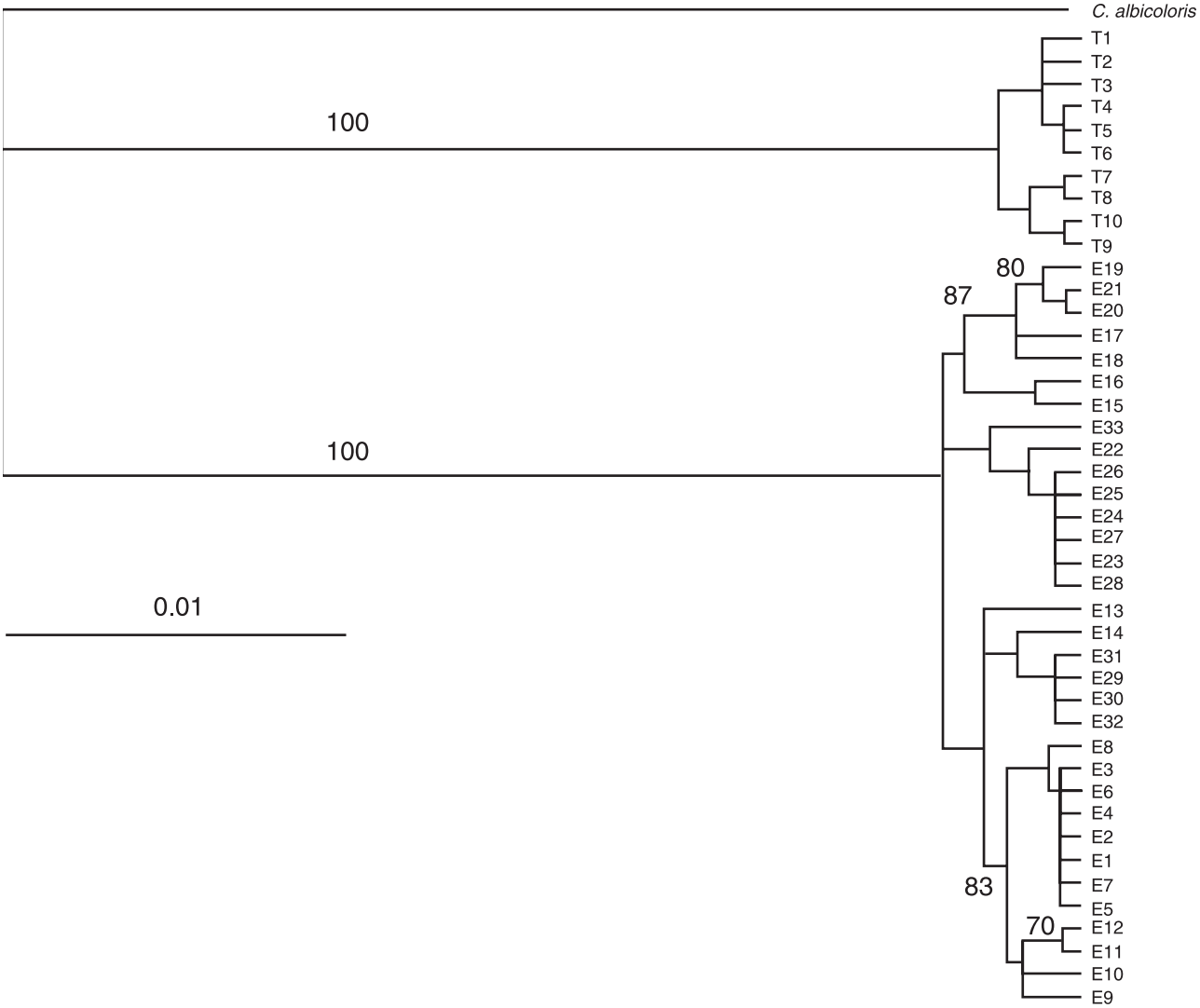


Fig. 3 Maximum-likelihood (ML) tree showing phylogenetic relationships among haplotypes encountered in this study. Branches that obtained bootstrap support equal to or higher than 70% in the neighbour-joining defined the same clades as in ML and are indicated by numerals above them. Two major clades comprising haplotypes E1–E33 and T1–T10 correspond to clades A and B from Janko *et al*. (2003), respectively.

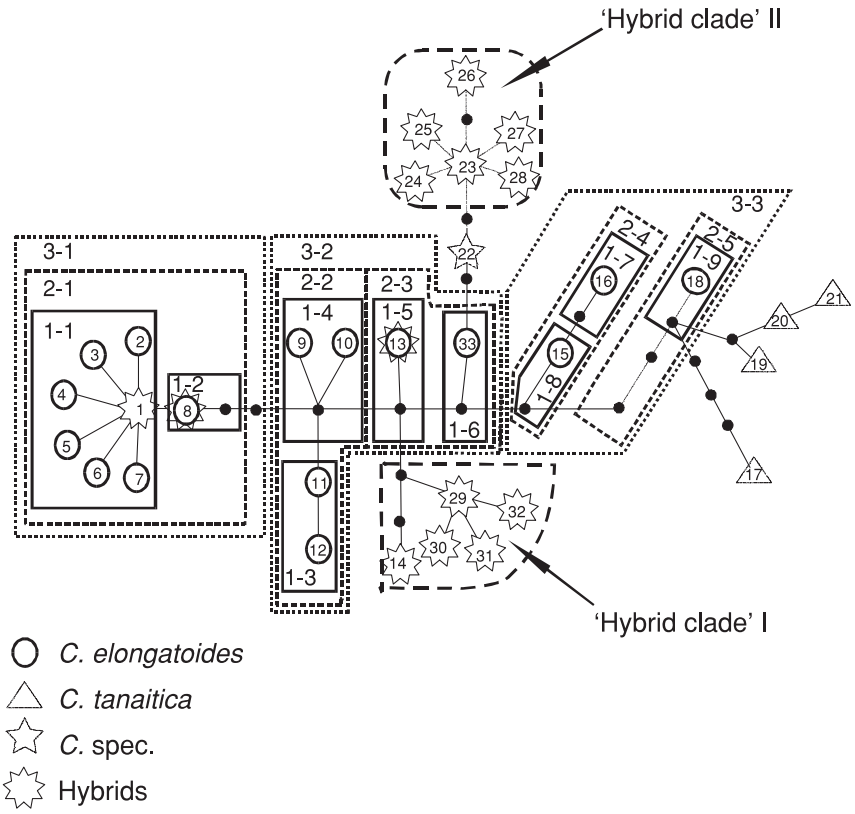
called 'hybrid' clade I (haplotypes E14, E29–32) and 'hybrid' clade II (haplotypes E23–28).

Nested clade phylogeographical analysis of Cobitis elongatoides *data*

The nesting design of the *C. elongatoides* data consisted of four levels (Fig. 4). The null hypothesis of no association of the genealogical position of the clades with the geographical distribution was rejected for at least one clade at each level (Table 2). Contiguous range expansion best explained the pattern observed among the clades nested within clade 2-1 distributed in the upper Danube, Elbe and Odra River basins. Low genetic resolution prevented the distinction between range expansion and restricted dispersal among the clades nested within clade 2-2 and the lack of samples from the central part of the Pannonian Basin disabled the discrimination between long- vs. short-distance movements in this clade. Allopatric fragmentation best explained the pattern observed within clade 3-2 distributed on both sides of the Carpathian Mountains. At the highest nesting level, restricted gene flow with some long-distance dispersal was consistent with the distribution of the clades (Table 2).

Dispersal rate estimates

Tables 3 and 4 show the geographical variance estimates (σ^2_H) and standard dispersal distances (δ) for lineages in the 5



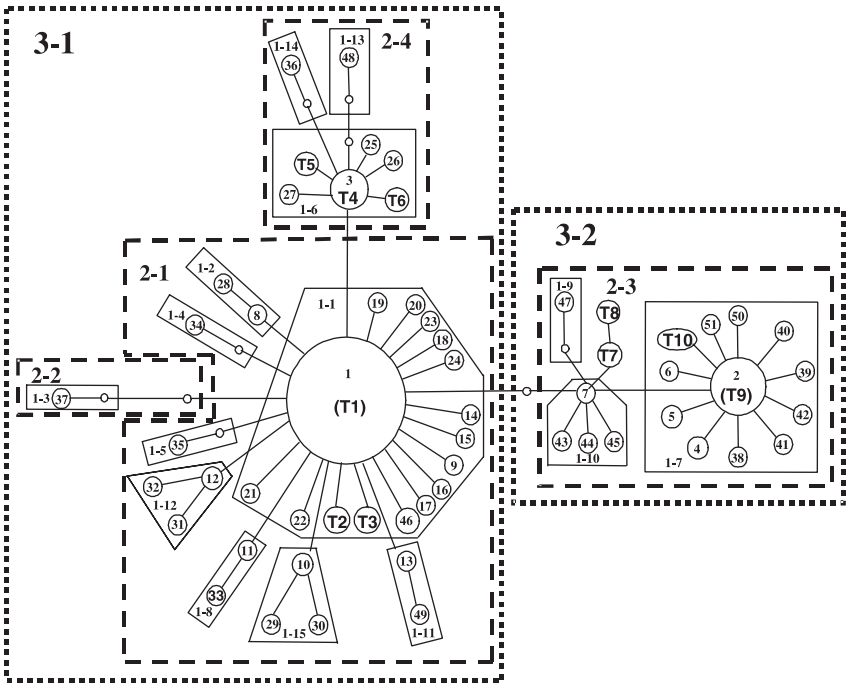


Fig. 4 Unrooted trees constructed by statistical parsimony and corresponding to the two major clades. (a) Clade A: haplotypes sampled in *Cobitis elongatoides* were used for subsequent NCPA with the indicated nested clade design. Dotted lines delimit 'hybrid' clades I (haplotypes E14, E29–E31) and II (E23–E28); (b) clade B: nested design of *Cobitis taenia* haplotypes taken from Culling *et al*. (submitted) with additional haplotypes encountered in hybrids (T1–T4, T6, T9–T10) and *C*. spec. (T5, T7–T8) corresponding to clade B.

and 10 age classes of *C. elongatoides* and *C. taenia*, respectively, as well as the Spearman rank correlation coefficients indicating a relationship between the estimated parameter values and lineage ages. The strength of the test was decreased by the low number of assessed lineages and we found only one significant relationship for the negative correlation of the latitudinal component of δ in *C. taenia*. We nonetheless observed remarkable trends of negative

Table 2 Nested clade phylogeographical analysis of *Cobitis* mtDNA data. For each haplotype/clade, D_c and D_n values are given with subscripts L and S indicating values significantly large and small at the 0.05 level, respectively. The average differences between interior (bold) and tip clades in both measures are given (I–T). For each clade with at least one significant value in the nested series, we indicate the inference chain followed by a biological interpretation according to Templeton (2004)

Haplotypes			One-step clades			Two-step clades			Three-step clades		
No.	D_c	D_n	No.	D_c	D_n	No.	D_c	D_n	No.	D_c	D_n
E2	285	353									
E3	0	81^S									
E4	0	221									
E5	0	178									
E6	0	432^L									
E7	0	178	1-1	275	295						
			1-2: I–T undetermined: inconclusive								
E8	—	—	**1-2**	130^S	245	2-1	—	—			
			I–T	–146	–49	1-2-11-12 no-contiguous range expansion			3-1	276^S	367
E9	0	73									
E10	0	217	**1-4**	109^S	326						
E11	—	—									
E12	—	—	1-3	0^S	407^L	**2-2**	361	360			
			I–T	109^L	-81^S	1-2-3-5-6: range expansion or restricted geneflow					
E13	—	—	**1-5**	35	38						
E33	—	—	**1-6**	0	39	**2-3**	39^S	299	**3-2**	332	402
									1-19 no: allopatric fragmentation		
E15	—	—	**1-8**	—	—						
E16	—	—	1-7	—	—	**2-4**	0	135			
E18	—	—	1-9	—	—	2-5	0	134	3-3	135^S	540^L
						I–T	0	0.7	I–T	86	–1

Table 3 Estimates of distance variances and standard dispersal distances from *Cobitis taenia* mtDNA data. *D* is the upper limit of sequence divergence defining the lineage age, *n* is the median age of lineages in a given age-class in generations and, finally, estimated parameters are given for the longitudinal (*x*) and latitudinal (*y*) component. For each parameter, Spearman rank correlation coefficient with corresponding *P* values in parentheses for correlation with time are given in italics

D	*n*	σ_x^2 (in km²)	δ_x (in km)	σ_Y^2 (in km²)	δ_Y (in km)
0.00125	143 750	253 539	1.3	98 052	0.8
0.00158	173 452	388 711	1.5	86 512	0.7
0.00196	205 476	413 546	1.4	131 350	0.8
0.002247	266 666	435 150	1.27	7 511	0.17
0.00258	294 583	205 465	0.8	116 882	0.63
0.0029	328 809	206 419	0.8	32 913	0.3
0.00324	348 937	783 727	1.5	54 630	0.4
0.0036	402 261	279 803	0.8	165 940	0.64
0.0039	456 547	634 632	1.18	45 406	0.3
0.00424	504 642	581 349	1.07	149 657	0.54
		0.44	*–0.47*	*0.16*	*–0.55*
		(0.1)	*(0.08)*	*(0.33)*	*(0.049)*

Table 4 Estimates of distance variances and standard dispersal distances from *Cobitis elongatoides* mtDNA data. *D* is the upper limit of sequence divergence defining the lineage age, *n* is the median age of lineages in a given age-class in generations and, finally, estimated parameters are given for the longitudinal (*x*) and latitudinal (*y*) component. For each parameter, Spearman rank correlation coefficient with corresponding *P* values in parentheses for correlation with time are given in italics

D	*n*	σ_x^2 (in km²)	δ_x (in km)	σ_Y^2 (in km²)	δ_Y (in km)
0.0023	246 875	57 460	0.48	15 058	0.24
0.003697	440 119	115 382	0.5	2 405	0.01
0.00524	623 809	118 336	0.44	12 276	0.14
0.006674	794 523	98 345	0.35	30 709	0.2
0.008654	1 019 524	131 044	0.35	32 616	0.17
		0.7	*–0.8*	*0.7*	*–0.1*
		(0.094)	*(0.052)*	*(0.094)*	*(0.43)*

correlation of the longitudinal component of δ in *C. elongatoides* as well as positive correlations of both the latitudinal and longitudinal components of σ_H^2 in this species as suggested by the high absolute values of the correlation coefficients. On the other hand, low parameter values indicated that the latitudinal components of δ in *C. elongatoides* and σ_H^2 in *C. taenia* are independent of lineage age. We chose the 'hybrid' clade I to estimate the dispersal potential of the hybrid lineages since it is a monophyletic assemblage of asexual lineages likely having originated in a single location, thus fulfilling the assumption of Neigel's approach.

This estimate equalled 1 km per year for the longitudinal and 0.5 km for the latitudinal components for the monophyletic lineage composed of haplotypes E29–E31.

Discussion

Mothers and fathers — parental species

In agreement with our previous results (Janko *et al*. 2003), females from both *C. elongatoides* and *C. taenia* have been documented to take part in hybridizations leading to *elongatoides–taenia* clonal lineages. The maternal ancestor of the hybrids clustering in the 'hybrid' clade II most likely was *C.* sp., since their mtDNA is derived from the Bulgarian population of this species (Veleka River; site 29) and its chromosome set was present in one copy in nuclear genome of these hybrids. Despite the close affinity of the nuclear genome of *C.* sp. to *C. taenia* (Janko unpublished data), individuals of this species possessed not only *taenia*-type haplotypes but also *elongatoides*-type mtDNA. Although the reason for the polyphyly of *C.* sp. mtDNA is not clear, for the purpose of this study it is important that we never observed the *C.* sp. nuclear genome in hybrids with *taenia*-type mtDNA, suggesting that only the Bulgarian population of *C.* sp. was involved in the origin of asexual lineages. Females of *C. tanaitica* are unlikely to have taken part in the hybridization since no hybrids clustered in the clade formed by *C. tanaitica* haplotypes.

Inference of the geographical structuring of the maternal ancestors is crucial for assessments of the geographical origins of asexual lineages. Culling *et al*. (submitted) described significant geographical structuring of *C. taenia*. In brief, authors found that Europe was recolonized by two lineages with a significant overlap originating in separate refuges, one in the middle or upper part of the southern Bug River (clade 2-1), the other (clade 2-3) located to the Dnieper River or the Volga River basins. Since the latter lineage is distributed in northern Europe, populations from the southern Bug River most likely dispersed westwards, whereas the eastern populations first colonized Europe northwards and then dispersed westward making use of the freshwater stage of the Baltic Sea (Culling *et al*. submitted). A third lineage was found in the lower southern Bug River (clade 2-4), which apparently did not contribute to the postglacial colonization of Europe.

The range of *C. elongatoides* is inhabited by three lineages with virtually no geographical overlap corresponding to the distribution of the three-step nesting clades. Significantly small D_c and high D_n values for tip clades at the highest nesting level suggest limited gene flow among the regions. The northernmost clade 2-1 found in the middle Danube, Odra and Elbe River basins showed the signal of a contiguous range expansion (Table 2). Drainage of the middle Danube River may have provided a refuge for this clade during the last glacial maximum (LGM), because it provided a more suitable habitat than the northern slopes of the Carpathians or adjacent Bohemian Massif (Adams & Faure 1997). North to northwestern expansion to the upper Danube, Elbe and Odra River basins would then be expected based on the distribution of tip clade 1-1 with a starlike structure. Changes to the hydrological network in this part of Europe during the Holocene (Bănărescu 1990) probably allowed *C. elongatoides* to cross the watersheds by the mechanism of river capture. At the upper Tisza River tributaries, a secondary contact between the northern lineage and clade 2-3 was detected. This clade was further distributed in the Mur River and Transylvanian populations on the other sides of the Danube River basin. The lack of samples from the Pannonian Basin prevented us from inferring whether this part of the Danube River basin was recolonized from a restricted refuge or whether it may have supported a large population less affected by the climatic perturbations. At a higher nesting level (clade 3-2) this lineage clustered with populations from the Moldavian river Siret on the opposite side of the Carpathian Mountains yielding a distribution pattern consistent with allopatric fragmentation across this mountain range. It is interesting that the Transylvanian populations possess evolutionary younger haplotypes relative to the Siret River, suggesting that they might come from a Moldavian ancestor having crossed the Carpathian Mountains, rather than having expanded via the lower Danube River, which is inhabited by a monophyletic lineage (clade 3-3) not sampled inside the Carpathian arch. Nonetheless, without samples from the Sava and Morava rivers draining the Dinaric Mountains into the Danube, which were shown to have played a significant role in the Quaternary maintenance of freshwater biota (Kotlík & Berrebi 2002), strong conclusions about *C. elongatoides* population history in the central part of its range may not be drawn. On the other hand, our data strongly indicate the long-term separation of the lowest part of the Danube River basin as indicated by the distribution of clade 3-3. Substantial differences among haplotypes indicate a high long-term effective population size and suggest that local populations have not undergone founder-flush cycles characteristic of areas strongly influenced by glacial cycles (Hewitt 2000).

Altogether, the current data suggest that *C. taenia* and *C. elongatoides* used different routes to disperse through the deglaciated parts of Europe from refugia located in the northern Black Sea drainages and the Danube River basin south of the Carpathians, respectively. The postglacial history of *C. tanaitica* remains unclear, but its recent finding in the Odra River basin suggests it was able to disperse northward around the Carpathians, probably via the Dniester River. The fourth species in the complex, *C.* spec., has a disjunct distribution similar to *Gobio crymensis* (Vasil'eva &

Kuga 2004) and the *Barbus escherichii* lineage I (Kotlík *et al*. 2004), which likely resulted from Quaternary water level and salinity changes of the Black Sea. Much of its north-eastern shelf has relatively recently been exposed, enabling riverine fish migration during the freshwater phase of the Black Sea (Kotlík *et al*. 2004).

Evolution of asexuality

Origins. The overlay of the phylogenetic relationships of the hybrids to the parental species onto the geographical distribution of the mtDNA lineages indicated several candidate areas for the origin of asexuality. The *elongatoides–taenia* hybrids possessing haplotypes E8, T1–T3 derived from maternal clades showing the signal of postglacial expansion into central Europe likely originated in this area, namely in the upper Odra and Elbe River basins. On the other side, the low-sea stand of the Black Sea in the early Holocene (Ryan *et al*. 1997) likely promoted reproductive contact between the parental species along the northern Black Sea shelf, giving rise to hybrid lineages with T3 and T4 haplotypes restricted within the range of their ancestral *C. taenia* clade 2-4. The hybrid lineages T9 and T10, with a distribution limited in the Dnieper River, descending from *C. taenia* clade 2-3 that has a range from the Atlantic coasts to the Crimean Peninsula and Kazakhstan, is best explained by their origin in the Ponto–Caspian contact zone followed by a northward dispersal into the Dnieper River.

At least two independent origins of the *elongatoides–tanaitica* hybrids are suggested by our data. One took place in the lower Danube River basin as evidenced by the haplotype E13 shared with the Siret River population of *C. elongatoides*, the other occurring within the range of *C. elongatoides* clade 2-1, possibly involving *C. tanaitica* populations from the Odra River.

In addition to these recent hybridization events, two monophyletic clusters of hybrids with substantial intra-lineage diversity suggesting a higher evolutionary age were sampled ('hybrid' clades I and II). The origin of clade II distributed in the southern Bug River likely involved a *C.* sp. female of the Bulgarian lineage and a *C. elongatoides* male and took place 118 000 ± 84 000 BP estimated by the Saillard *et al*. (2000) method or even 219 000 BP when considering the mean pairwise within-clade divergence. The estimated age of the clade I is even higher, about 342 000 ± 199 000 and 263 000 BP, respectively. Due to the incomplete sampling of *C. elongatoides* range we cannot reject the hypothesis that haplotypes included within 'hybrid' clade I come from this species and thus did not originate exclusively by postformational mutations; however, this explanation seems unlikely since *C. elongatoides* populations in the area of contact with *C. tanaitica* were reasonably well sampled. Furthermore, hybrids from this clade carried one genome of *C. elongatoides*, one of *C. tanaitica* and an additional third genome from either *C. elongatoides* or *C. taenia*, which suggests that the original hybridization was followed by triploidization through either *elongatoides* or *taenia* sperm. Therefore, the single ancient origin of the clade is the most plausible explanation. Despite the perhaps controversial usage of molecular clocks and the significant error associated with age estimates, our data suggests in general, that the asexual loach lineages are not only the result of Holocene reproductive contact among the parental species, but that they coexisted in the case of clades I and II with the parentals over one or more glacial cycles and faced the same ecological and geophysical challenges. We therefore suggest the comparable adaptive potential of both sexual and clonal populations to Quaternary climatic oscillations.

Our data are not concordant with any obvious constraint on the origin of asexuality within the *Cobitis* hybrid complex, but rather indicate that asexual lineages arise frequently, whenever parental species come into reproductive contact. Since several distinct parental lineages were involved in the origin of the clones, there seem to be no differences in the genetic predispositions for the origin of asexuality among geographical populations of the parental species as for example in the *Rana esculenta* (Hotz *et al*. 1985) or *Poeciliopsis monacha–lucida* complexes (Quattro *et al*. 1991).

Dispersal. After their origin, sperm parasites may disperse either by invading the established host populations, or by following the host's range expansion. Both mechanisms are suggested by our data. Some lineages (e.g. those possessing haplotypes E1, E13, T4, T6, T9, T10 and 'hybrid' clade II) likely dispersed by invading new host populations, since they are distributed across refuge areas, which have likely been populated by the sexual progenitors prior to the origin of the clones. Furthermore, in some cases (E1), host populations were invaded across watersheds suggesting the hybrids used similar dispersion mechanisms as the parentals. Hybrids with T1–T3 haplotypes originated in central Europe during westward expansion of *C. taenia*, but their absence from England and Sweden suggests they were not present at the expanding edge of the parental populations, and have reached their range limits later, probably after the opening of the Channel and Baltic Sea, taking place about 7500 (Wheeler 1977) and 4000–8000 BP (Donner 1995), respectively. On the other hand, the distribution of 'hybrid' clade I suggests they have survived the LGM together with *C. elongatoides* in refuge areas, from which they expanded postglacially together with its host species. The hybrids thus probably followed *C. elongatoides* to the upper Odra and Danube rivers and were able to cross the Danube–Rhine watershed and invade the local populations of *C. taenia*. The inferred dispersal potential of the hybrid lineages, which is comparable or even superior to the parental species, supports this scenario suggesting

that sperm parasites invade bisexual populations at rates similar to the expansion of the parental species into newly available areas and may easily follow such a parental expansion.

Unlike true parthenogens, where increased dispersal represents strong adaptive advantage minimizing the parasite-load (see Pongratz *et al*. 2003) or competition with ancestors, sperm-dependent asexuals are generally restricted within the ranges of ancestral species due to their dependence on sperm donors. It has been proposed that such organisms tend to adapt to local conditions giving them a chance of establishment in a given subniche, for which they are better specialized than their parents (Lynch 1984; Vrijenhoek 1998). To date phylogeographical studies of asexual complexes, although not numerous, generally confirm the difference between true and sperm-dependent parthenogenetic populations. Whereas former ones have generally large geographical distributions even across apparent barriers (Avise *et al*. 1992; Johnson & Leefe 1999; Pongratz *et al*. 2003), latter ones generally consist of many clonal lineages of polyphyletic origin, with a rather endemic distribution restricted, in the case of fishes, to the river system of the original hybridization (Quattro *et al*. 1991, 1992a; Cunha *et al*. 2004; but see Quattro *et al*. 1992b). Our data nonetheless indicate the significant role of dispersal in the evolution of sperm-dependent parthenogenesis in loaches. We confirm that extrinsic barriers likely responsible for the population structuring of the parental species can easily be overcome by clones. The considerable dispersal potential of the hybrids further led in both 'hybrid' clades to the invasion of territory uninhabited by the original parental species by switching to a new host, thus blurring the distinction between true parthenogens and sperm-dependent clonals. A growing body of studies focusing on the metapopulation structure of pseudogamous lineages suggests that they undergo population density cycles according to sperm availability leading in extreme cases to local extinctions (Moore & McKay 1971; Hellriegel & Reyer 2000; Bobyrev *et al*. 2003). The increased dispersal of clones, just as in 'normal' parasites, may therefore represent a selective advantage augmenting a chance of invading unparasitized host populations (reviewed in Rózsa 1999).

With this in mind, it is interesting why *C. elongatoides* and *C. taenia*, given their phylogenetic proximity, similarity in morphology and ecological requirements, differ so remarkably in their dispersal potential. Since Neigel's method estimates the actual values of δ from the present distribution of the clades reflecting historical, rather than contemporary events, lower δ in *C. elongatoides* may be influenced by its smaller distribution range caused either by geographical barriers or by competitive exclusion by *C. taenia*. Nonetheless, the indicated (although not statistically significant) positive correlation of the geographical distribution and the age of lineages, suggests that range limits did not affect the estimate of standard dispersal distance of *C. elongatoides*. On the contrary, the significant negative correlation of the latitudinal component of δ of *C. taenia* and the independence of σ^2_H on time indicates it is closer to the migration–drift equilibrium. Inferred differences in the estimates of δ therefore are not artefacts of different distribution ranges, but reflect better dispersal potential of *C. taenia*. From a long-term point of view, the dispersal of a sexual lineage and its settlement at a given site depend on the ability of migrating individuals to find a mate. Given that asexuals compete with host species for resources, thus ultimately decreasing its population densities, we suggest that coexistence with sperm parasites reduces the dispersal potential of the bisexual population. The inferred difference in dispersal potential of both species may thus reflect that the expanding *C. taenia* populations were free of 'sex-parasites', whereas the expansion of *C. elongatoides* was followed by coexisting clones. This may explain why the Danube did not play a significant role in the colonization of northern and western Europe by loaches, contrary to the situation in other fish species such as *Barbus barbus* (Kotlík & Berrebi 2001), *Perca fluviatilis* (Nesbø *et al*. 1999), or *Leuciscus cephalus* (Durand *et al*. 1999), although *C. elongatoides* evidently was able to cross the Danube–Elbe and Danube–Odra watersheds.

Conclusions

This study revealed contrasting Quaternary histories for parental species within the loach asexual complex. *Cobitis taenia* survived the Pleistocene glaciations in at least three refugia, two of which served as the source for a postglacial expansion followed by reproductive contact in central Europe with *C. elongatoides* that dispersed from Danubian refuges. The subsequent hybridization of these species led to the emergence of *elongatoides–taenia* clonal lineages, which invaded the ranges of both parental taxa. *Cobitis tanaitica* probably originated in the Ponto–Caspian basin and dispersed into central Europe around the Carpathians, whereas *C.* sp. have remained restricted to the northwestern coast of the Black Sea. Holocene reproductive contact between *C. elongatoides* and *C. tanaitica* resulted in triploid clonal lineages distributed mainly in the Danube River basin. Two putatively ancient clonal lineages were discovered that originated not later than during the last interglacial by hybridization of *C. elongatoides* with *C. tanaitica* and *C.* sp., respectively. The former lineage survived the LGM in refuges with *C. elongatoides* and followed its postglacial expansion into central Europe, where it invaded local populations of *C. taenia*, thus switching to a new host species. Unlike other freshwater fishes, Danubian populations of loaches apparently failed to colonize northwestern Europe. We suggest that such a pattern may be a consequence of

their long-term coexistence with clonal lineages, which reduced their colonization ability.

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