European colonization by the spined loach (*Cobitis taenia*) from Ponto-Caspian refugia based on mitochondrial DNA variation

MARK A. CULLING,* KAREL JANKO,† ALICJA BOROŃ,‡ VICTOR P. VASIL'EV,§
ISABELLE M. CÔTÉ* and GODFREY M. HEWITT*

**School of Biological Sciences, Centre for Ecology, Evolution and Conservation, University of East Anglia, Norwich NR4 7TJ, UK,
†Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburská 89,
27721 Libechov, Czech Republic, and Biodiversity Research Group, Department of Zoology, Charles University, Vini#269:ńa 7, 128 44,
Pragu-2, Czech Republic, ‡Department of Zoology, Faculty of Biology, University of Warmia and Mazury in Olsztyn, 5 Oczapowski
St., 10-718 Olsztyn, Poland, §Institute of Ecology and Evolution, RAS, Leninskyi prospect, 33, Moscow 119071, Russia*

Abstract

In the last 20 years, new species, asexual reproduction, polyploidy and hybridization have all been reported within the genus *Cobitis*. An understanding of the current distribution and baseline phylogeographical history of 'true' nonhybrid *Cobitis* species is crucial in order to unravel these discoveries. In the present work, we investigated the phylogeography of the spined loach, *Cobitis taenia*, using 1126 bp of the mitochondrial cytochrome *b* gene from 174 individuals collected at 47 sites. In total, 51 haplotypes that differed at 49 positions (4.35%) were detected. We deduce that *C. taenia* survived European glaciations in at least three refuges in the Ponto-Caspian area. Two of these refuges each provided a major lineage that recolonized Europe in separate directions: one westward to England and the other spreading north into Russia before moving west. A third (minor) lineage that contributed little to the recolonization of Europe was also revealed — remaining near its Black Sea refuge. However, more recent history was difficult to resolve with colonization from a more western refugium during the last glacial maximum (LGM) a distinct possibility. Nested clade analysis indicates a pattern of restricted gene flow with isolation by distance at the first two levels and overall. Unlike many other European freshwater fish species, the Danube is not part of the current distribution of *C. taenia*, nor was it used as either a refuge or a source of colonization of Europe. Low genetic diversity within *C. taenia* suggests that its colonization of Europe is relatively recent. Demographic analyses revealed a history of recent expansion and isolation by distance.

Keywords: colonization, glacial refugia, mitochondrial DNA, nested clade phylogeographical analysis, Pleistocene

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Introduction

Climatic and environmental changes during the Pleistocene have had significant impacts upon the habitat and natural range of many species (Hewitt 1993, 2000). In temperate latitudes of the Northern Hemisphere southward range shifts and contractions during cold periods into so-called glacial refugia have been followed during warmer interglacials by northerly range shifts and recolonizations.

Quaternary glacial events have left a signature upon the genome of many species that has been reconstructed by phylogeographical analyses (Hewitt 1996, 1999, 2001; Taberlet *et al*. 1998; Avise 2000). Most phylogeographical analyses have focused on terrestrial animals (but see Bernatchez & Wilson 1998 for a compilation of fish studies from North America), such as the grasshopper *Chorthippus parallelus* (Cooper *et al*. 1995), the hedgehog *Erinaceus europaeus/concolour* (Santucci *et al*. 1998; Seddon *et al*. 2001) and the bear *Ursus arctos* (Taberlet & Bouvet 1994). Together, these studies have revealed the rapid postglacial northward colonization of Europe from four major glacial

Correspondence: Mark A Culling, Fax: +44 (0) 1603 592250; E-mail:
m.culling@uea.ac.uk

refugia in Iberia, Italy, the Balkans-Greece and the Caspian/Caucasus regions (Hewitt 2004).

Freshwater organisms are likely to show patterns of range contractions and recolonization routes after glaciation, which differ from those of terrestrial organisms due to their reliance on waterways. The dispersal of such organisms that are tied to drainages may therefore reflect historical causes more closely than less confined species (Avise 2000). For example, in European freshwater fish studied up until now, mitochondrial DNA (mtDNA) and allozyme studies show a range of phylogeographical structure from two lineages in barbel *Barbus barbus* (Kotlik & Berrebi 2001) up to seven lineages in the bullhead *Cottus gobio* (Hänfling *et al.* 2002; Volckaert *et al.* 2002). Furthermore, deduced patterns of colonization from glacial refugia vary among species. For example, there is evidence of recolonization from refugia in the Balkans, Danube and Black Sea basins via the Danube and Dnieper in the chub *Leuciscus cephalus* (Durand *et al.* 1999), from the Dnieper, Danube and Black Sea basins via the Dnieper, Rhine and Baltic Sea basins in the perch *Perca fluviatilis* (Nesbø *et al.* 1999) and from the Mediterranean, Atlantic and Caspian Sea areas via the north Atlantic, Adriatic and the Black Sea drainages in the brown trout *Salmo trutta* (Bernatchez 2001).

However, there are some problems with studies of the phylogeography of aquatic species. Anthropogenic movements of fish (Bernatchez & Osinov 1995; Brito *et al.* 1997; Hänfling *et al.* 2002), due to angling, attractiveness of species to aquarists, river connections across watersheds via canals, fish breeding and restocking (Kotlik & Berrebi 2001; Triantafyllidis *et al.* 2002; Ludwig *et al.* 2003; Salzburger *et al.* 2003), can confuse species' historical distribution, and hence its accurate sampling and subsequent interpretation of phylogeographical analysis.

In the last 20 years, the increase in genetic studies of aquatic species has led not only to an increase in the recognized number of freshwater fish species in Europe, but also to a realization of the complexity of their reproduction (Kottelat 1997; Bohlen & Ráb 2001). For example, the spined loach, *Cobitis taenia* Linnaeus, 1758, protected under Annex II of the EC Habitats and Species Directive, was until recently considered to be a widely distributed polymorphic species distributed from Japan to Europe (Berg 1949; Bănărescu 1990). However, new species, unisexual reproduction, polyploidy and hybridization have all recently been identified within the genus *Cobitis* (Vasil'ev 1985; Vasil'ev *et al.* 1989, 1990a, b; Boroń 1992; Ráb & Slavik 1996; Bohlen & Ráb 2001). Over a considerable part of the known cobitid distribution range, diploid, triploid and tetraploid, almost all female hybrids between *C. taenia* and *Cobitis elongatoides*, as well as triploid and tetraploid all-female hybrids between *C. elongatoides* and *Cobitis tanaitica* have been identified that co-occur sympatrically with the parental species (Šlechtová *et al.* 2000; Bohlen & Ráb 2001;

Persat *et al.* 2002; Boroń 2003). Most populations are dominated by triploids, except in the Odra and Elbe River basins where diploid hybrids are common (Bohlen *et al.* 2002). Gynogenesis has been suggested as the mode of reproduction for both the *C. elongatoides–taenia* and the *C. elongatoides–tanaitica* hybrid complexes (Vasil'ev *et al.* 1989; Saat 1991; Bohlen & Ráb 2001). However, it will be difficult to unravel where, when and how the parental cobitid species involved have hybridized without first understanding the current distribution and baseline phylogeographical history of the 'true' diploid species (we define 'true' here as nonhybrids, corresponding to the *C. taenia* karyotype 2n = 48 chromosomes and other cytogenetic and genetic features defined by Vasil'ev *et al.* 1989; Ráb *et al.* 2000; Šlechtová *et al.* 2000). This information is vital to understand the current distribution of *C. taenia* throughout Europe, to study its entities properly, and to deduce how such complex species structures evolved. It may serve as an example for other (aquatic) species where complex reproductive relationships occur.

The spined loach, *C. taenia*, has long been thought to be present throughout northern, central and southern Europe including the Danube system (Berg 1949; Lelek 1987; Ahnelt & Tiefenbach 1994; Spindler 1997; Angermeier & Davideanu 2004). However, this supposed distribution was largely based upon qualitative visual assessment or coarse morphological analyses despite the fact that several *Cobitis* species vary little in morphology (Nalbant 1993; Vasil'ev & Vasil'eva 1994; Vasil'eva & Vasil'ev 1998; Bohlen & Ráb 2001). According to Vasil'eva (2000), any descriptions of *Cobitis* species based upon weak morphological differences without genetic analyses are unreliable. The lack of detailed genetic knowledge about the current distribution and baseline phylogeographical history of the protected spined loach, *C. taenia*, can have profound consequences for the effective conservation management of its populations.

Cobitis taenia is a highly suitable organism for phylogeographical investigation due to ecological traits such as bottom-dwelling (Sawada 1982), with a tendency to burrow (Culling *et al.* 2003) and poor swimming ability that give it poor dispersal capability. Furthermore, an apparent lack of economic importance and use as a bait fish by anglers in the past, make it unlikely that spined loach populations have been altered by human-induced translocations.

In this study, we specifically aimed to infer the phylogeographical patterns of the 'true' diploid *C. taenia* in order to assess its refugia during ice ages and postglacial colonization routes and provide the necessary basis for a more detailed understanding of the evolution of asexual reproduction and hybridization in the *Cobitis* complex using mtDNA analyses of identified *C. taenia* populations. Our analysis is based on DNA sequences of the mitochondrial gene cytochrome *b*, using samples from 47 populations located throughout the distribution range of the species.

Materials and methods

Sampling

A total of 320 individual specimens of *Cobitis* were obtained from 68 locations across Central and Eastern Europe, including the Danube (see also Janko *et al.* 2005). Allozyme and karyotype analyses were carried out to confirm the identity of diploid 'true' *Cobitis taenia*. This initial step was necessary because in this difficult-to-identify genus, the karyotype combined with allozyme data is the only reliable method for the identification of species and hybrids (Bohlen & Ráb 2001). Specimens analysed for karyotype analysis were dissected and the kidneys used for chromosome preparations according to the protocols of Vasil'ev *et al.* (1989), Ráb *et al.* (2000) and Boroń *et al.* (2003). Allozyme analyses were carried out on previously identified variable loci (both within and between species) using standard protocols (Šlechtová *et al.* 2000). In total, 174 samples from 47 locations were identified as diploid 'true' *C. taenia* and were used in this study (Fig. 1, Table 1). We took a similar approach to that of Kotlik & Berrebi (2001) of increasing the number of sampling locations and geographical coverage at the expense of within-population sample sizes to fit in with our aim of understanding the phylogeography of this species. This has enabled us to include samples from most of the major European drainages within the species' range and allowed coverage of potential refugial areas and colonization routes located within Northern and Central Europe. However, despite the large sampling effort, our sample sizes are not uniform due to difficulties in obtaining the loaches from some parts of Europe and therefore we have had to take a conservative approach to the interpretation of our data. To confirm the monophyly of the investigated *C. taenia*, eight samples of *C. elongatoides*, and two of *Cobitis vardarensis* (GenBank Accession nos AF263079 and AF263080, Perdices & Doadrio 2001) belonging to the same subgenus *Cobitis sensu stricto* were included in the phylogenetic analysis along with an
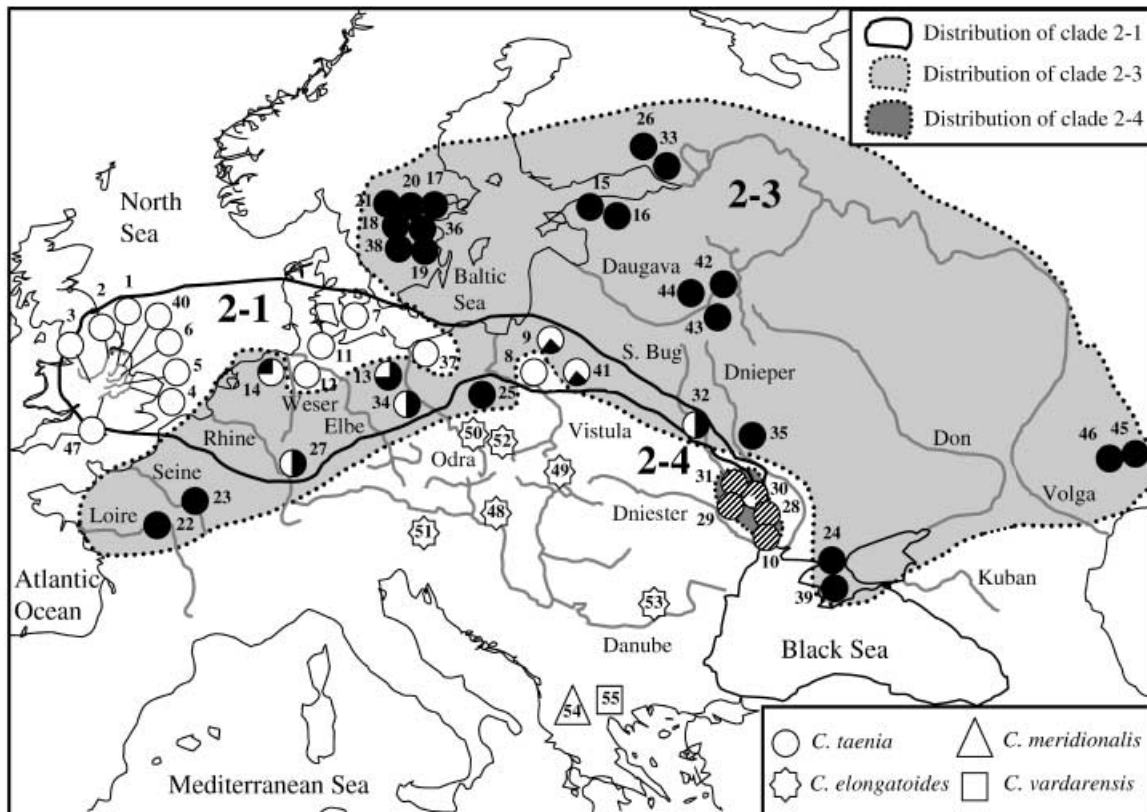

Fig. 1 Map illustrating the location of samples identified as 'true' *Cobitis taenia*, and the distribution and relative frequencies of the haplotypes that are affiliated to the NCA identified clades 2-1, 2-3 and 2-4 (a detailed distribution of all haplotypes is described in Table 1). The numbers correspond to location numbers listed in Table 1. The distribution of Haplotype 1 (white circle) also includes derivatives H8–24, H28–35, H46, H49; Haplotype 2 (black circle) includes derivatives H4–6, H38–42, H50–51 and Haplotype 3 (diagonal striped circle) includes derivatives H25–27, H36, H48. Clade 2-2 (not shown) occurred only in the River Elbe.

Table 1 Geographical origin and number of individuals of *Cobitis* used in the phylogeographical analysis. Location codes are shown in Fig. 1. Haplotype numbers refer to the NCA (Fig. 4), with absolute sample frequencies given in parentheses. NRM refers to voucher numbers of the Swedish Museum of Natural History

Code	Locality	Biotype	N	Coordinates	Cyt *b* haplotypes
1	R. Witham at Cannick, ENG	*C. taenia*	12	53°13′35″N, 00°28′49″W	H1(7) H13(1) H18(2) H24(1) H49(1)
2	R. Trent at Stoke Bardolph, ENG	*C. taenia*	4	52 °57′37″N, 01°02′04″W	H1(2) H9(1) H15(1)
3	R. Trent trib., R. Mease, ENG	*C. taenia*	8	52 °43′24″N, 01°43′02″W	H1(8)
4	Gt. Ouse at Ouse Washes, ENG	*C. taenia*	17	52 °07′49″N, 00°08′42″E	H1(12) H10(1) H17(1) H21(1) H29(1) H30(1)
5	R. Nene at Mortons Leam, ENG	*C. taenia*	12	52°34′25″N, 00°03′10″W	H1(10) H10(1) H33(1)
6	R. Welland at Baston Fen, ENG	*C. taenia*	12	52°44′43″N, 00°18′19″W	H1(8) H11(1) H28(1) H31(1) H32(1)
7	R. Susaa, DEN	*C. taenia*	4	55°16′00″N, 11°43′00″E	H1(1) H12(1) H16(1) H34(1)
8	R. Vistula at Zegrzynski Res., PL	*C. taenia*	3	52°27′00″N, 21°02′00″E	H1(3)
9	R. Vistula at Wigry Lake, PL	*C. taenia*	3	54°01′58″N, 23°06′29″E	H1(2) H2(1)
10	Southern Bug R., UKR	*C. taenia*	2	47°08′10″N, 31°25′22″E	H3(2)
11	L. Ploen in the Baltic, D	*C. taenia*	3	54°10′00″N, 10°25′00″E	H1(3)
12	R. Elbe trib., R. Ilav, D	*C. taenia*	4	53°23′00″N, 10°25′00″E	H1(1) H8(1) H14(1) H37(1)
13	R. Odra, D	*C. taenia*	4	52°34′07″N, 14°36′16″E	H1(1) H2(1) H5(2)
14	R. Weser trib., Haaren Crk, D	*C. taenia*	4	53°05′00″N, 07°50′00″E	H1(1) H2(1) H20(1) H23(1)
15	R. Jagala, EST	*C. taenia*	3	59°28′33″N, 25°09′04″E	H2(2) H40(1)
16	R. Loabu, EST	*C. taenia*	3	59°33′59″N, 25°48′00″E	H2(3)
17	L. Malaren, Bockholmsundet (NRM 44662-3) SV	*C. taenia*	2	59°16′32″N, 17°39′34″E	H2(2)
18	R. Soderkopingsan (NRM 46981) SV	*C. taenia*	1	58°22′00″N, 16°27′00″E	H2(1)
19	R. Vindan, L. Vindommen (NRM 46982), SV	*C. taenia*	1	58°08′36″N, 16°24′28″E	H2(1)
20	L. Karringfisket (NRM 46983) SV	*C. taenia*	1	58°44′22″N, 15°49′33″E	H2(1)
21	Kilaan Cr'k, Albergaan Cr'k (NRM 41778) SV	*C. taenia*	1	58°43′36″N, 16°31′44″E	H2(1)
22	R. Loire nr Orleans, F	*C. taenia*	2	47°16′00″N, 02°11′00″W	H2(1) H39(1)
23	R. Seine nr Troyes, F	*C. taenia*	4	48°18′00″N, 04°05′00″E	H2(4)
24	R. Alma nr the Black Sea, CR	*C. taenia*	2	44°08′10″N, 31°51′00″E	H2(2)
25	R. Odra at Slesinski Channel, PL	*C. taenia*	2	52°23′00″N, 18°20′00″E	H2(1) H43(1)
26	R. Kangaskoski, FI	*C. taenia*	5	61°25′00″N, 29°25′00″E	H2(2) H4(1) H6(2)
27	R. Rhine, D	*C. taenia*	2	49°38′08″N, 08:21′35″E	H2(1) H35(1)
28	Southern Bug R., UKR	*C. taenia*	3	47°30′34″N, 31°25′22″E	H3(1) H36(1) H48(1)
29	Southern Bug R., Kodyma R., UKR	*C. taenia*	2	47°56′10″N, 30°45′53″E	H3(1) H26(1)
30	Southern Bug R., Savranka R., UKR	*C. taenia*	5	48°07′34″N, 29°44′12″E	H3(3) H22(1) H27(1)
31	Southern Bug R., UKR	*C. taenia*	4	48°57′33″N, 28°41′02″E	H3(3) H25(1)
32	R. Dnieper trib., Sluch R., UKR	*C. taenia*	2	50°39′29″N, 27°37′53″E	H1(1) H6(1)
33	R. Sahakoski, FI	*C. taenia*	2	61°50′00″N, 24°30′00″E	H6(2)
34	R. Ems trib., Hase R., D	*C. taenia*	4	51°09′00″N, 09°26′00″E	H5(1) H8(1) H19(1) H42(1)
35	R. Dnieper at Bicianka Cr'k, UKR	*C. taenia*	2	50°26′00″N, 30°31′00″E	H7(1) H45(1)
36	R. Stangan (NRM 46980) SV	*C. taenia*	1	57°39′00″N, 15°36′00″E	H38(1)
37	R. Odra at Glebokie Lake, PL	*C. taenia*	2	53°40′00″N, 15°30′00″E	H1(2)
38	Kapellan Stream (NRM 46969) SV	*C. taenia*	1	58°24′07″N, 15°28′58″E	H41(1)
39	R. Cornaya Reka, CR	*C. taenia*	2	44°34′09″N, 33°38′23″E	H44(1) H47(1)
40	R. Witham, Metheringham D'ph ENG	*C. taenia*	5	53°13′35″N, 00°18′19″W	H1(3) H9(1) H12(1)
41	R. Bug, PL	*C. taenia*	3	52°31′58″N, 21°05′00″E	H1(2) H7(1)
42	Volga R., Moscow R., RU	*C. taenia*	2	55°24′31″N, 37°33′18″E	H2(1) H51(1)
43	R. Dnieper at Smolensk, RU	*C. taenia*	2	55°34′22″N, 33°08′12″E	H2(2)
44	Zapadnaya, R. Dvina, Velizh City, RU	*C. taenia*	2	56°16′35″N, 32°03′45″E	H2(2)
45	R. Bol'shoy Uzen', KZ	*C. taenia*	2	48°50′00″N, 49°40′00″E	H2(1) H50(1)
46	R. Malyy Uzen', KZ	*C. taenia*	2	48°50′00″N, 49°39′00″E	H2(2)
47	Padbury Brook, Gt. Ouse, ENG	*C. taenia*	4	51°58′00″N, 00°58′03″W	H46(4)
48	Szodrakosz creek, Danube, H	*C. elongatoides*	1		E3
49	Zierná Voda River, Danube, SK	*C. elongatoides*	1		E5
50	Polska Woda River, Odra, PL	*C. elongatoides*	1		E8
51	Mur River, Danube, A	*C. elongatoides*	1		E11
52	Polska Woda River, Odra, PL	*C. elongatoides*	2		502, 516
53	Timis/Albina River, Danube, RO	*C. elongatoides*	2		81, 82
54	Lake Prespa/Psarades, GR	*C. meriodionalis*	2		83, 84
55	Agiaki/Kastanies River, Vardar, GR	*C. vardarensis*	2		79, 80

CR, Crimea; D, Germany; DEN; Denmark; ENG, England; EST, Estonia; F, France; FI, Finland; GR, Greece; KZ, Kazakhstan; PL, Poland; RO, Romania; RU, Russia; SK, Slovakia; SV, Sweden; UKR, Ukraine.

outgroup of two samples of *Cobitis meridionalis* (GenBank Accession nos AF263083 and AF263084, Perdices & Doadrio 2001) from the subgenus *Bicanestrinia*, which was justified by the relationships within the genus established by Perdices & Doadrio (2001).

MtDNA amplification and sequencing

DNA was extracted from fin clips preserved in ethanol (99%), either by phenol–chloroform extraction (Sambrook *et al.* 1989), or by salt extraction after Sunnucks & Hales (1996) and Aljanabi & Martinez (1997). The entire cytochrome *b* gene (1140 bp) was amplified with the primers GluDG.L (5′-TGACTTGAARAACCA-3′; Palumbi 1996) and H16460 (5′-CGAYCTTCGGATTAACAAGACCG-3′; http://nmg.si.edu/bermlab.html/). Polymerase chain reactions (PCR) were performed in a 55-μL volume containing 2 μL (approximately 80 ng) of genomic DNA, 2 μL of each primer (0.01 M), 0.75 μL MgCl$_2$ (0.05 M), 4 μL of $10 \times Taq$ polymerase buffer, 1 μL each of A, T, C, G nucleotide mix (0.01 M), and 0.25 U of *Taq* polymerase (Bioline). A negative control (no DNA) was included in each run. PCR was carried out with the following conditions: an initial cycle of 94 °C denaturation for 1 min 20 s, followed by 5 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s and extension at 72 °C for 1 min 30 s. This was followed by 29 cycles of denaturation at 94 °C for 45 s and annealing at 58 °C for 45 s. A final cycle of extension at 72 °C for 1 min 30 s was carried out.

The primers used for sequencing were the same as for PCR plus internal primers INT1R (5′-TCGCCCGAGGA-CTATATTAT-3′) and INT2R (5′-TAGTACCTATTCTT-CATACGTC-3′), designed for loach cytochrome *b*. The amplified fragment was visualized on a 1.5% agarose gel and purified by filtration through a QIAquick column (QIAGEN). Direct sequencing of the purified double-strand PCR product was performed using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 2.0 to version 3.1 (Applied Biosystems) on a 377 DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. All 174 *C. taenia* sequences were deposited in the GenBank database under accession numbers AY35185 to AY35233, AY940213 to AY940214, and DQ175659 to DQ175781.

Sequence alignment and phylogenetic analyses

Cobitis taenia cytochrome *b* sequences were edited manually with the SEQMAN II program version 4.0.0 of DNASTAR, and aligned to each other and to the published cyt *b* sequences of *C. taenia* from the River Weser in Germany (GenBank Accession nos AF263077–78; Perdices & Doadrio 2001) using Sequence Alignment Editor© version 1.0 alpha 1 (Rambaut 1996), and corrected by visual inspection for phylogenetic analyses.

The phylogenetic relationships of all the haplotypes were inferred employing a neighbour-joining (NJ) algorithm and a maximum-likelihood method (ML) using the PAUP* software package version 4.0b2 (Swofford 1999). Statistical support for branching patterns was estimated by bootstrap replication (NJ: 1000 replicates, ML: 100 replicates). The HKY 85 + gamma model of DNA substitution (Hasegawa *et al.* 1985) was determined to be the most appropriate model for the analyses by applying MODEL-TEST (version 3.06, Posada & Crandall 1998) to the data set (base frequencies: A: 0.2775, C: 0.2415, G: 0.1564 and T: 0.3246. transition/transversion ratio = 3.2182, proportion of invariable sites = 0, and gamma distribution shape parameter = 0.3968). To reconstruct a minimum spanning haplotype network (MSN), we employed the statistical parsimony of Templeton *et al.* (1992) implemented in the TCS program version 1.06 (Clement *et al.* 2000).

To be able to date the cladogenetic events, we first tested the rate homogeneity of the cyt *b* gene with a log-likelihood ratio test (Page & Holmes 1998) performed in PAUP*. Under the assumption of a constant rate of nucleotide substitution, the age of clades was estimated in two ways. First, the mean pairwise within-clade divergences were calculated using the previous calibration for the genus *Cobitis* (Perdices & Doadrio 2001). This calibration for cyt *b* mtDNA corresponds to 0.84% sequence divergence per million years (calculated using HKY 85 + gamma) for the *Cobitis* subgenera based upon the opening of the Strait of Gibraltar after the Messinian salinity crisis (5.5 million years ago) (Krijgsman *et al.* 1999). The variance of the age estimates was calculated from the variance of the mean pairwise within clade differences also using the same calibration (Perdices & Doadrio 2001). In a second approach, the average distance of the members of a given clade (ρ) was inferred from the most recent common ancestor (MRCA) of the clade as expressed in the number of mutation steps using the formula $\rho = \{n_1 l_1 + \ldots + n_k l_1\}/n$, where *n* is the number of individuals and *l* is the length of the *k*th branch expressed in mutation steps, and the variance of the inference (σ_H^2) is expressed as $\sigma_H^2 = \{n_1^2 l_1 + \ldots + n_k^2 l_1\}/n^2$ (Saillard *et al.* 2000). In the second approach, the absolute timing was calculated by multiplying the observed values by a mutation rate of 0.0042 mutations per site per million years.

To test for isolation by distance (Slatkin 1993), the independence between geographical and genetic distances was analysed using transformed F_{ST} and geographical distance $[F_{ST}/(1 - F_{ST})$ and natural logarithm, respectively] in ARLEQUIN version 2.000 (Schneider *et al.* 2000). Geographical Euclidean distances (in kilometres) between samples were calculated from latitude and longitude measurements using a JAVASCRIPT distance converter (Williams 1975). A Mantel test (1000 permutations) was used to assess the significance of any correlation between genetic distance and geographical distance using ARLEQUIN version 2.000.

Mismatch analysis

The demographic history of *C. taenia* was explored using mismatch analysis (Slatkin & Hudson 1991; Rogers & Harpending 1992) of cyt *b* mitochondrial sequences. This method is based on the premise that compared with constant population size, population growth or decline leaves distinctive signatures in DNA sequences. A smooth and unimodal distribution is the signal of a population that has experienced recent population expansion (Rogers & Harpending 1992). The observed distribution was compared with the expected distribution under a model of sudden expansion (Rogers 1995). Parametric bootstrap (1000 replicates), using ARLEQUIN version 2.000, produced confidence intervals for the distribution parameters. The validity of the stepwise expansion model was tested using parametric bootstrap (1000 replicates) of the goodness-of-fit statistic (P), which represents the probability that the variance of the statistic in the simulated data is equal to or greater than that in the observed data. The significance of the raggedness index (*rg*), which measures the smoothness of the mismatch distribution, were also calculated in ARLEQUIN version 2.000. Rogers & Harpending (1992) have shown that the time at which population expansion started can be estimated from the peak of the mismatch distribution curve. The time is expressed as τ, or units of 0.5μ generations (where μ is the mutation rate per locus per generation). We used the relationship between absolute time (t_e) in years, and $\tau(t_e = \tau/2\mu_y)$ to compare the timing of possible demographic expansion among *C. taenia* clades. Values of the estimator of θ before (θ_0) and after (θ_1) expansion, and their approximate confidence intervals were obtained through parametric bootstrapping (1000 replicates). This approach gives conservative and overly large confidence intervals for values of θ (Schneider & Excoffier 1999). To further investigate the population history of *C. taenia*, Fu (1996) and Tajima (1989) tests of neutrality (Bertorelle & Slatkin 1995; Aris-Brosou & Excoffier 1996) (ARLEQUIN version 2.000) were calculated. If there is an indication of a surfeit of recent mutations then Fu's and Tajima's neutrality test statistics are predicted to be high and negative, an outcome that is indicative of recent population growth or selection (Fu 1997).

Nested clade analysis (NCA)

Nested clade analysis allows a statistical testing for nonrandom geographical grouping of haplotypes and an inference of demographic processes as the cause of the geographical associations (Templeton *et al.* 1995; Templeton 1998). A nested clade analysis with 10 000 permutations was performed using the program GEODIS 2.1 (Posada *et al.* 2000). For the first step, we constructed a nested series of clades from the haplotype network using the nesting rules described in Templeton *et al.* (1987) and Templeton & Sing (1993). Moving from the tip to the centre of the tree, individual haplotypes found at the tip were connected to an interior node one step away and unified into 'one-step clades'. This was repeated until all haplotypes and interior nodes were unified in one-step clades. These clades were then treated as single units in the tree and were unified in the same manner as above into 'two-step clades'. This process was repeated until the entire haplotype network was encompassed in a single clade. To resolve any ambiguity in the haplotype network, rare haplotypes were deemed more likely to be found at the tip, and common haplotypes at interior nodes. In addition, a haplotype represented by a single individual was deemed more likely to be connected to haplotypes from the same population than to haplotypes from different populations, following the criteria of Crandall & Templeton (1993).

We then quantified the geographical data by measuring the geographical range of each clade (D_c) and how each clade was distributed geographically relative to the higher-level clade within which that clade was nested (D_n). In addition, the average distance between tip and interior clades within the nested group (Int–Tip$_c$) and the tip to interior distance for the nesting clade (Int–Tip$_n$) were measured. Recalculation of all distances over 10 000 random permutations of clades against sampling locality was performed to determine the distribution of all distance measures. Interpretation of these contingency tests employed the latest (more cautious) version of the inference key of Templeton (2004) (http://bioag.byu.edu/zoology/crandall_lab/geodis.htm) that takes into account some recent criticisms (cf. Knowles & Maddison 2002).

Results

mtDNA genotypes

The complete nucleotide sequence was determined for 1126 bp of the cyt *b* gene for 186 *Cobitis* individuals. Alignment of all cyt *b* gene sequences revealed a total of 51 unique haplotypes within which a total of 208 positions (18.47% of total sites) were polymorphic (Table 2). Of these, 175 positions (15.54% of total sites) were parsimony informative. Within the ingroup, 49 positions were polymorphic (4.35%), 14 of which were parsimony informative (1.24%). Low mean pairwise differences and nucleotide diversity were found among all the *Cobitis taenia* haplotypes (3.295 ± 1.703; 0.003 ± 0.0017). With the absence of stop codons or indels, the sequence characteristics matched the general properties of the *C. taenia* cyt *b* gene (Perdices & Doadrio 2001; Janko *et al.* 2003), which suggests a functional mtDNA cyt *b* gene and not a nuclear pseudogene (Zhang & Hewitt 1996).

<div style="float:right; width:30%;">

Table 2 Alignment of all obtained *Cobitis taenia* cytochrome *b* mtDNA haplotypes showing the variable sites only. Numbers in rows refer to variable nucleotide positions in the 1126-bp cyt *b* sequences. 'H' refers to the haplotype and each haplotype is affiliated to the NCA (Fig. 4)

</div>

```
                                 111111111
        1111122233333344455555556667778888889999000000001
        3011386792335714755568813802900589913451233345581
        6201335545758032823791380923373580475731430153411
H1      CGATGAGTTTAGGCTGTGTGGCCATTTGCGTTTCTTCCAATCTATTTAG
H2      ................C.........A..............G.....
H3      .........C....................................
H4      ................C.........A.............G..G..
H5      ....A...........C.........A.............G.....
H6      ................C.........A..G..........G.....
H7      ................C.........A...................
H8      ......G.......................................
H9      ..................................T...........
H10     .........G....................................
H11     ..........T...................................
H12     .......................................G...
H13     ..................................G...........
H14     ..................................A...........
H15     .......................................G.....
H16     ..................................G...........
H17     ...A..........................................
H18     ...............................C..........
H19     ..................................G...........
H20     .............................A................
H21     ....................A.........................
H22     ......A.......................................
H23     ..............A...............................
H24     ..G...........................................
H25     .A......C.....................................
H26     ........C......A..............................
H27     ......G.C.....................................
H28     ......G..........A............................
H29     .........G..................G.................
H30     ......GG......................................
H31     ....................A..................G...
H32     ...........A...........................G...
H33     .......G.....T................................
H34     .....C.................G......................
H35     T..............................G.....
H36     ........C..................G.............C.
H37     ................GTC...........................
H38     ..............C....GA...........G.....
H39     ..............C.........A.....G.....G.....
H40     ..............C.........A.........G.G.....
H41     ..............C.C.......A...........G.....
H42     .............C.C.......A...........G.....
H43     ..............C.........A...........A.....
H44     ........A...C.........A.......................
H45     ..............C.........A.....T...............
H46     ..............................C...............
H47     ......A.......C.........A.........T.........
H48     ........C...............................C....G.A
H49     ...................G.........G...............
H50     .......C......C.........A.............G.....
H51     ..............C.........AT.............G.....
```
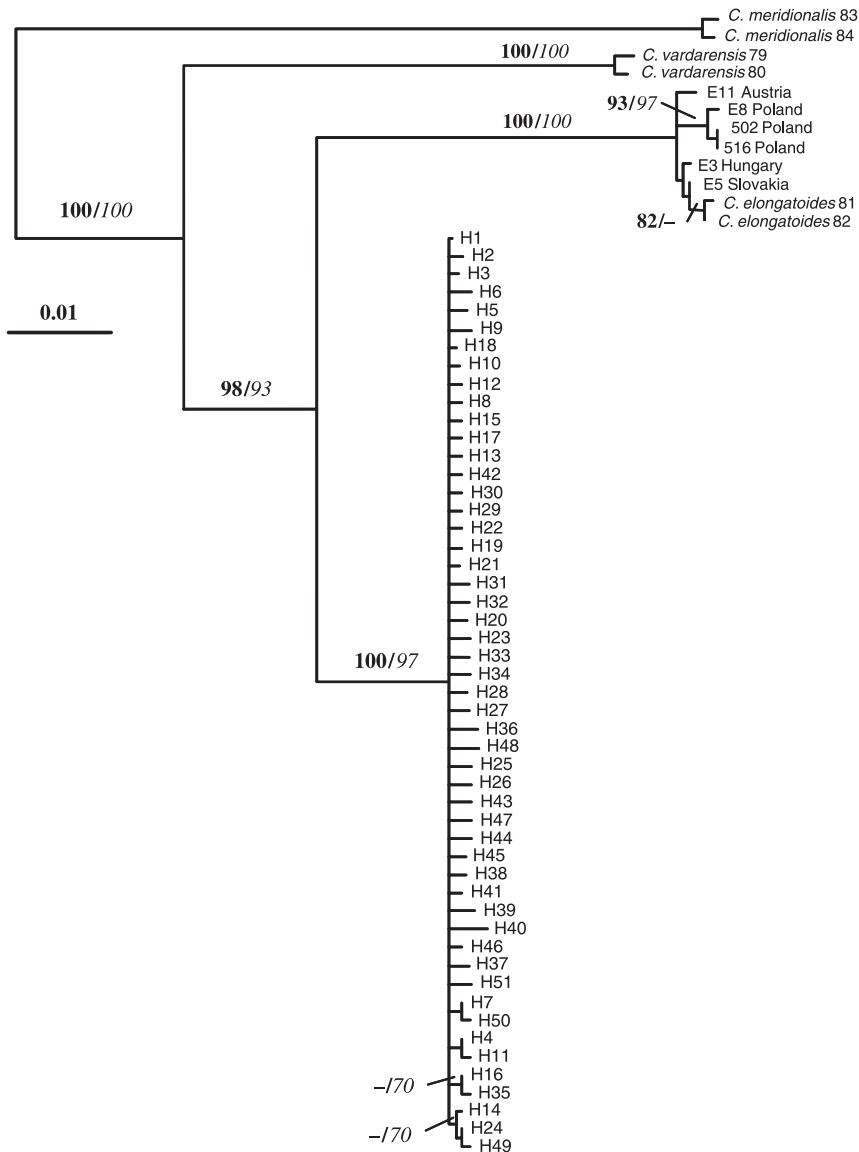
Fig. 2 Phylogenetic relationships among *Cobitis taenia* haplotypes reconstructed using the neighbour-joining (NJ) and maximum-likelihood (ML) analyses. The tree is rooted with *Cobitis meridionalis* and includes *Cobitis vardarensis* and *Cobitis elongatoides* haplotypes from Perdices & Doadrio (2001) and Janko *et al*. (2005). Numbers along the branches indicate the percentage bootstrap support obtained in the NJ (**bold**) and ML analyses (*italics*).

A log-likelihood ratio test based on the 63 haplotypes presented in Fig. 2, failed to reject the null hypothesis that the rate of evolution was constant among all branches in the phylogeny. In the analysis, searches with and without a molecular clock each yielded one likely reconstruction with –*ln* = 3073.05 and 3043.84, respectively. A chi-squared (χ^2) test with 61 degrees of freedom (*d.f.*) indicated no significant difference ($P > 0.05$) between the reconstructions. Therefore, a molecular clock for *C. taenia* was calibrated to 0.84% sequence divergence per million years, under the assumption of a homogeneous rate of nucleotide substitution.

Phylogenetic analyses

The NJ and ML tree topologies revealed four main groups: not surprisingly, the first group contained the two outgroup haplotypes of *Cobitis meridionalis*; the second group contained two haplotypes of *Cobitis vardarensis*; the third comprised the eight *Cobitis elongatoides* haplotypes; and finally, the fourth group completely comprised the 51 *C. taenia* haplotypes (Fig. 2). There was over 98% (NJ) and 93% (ML) bootstrap support for these four groupings, respectively. This analysis corresponds to the groupings found by Perdices & Doadrio 2001) for the genus *Cobitis* that included two *C. taenia* individuals from the River Weser in Germany.

Divergence times

The original (Penck & Brückner 1901–1909) classic division of Alpine Ice Ages into four Pleistocene cold periods named Günz, Mindel, Riss and Würm no longer corresponds to the results of recent international Quaternary research (Ehlers & Gibbard 2003; Guiter *et al.*

2003). An almost complete Quaternary stratigraphy of marine isotope stages (MIS) which provides a timescale for the period to which the continental stratigraphies must be fixed along with $^{16}O/^{18}O$ ratios in cores through the Greenland and Antarctic ice sheets as well as high resolution marine and lake cores are now used (Ehlers & Gibbard 2003).

We have dated *C. taenia* divergence to the MIS (Imbrie *et al.* 1984) because many glacial (and interglacial) periods have different names in the literature, with only the same MIS common to all. For example, Ribains, VdC-10, Metsovon and Pangaion (Tzedakis *et al.* 2001) are all names for the same temperate stage (110 000–128 000 BP) called MIS 5e in the MIS chronology. Therefore, using currently accepted MIS chronology (Imbrie *et al.* 1984; de Beaulieu *et al.* 2001; Tzedakis *et al.* 2001; Guiter *et al.* 2003; Lisiecki & Raymo 2005) the time to the most recent common ancestor (MRCA) of clade 2-1 corresponds to the MIS 5c interstadial (87 000–99 000 BP) when estimated by the Saillard *et al.* (2000) method (93 527 ± 18 275 years BP) or even the MIS 6 glacial period (128 000–186 000 BP) when considering the mean pairwise within-clade divergence (183 899 ± 107 869 years BP). There was congruence with both methods respectively when dating the MRCA even further back into the MIS 8 glacial period (245 000–303 000 BP) for clade 2–3 (271 297 ± 141 948 or 277 432 ± 141 948 years BP). Conversely, however, both methods varied extensively in dating the MRCA for clade 2-4, that was solely located in the Southern Bug River, to 112 775 ± 131 570 years BP which corresponds to the MIS 5e interstadial (110 000–128 000 BP) or 482 111 ± 275 839 years BP that is in the MIS 13 interglacial period (478 000–524 000 BP). Overall, both methods showed some congruence in estimating the time to the MRCA from the MIS 8 glacial period (240 000–303 000 BP) to the MIS 10 glacial period (339 000–362 000 BP) for all the *C. taenia* clades (299 455 ± 50 155 or 348 353 ± 180 040 years BP).

Mismatch analysis

Predictably, from the starlike statistical parsimony network (Fig. 4), the mismatch distribution of all *C. taenia* haplotype sequences was consistent with a model of population expansion (Fig. 3a), with both the variance (SSD) and raggedness index (rg) tests suggesting that the curves did not significantly differ from the expected distribution under a model of population expansion (total data set $P_{SSD} = 0.216$ and $P_{rg} = 0.07$). However, the distribution produced was not entirely smooth, with a single peak as expected, and the raggedness index showed a clear trend being close to significantly different from expectation under a model of sudden expansion. The neutrality tests of Tajima (1989, 1996) and Fu (1996) resulted in negative D (–1.79) and F_S (–34.02) statistics that were significantly different ($P < 0.001$) from expectation. The mismatch distribution for clades 1-1 and 1-7 ($P_{SSD} = 0.315$ and $P_{rg} = 0.573$ and $P_{SSD} = 0.522$ and $P_{rg} = 0.571$) did not significantly differ from the expected distribution under a model of population expansion (Fig. 3b, c). The neutrality tests of these clades resulted in negative D (–1.81, –1.55) and F_S (–9.72, –3.01) statistics that were significant ($P < 0.001$) in all cases. Older clades 2-1, 2-3 and 2-4 also, did not deviate significantly from the expected distribution under a model of sudden expansion (Fig. 3d–f) (clade 2-1 $P_{SSD} = 0.724$ and $P_{rg} = 0.713$; clade 2-3 $P_{SSD} = 0.657$ and $P_{rg} = 0.06$; clade 2-4 $P_{SSD} = 0.088$ and $P_{rg} = 0.174$). The neutrality tests of these clades resulted in mainly negative D (–2.04, –1.41, 0.25) and F_S (–13.02, –5.56, 1.01) statistics that were significant ($P < 0.001$) in all cases (except F_S for clade 2-4). The observed values of the age expansion parameter (t_e) differed among the younger one-step clades (Table 3), with clade 1-1 beginning expansion earlier than clade 1-7 (0.0169 and 0.00148 million years). While in contrast, among the older two-step clades, clade 2-3 that contains both clades 1-7 and 1-10, began expansion earlier than clade 2-1 (0.0.0215 and 0.00581 million years).

Lineage	S	95% CI	τ	t_e	θ_0	θ_1
Clade 1-1	3	0–8	0.3	16913	0.1 (0–0.5)	171.1 (0–1910.6)
Clade 1-7	5	1–11	0.02	1479	0.17 (0–1.1)	190.7 (0.1–1925.1)
Clade 2-1	7	2–14	0.1	5813	0.4 (0–1.7)	23.4 (0.1–52.8)
Clade 2-3	10	4–19	0.4	21511	0.5 (0–2.4)	12.6 (0.5–29.9)
Clade 2-4	7	2–15	0.6	28822	0.8 (0–2.9)	15.0 (0.4–20.7)
All data	24	14–35	1.2	62737	0.1 (0–1.2)	112.7 (2.7–1906.1)

Table 3 The results of demographic analyses for *Cobitis taenia* cyt *b* mtDNA sequences from clades 1-1, 1-7, 2-1, 2-3, 2-4 and all data

S, number of polymorphic sites; CI, confidence intervals; τ, time since expansion measured in mutational time units or units of 0.5 μ generations (where μ is the mutation rate per locus per generation); t_e, absolute time in years (where the relationship between absolute time and τ is $t_e = \tau/2\mu_y$); θ_0 and θ_1 are the theta values before and after expansion, respectively.
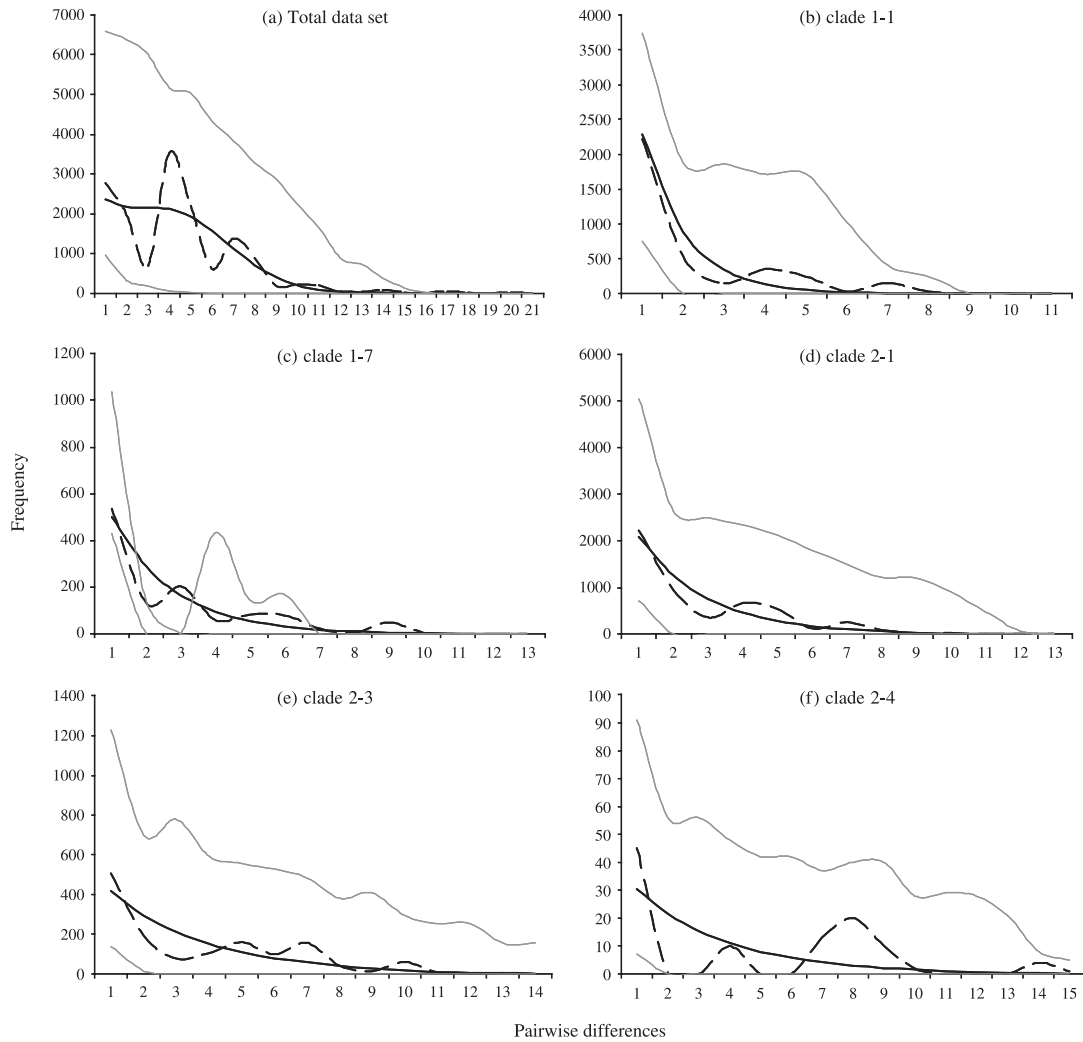
Fig. 3 The observed pairwise sequence difference (dashed line), and the expected mismatch distribution under the model of population expansion (solid line) of cytochrome *b* (1126 bp) haplotypes of spined loach (*Cobitis taenia*); (a) complete data set, (b) clade 1-1 (c) clade 1-7 (d) clade 2-1, (e) clade 2-3 and (f) clade 2-4. Confidence intervals (95%) are in grey.

Nested clade analysis

The nested clade configuration of cyt *b* sequences of *C. taenia*, constructed from the haplotype network, consisted of four levels (Fig. 4). Low mean pairwise differences and nucleotide diversity were found within all the *C. taenia* major clades (clade 2-1: 1.74 ± 1.0203, 0.0015 ± 0.001; clade 2-3: 2.62 ± 1.43, 0.0023 ± 0.0014; clade 2-4: 4.56 ± 2.609, 0.0041 ± 0.0027). Mapping the distribution of the major clades found in Fig. 4 (2-1, 2-3 and 2-4) revealed that they were all rooted around the Black Sea Basin with clade 2-1 rooted in the Savranka River, a tributary of the South Bug River in the Ukraine (Fig. 5). A minor clade (2-2) consisted of a single haplotype from the Elbe River that was a single mutation outside of clade 2-1. The nested clade analyses revealed significant geographical associations for

clades 1-1 ($\chi^2 = 387.27$, $P = 0.003$), 3-1 ($\chi^2 = 138.22$, $P = 0.009$), and the total cladogram ($\chi^2 = 146.98$, $P = 0.001$), and nearly significant associations for clade 2-1 ($\chi^2 = 208.22$, $P = 0.07$) (Fig. 4). Interpretations of these statistical results (Table 4), employing the latest inference key from Templeton (2004), was of restricted gene flow with isolation by distance in clades 1-1, 2-1 and the total cladogram (significantly large D_n and/or significantly large I-T D_c), and a lack of samples from the middle Dnieper/Pritpjat Swamps area terminated the discrimination between restricted gene flow with isolation by distance and other demographic processes in clades 1-7 and 3-1. The pattern of restricted gene flow is further corroborated by the Mantel test that significantly rejected the hypothesis of independence between geographical and genetic distances ($P = 0.006$), indicating a pattern of isolation by distance.
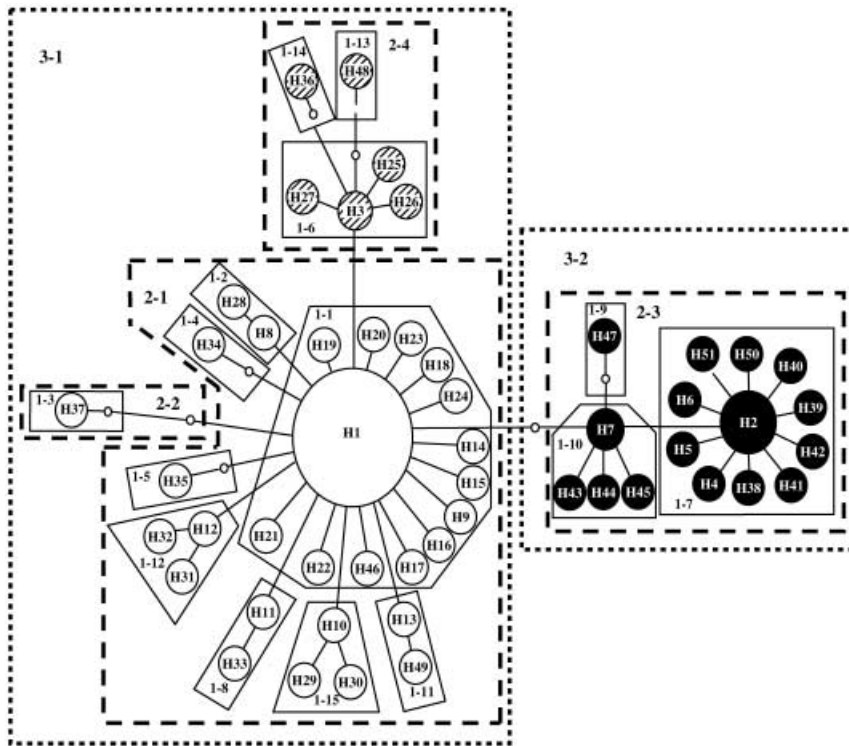
Fig. 4 *Cobitis taenia* cytochrome *b* nested clade design. Each solid line in the network represents a single mutational change. A haplotype is represented by a circle, the surface area of which is proportional to the number of individuals bearing this particular haplotype. Each haplotype is identified by a number. Small, empty circles indicate intermediate haplotypes that are not present in the sample, but are necessary to link all observed haplotypes to the network. The shading patterns correspond to the location of each identified second-level clade on a *C. taenia* sampling locations map (Fig. 1). Clade 2-2 contains a single, Elbe River haplotype and is not located on Fig. 1.

Table 4 Interpretation of the results of the nested clade analysis using the inference key of Templeton (2004). Clades with a significant or near significant association are shown

Clade	Chain of inference	Demographic event inferred
1-1*	1-2-3-4–NO	Restricted gene flow with isolation by distance
1-7	1-2-3-5-6-7-8–NO	Sampling design inadequate to discriminate between isolation by distance (short-distance movements) vs. long-distance dispersal
2-1	1-2-3-4–NO	Restricted gene flow with isolation by distance
3-1*	1-2-3-5-15-16-18–NO	Geographical sampling(s) inadequate to discriminate between fragmentation, range expansion and isolation by distance
Total cladogram*	1-2-3-4–NO	Restricted gene flow with isolation by distance

*Denotes significant association.

Discussion

Glacial refugia

Phylogenetic analysis (Fig. 2) confirmed the monophyly of the investigated *Cobitis taenia* and revealed low levels of differentiation among their mtDNA cyt *b* haplotypes. However, a significant association between genetic variability and geographical distribution of *C. taenia* lineages was revealed by the NCA. The centre of variability, the root of all recovered lineages and therefore, probably the oldest population structure for *C. taenia* in Europe, were found to be in a limited area on the northern side of the Black Sea Basin (Fig. 5). Furthermore, Black Sea Basin

haplotypes were present in the spine (clades 1-1, 1-6, 1-7 and 1-10) of the NCA network (Fig. 4), supporting the conclusion that the Ponto-Caspian area provided shelter for *C. taenia* in Europe during the glaciations. According to the distribution of the major clades in this region, several isolated areas seem to have harboured *C. taenia* refugial populations during the glacial periods. For clade 2-4, the lower Southern Bug River was likely such a place and the Crimean peninsula, lower Dnieper River and/or rivers east to the Dnieper harboured clade 2-3. However, the distribution of clade 2-1 is mostly restricted to areas more to the northwest, not close to what are thought of as traditional refugial regions. The easternmost locality that we documented this clade in was the upper and middle
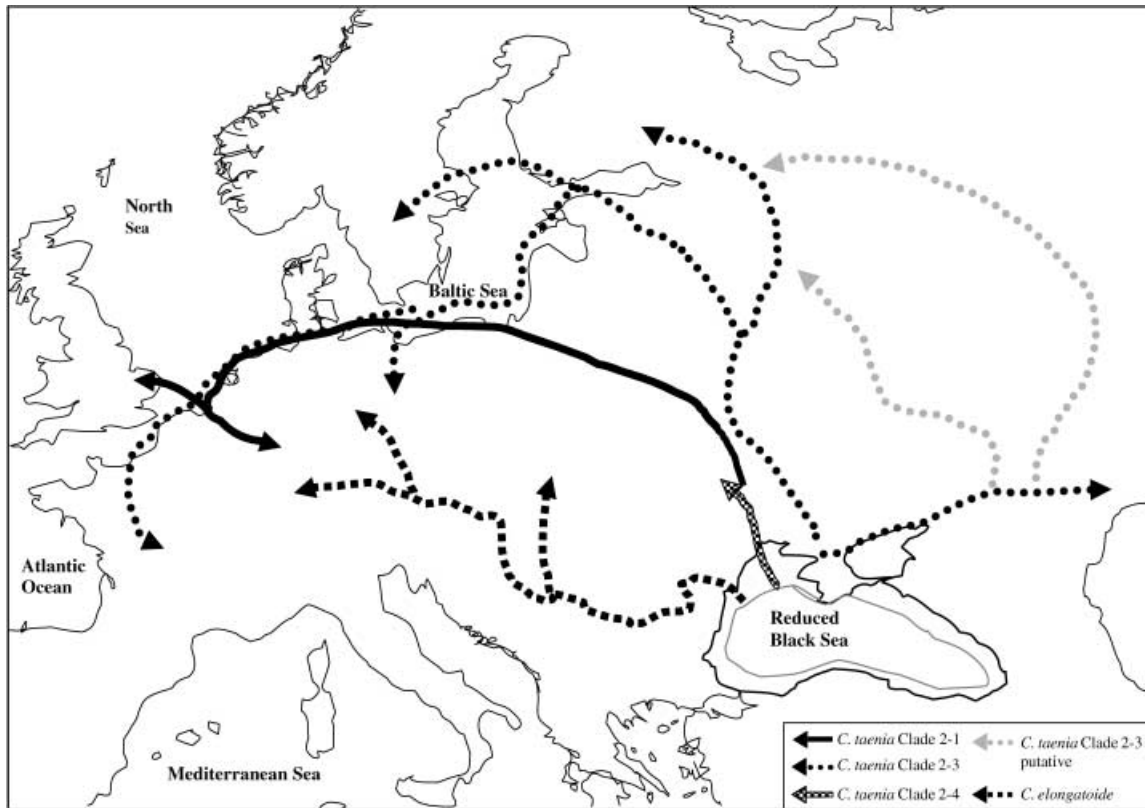
Fig. 5 Possible recolonization pathways of *Cobitis taenia* (the major clades revealed in the NCA) and *Cobitis elongatoides* (see Janko *et al.* 2005). Clade 2-2 (not shown) occurred only in the River Elbe.

Southern Bug River, which indeed was suitable to maintain *Cobitis* populations during glacial periods as seen in the example of clade 2-4. Since we were not able to analyse samples from the Pripjat swamp area however, it is not possible to refute the possibility that the origin of this clade was more to the north.

It is difficult to ascertain a more recent history in detail, since our data, despite providing evidence for the eastern origin of all clades, indicate the possibility that at least during the last glacial maximum (LGM), Western Europe might have also provided shelter for *C. taenia*. Clades 1-2, 1-4, 1-5, 1-8 and 1-12, nested within clade 2-1 distributed uniquely in Central and Western Europe, are exclusive to northern Germany, Denmark and southeastern England, which are areas known to have been colonized from separate western refugia in some species such as *Cottus gobio* and *Perca fluviatilis* (Nesbø *et al.* 1999; Volckaert *et al.* 2002). This is an area which had been thought of as unsuitable for habitation and reproduction due to the presence of the Scandinavian ice sheet that reached its LGM (52°N latitude with permafrost to 50°N), at approximately 22 000 BP in Poland (Huijzer & Vandenberghe 1998; Mol *et al.* 2000; Marks 2002, 2004). However, the populations within clade 2-1, from northern Germany, southeastern England and Denmark, contain greater diversity relative to other central

and western European areas, suggesting they might have survived there or more likely were recolonized from a nearby refugium located in Western Europe.

Similarly, the unique distribution of clade 2-3 in France may also indicate a western refugium. On the other hand, we argue that in such a case, clade 2-3 would be expected to have colonized southeastern England when the land bridge connection to Europe was present as has again been shown in *Cottus gobio* and *P. fluviatilis* (Nesbø *et al.* 1999; Volckaert *et al.* 2002). Furthermore, clade 2-3 is composed of two lineages (1-7 and 1-10), of which only one, clade 1-7, colonized Europe. The expansion of this lineage was relatively recent, dated according to mismatch analysis to the last few thousand years, which indicates the later arrival of this lineage to Western Europe. Such westward expansion was likely facilitated through damming by the retreating Scandinavian ice sheet that resulted in development of southward flowing meltwater valley trains and ice-marginal spillways running westwards. The Warsaw-Berlin and Warsaw-Torun-Eberswalde spillways collected proglacial and extraglacial water from the Neman, Vistula, Odra and Elbe drainage basins and joined vast ice-dammed lakes in the Gdansk Basin, Dnieper Basin and in the Lebork Lowland (Marks 2002, 2004; Mangerud *et al.* 2004) and would have facilitated the spread of clade 1-7

throughout Western Europe. Unfortunately, detailed assessment of the most recent history of *C. taenia* in Central and Western Europe is hampered by the lack of extensive sampling from Belarus, the Vistula-Dniester watershed and western European rivers. Nonetheless, the data already available suggests that at least clade 2-1 survived the LGM in more northerly located refugia than would be expected for this cold-intolerant species.

Previous phylogeographical analyses of other freshwater fish species in Europe such as chub *Leuciscus cephalus*, perch *P. fluviatilis* and barbel *Barbus barbus* (Durand *et al*. 1999; Nesbø *et al*. 1999; Kotlik & Berrebi 2001) revealed the Danube as an important glacial refugium and source for postglacial recolonization of Europe. Our genetic analyses have confirmed that *C. taenia* is completely absent from the Danube (see also Šlechtová *et al*. 2000; Bohlen & Ráb 2001; Janko *et al*. 2003, 2005; Luskova *et al*. 2004), and therefore did not utilize this drainage to recolonize Europe or escape glacial events. The cobitid sister genus *Sabanejewia* provides support for our findings, with these golden loaches from the Caspian and the eastern Black Sea basins (*S. baltica*, *S. kubanica*, *S. caucasica* and *S. aurata*) found to be the sister group to a Danubian-Balkanian lineage (*S. montana*, *S. bulgarica*, *S. balcanica*, *S. doiranica*, *S. radnensis*, *S. thrakica* and *S. vallachica*) (Perdices *et al*. 2003). Furthermore, as with *C. taenia*, haplotypes of the monophyletic lineage *S. baltica* from the Vistula and Dneister drainages were not found in the Danube and correspondingly haplotypes from the Danubian-Balkanian lineage were not found within the range of *S. baltica*, which suggests that the Carpathian Mountains provide a very effective barrier to their range expansions. The golden loaches inhabiting the Danube drainage were clearly related to the monophyletic lineages found in the Balkan Peninsula probably via Pliocene–Pleistocene direct connections from Danube tributaries to the River Vardar (Bănărescu 1990).

In a situation which parallels that seen with mtDNA lineages recovered in other fish species, e.g. the chub *Leuciscus cephalus* (cf. Hewitt 2004), *C. taenia* has used the Southern Bug River and the Dnieper for expansion, but these expanding populations appear to have reached far further westwards than any other fish species described to date. However, its close relative *Cobitis elongatoides* has used the Danube (Janko *et al*. 2005) and their secondary contact around the Crimea and in the Elbe and Odra rivers provided opportunity for hybridization resulting in nowadays widespread gynogenetic clones (Janko *et al*. 2005).

In general, our data suggest a late Pleistocene origin for the *C. taenia* lineages despite the perhaps contentious use of a molecular clock, the significant error associated with the subsequent age estimates and our heterogeneous sampling. Two clades, 2-1 and 2-3, recently underwent rapid population expansion during the Holocene as indicated by demographic analyses of their starlike haplotype networks

(Slatkin & Hudson 1991). The large discrepancy in the dating and variance for clade 2-4 and ambiguous results of the demographic analyses could be due to the small number of samples (15) compared to haplotypes (6) found within the clade and due to the fact that its structure was not starlike (Saillard *et al*. 2000). It is therefore likely that this lineage did not pass through founder-flush cycles in the Southern Bug River, but formed an evolutionary distinct lineage with limited gene exchange with the other geographically close lineages. The restricted geographical range of *C. taenia* from the lower Southern Bug River that were unable to recolonize Europe could be due to the fact that there were more northerly located populations. Alternatively it could have been as a result of long-term coexistence with gynogentic hybrids that according to Janko *et al*. (2005) may reduce the dispersal potential of the parental population via competition for sperm and resources.

The ranges of the time to the most recent common ancestor were greater than those found in *Cottus gobio* (Volckaert *et al*. 2002) although the more extended distribution of *Cottus* in France and Italy suggests it to be older there and to have survived the last glaciations, at least in Western Europe. There is little information however, on *Cottus gobio* from the Ponto-Caspian area; so these too may have a Ponto-Caspian origin, and the northwestern European refugia suggested for *Cottus gobio* may reflect later ice age refugia or secondary postglacial colonization.

A very low number (9) of intermediate haplotypes were missing from the entire haplotype network, suggesting that the present sampling contains most of the diversity of 'true' diploid *Cobitis taenia*. Therefore, it is likely that the inferred lack of geographical association of the genetic data (10 out of 13 clades) results from the low intraspecific genetic variability of *C. taenia*, which was also found in allozyme studies by Šlechtová *et al*. (2003), suggesting a small long-term effective population size within the range, as well as a recent expansion and recolonization of Europe. Kotlik & Berrebi (2001) found a similar low level of differentiation in *B. barbus*, which they suggested represented a recent expansion in Europe of barbel. It must nonetheless also be stated that the lack of *C. taenia* samples from several areas within the range have also negatively affected the power of NCA (Templeton *et al*. 1995).

The demographic events indicated by the nested clade analysis and the Mantel test imply restricted gene flow, with isolation by distance (clades 1-1, 2-1 and total cladogram). This is not surprising given the presence of effective barriers to gene flow in the studied area and the patchy distribution of habitats suitable for *C. taenia* both currently and historically, but it contrasts with the signal of population expansion obtained by the demographic analyses, which suggests a deviation from migration–drift equilibrium. Such a discrepancy may suggest that expansion did not take place from one refugium in the given clades but

rather from the wide margins of refugial populations which might themselves have been interconnected by limited gene flow, as proposed for *Cottus gobio* (Volckaert *et al.* 2002). Clearly, for more robust explanations, nested clade analyses should be combined with history, palaeobiology, climate and geography to reveal the phylogeography of a species. In this particular case, further sampling of the intermediate areas of Western Europe, the Don River and the Vistula/Dnieper watershed would aid understanding.

Current distribution and colonization

Two of the lineages inferred to have colonized deglaciated areas (clades 2-1 and 2-3) co-occur in the Vistula, Odra, Elbe, Weser and Rhine rivers, but appear to have initially recolonized Europe in different directions. Clade 2-1, exclusively present in Denmark and all of the English catchments, has expanded in Poland, Denmark, Germany and across to England, while clade 2-3, exclusively present in Estonia, France, Kazakhstan, Russia and Scandinavia, has likely expanded from Eastern Europe, given that it is rooted by clade 1-10, in a more northerly direction into Russia and Scandinavia (Fig. 1). Its colonization of the west in Poland, Germany and France is more difficult to assess, since it is uniquely present in France, pointing at the interesting possibility of a western refugium as mentioned above. However, its absence from England and the demographic analyses suggests that the expansion of its widespread nested clade 1-7 began as late as the last two thousands years. Although we take this estimate cautiously and expect rather large confidence intervals, it indicates that the mtDNA lineages nested within clade 2-3 reached Western Europe more recently than 7500 years ago, which was around the time when the land bridge between Europe and England disappeared (Wheeler 1977; Gibbard & Lautridou 2003; Woller & Long 2003) and therefore after clade 2-1. On the other hand, because English populations of stenohaline fishes became established having probably originated by colonization from the Rhine system while the land bridge was present, clade 2-1 most likely expanded in Western Europe earlier during the Late glacial (MIS 2, 12 000–24 000 BP) (last of the Upper Pleistocene, 195 000–12 000 BP). This scenario is also suggested by demographic analyses and by the fact that Central European populations of hybrid gynogens originated by *C. elongatoides–C. taenia* hybridization in the Elbe and Odra rivers possess uniquely mtDNA derived from clade 2-1 (Janko *et al.* 2005). The overlap of the two clades in the Vistula, Odra, Elbe, Weser and Rhine Rivers likely represents a post-Pleistocene secondary contact, corresponding to findings on other fishes (Durand *et al.* 1999; Kotlik & Berrebi 2001; Volckaert *et al.* 2002; Hewitt 2004), since Poland was covered by ice down to 52°N latitude during the Upper Pleniglacial (29 000–

15 000 BP), that ends in MIS 2 (12 000–24 000 BP), with permafrost to 50°N (Huijzer & Vandenberghe 1998; Mol *et al.* 2000).

This earlier expansion in Central and Western Europe of the first lineage could also explain why only clade 2-1 haplotypes occur on Jutland while clade 2-3 haplotypes occur in the rest of Scandinavia. *Cobitis taenia* is not a cold-tolerant, headwater species like *Cottus gobio* or *Barbatula barbatula* (Elliot *et al.* 1994, 1995). It is currently only found up to latitudes of 61°N compared to 65°N for the other two species (personal observations), and it requires temperatures above 18 °C to reproduce successfully (Bohlen 1999). These physiological constraints may have prevented the first lineage from colonizing Scandinavia northwards along the West Baltic coastline during the freshwater *Ancylus* stage, *c.* 10 800 BP (Koli 1969; Björck 1995), although it could have reached Jutland. However, when the climate became more suitable, salinity levels rose in the Baltic basin (4000–8000 BP) as the North Sea broke through the Danish Straits (Donner 1995), preventing the first lineage from taking a western route into Scandinavia, which is further corroborated by the absence of asexuals there. However, the second lineage could have colonized Scandinavia along the East Baltic coastline more recently, because although it cannot survive in open salt water, *C. taenia* has a higher tolerance of salinity in comparison with other primary freshwater fish species (Bohlen 1999) and currently occurs in the brackish areas of some rivers in parts of the Baltic coast (Winkler 1996). Therefore, a later coastal route into Scandinavia from the Eastern Baltic, which has lower salinity than the western part, where only the second lineage occurs, could have been possible and would explain the presence of the second lineage there exclusively.

The phylogeographical structure of European freshwater fishes studied to date has shown distinct clades in western (Atlantic), central (Danube) and eastern (Ponto-Caspian) Europe and indicated the important role of the Danube in postglacial recolonization of European waterways (Durand *et al.* 1999; Nesbø *et al.* 1999; Koskinen *et al.* 2000; Bernatchez 2001; Nilsson *et al.* 2001; Volckaert *et al.* 2002). The location of palaeorefugia of European freshwater fish and the subsequently deduced patterns of recolonization from these glacial refuges however, appear to vary with the life history traits, ecology, biology and physiology of species. The colonization of Europe by *C. taenia* has unusually been found not to occur along the Danube catchment, as in other European freshwater fish species, but by a more northerly route and originally from at least two eastern Ponto-Caspian refuges. This finding of non-Mediterranean refuges in *C. taenia* is one of a growing number of similar studies (Nesbø *et al.* 1999; Kotlik & Berrebi 2001; Hänfling *et al.* 2002; Kotlik *et al.* 2004) that firmly adds these eastern regions to the southern regions as sources of postglacial European recolonizations.

Conclusions

The phylogeographical analysis of *Cobitis taenia* populations reveals an intriguing picture of the colonization history of this species in Europe. *C. taenia* likely survived glaciations in three glacial refuges in the Ponto-Caspian area. From these refuges, two major lineages recolonized Europe in separate directions: one westward to England and the other spreading rather to the north. A minor lineage that did not contribute to the recolonization of Europe was also found. However, the more recent history is difficult to establish and the survival of at least one clade in Western Europe during the LGM is distinctly possible. Unusually, the Danube is not part of the current distribution of *C. taenia* nor was it used as either a refuge or a source of colonization of Europe, unlike other European freshwater fish species. Understanding of the phylogeography of 'true' diploid *C. taenia* provides a platform for further investigation of the patterns of asexual reproduction, polyploidy and hybridization within the *Cobitis* complexes. This may inform understanding of the evolution of such patterns in fish more generally.

Acknowledgements

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Mark Culling is a postdoctoral researcher in the Centre for Ecology, Evolution and Conservation of the University of East Anglia. His research interets in clude the distribution of genetic diversity, the processes of genetic divergence, and the conservation, ecology and phylogeography of fishes. Karel Janko is a postdoctoral fellow at the Laboratory of Fish Genetics. His research interest is in the understanding of the evolution of asexuality and the effects of the coexistence of sperm parthenogens on parental populations. Alicja Boroń is a Professor at Warmia and Mazury University in Poland. Her research interests include the structure, function and evolution of fish chromosomes. She is also interested in the occurrence, origin, mechanisms of reporduction and cytogenetic diversity of fish hybrids and natural polyploid forms. Dr Sci., Victor Vasil'ev, a leading scientist of the Institute of Problems of Ecology and Evolution RAS, is the one of the first investigators to conduct complex studies of clonal vertebrates. He has a particular interest in the unisexual-bisexual complexes in this group of animals. His research interests also include the evolutionary karyology, taxonomy and speciation of fishes. Isabelle Côté is Professor of Tropical Marine Ecology at Simon Fraser University. Her research ranges from pure behavioural ecology to applied ecology and conservation of organisms associated with tropical coastal ecosystems. professor Godfrey Hewitt, of the University of East Anglia, seeks to understand the distribution of genetic diversity and the processes of genetic divergence and speciation using DNA markers along with computer simulation analysis. He is particularly interested in the postglacial colonization of Europe and genetic diversity in domestic animals.