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Genetics and ecology of spined loach in England: implications for conservation management

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Steve Killeen

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Executive Summary

The spined loach *Cobitis taenia*, a small fish found in Northern Europe (Bohlen and Ráb, 2001), is protected under Annex II of the EC Habitats and Species Directive which gives species full protection. It is found in shallow, slow-flowing or stagnant water and lives primarily on the river bottom, which the fish sifts using a specialized feeding technique. This bottom-dwelling habit, combined with a tendency to burrow and a relatively poor swimming ability, limits the spined loach's potential to spread itself over long-distances. This in turn may limit genetic variation among UK populations and raises the question of how well the current network of Special Areas of Conservation (SACs) for spined loach can preserve its genetic diversity.

The Environment Agency studied this species in five catchments in England and confirmed for the first time that UK spined loach is the species *Cobitis taenia*. All the specimens examined in this report had a diploid chromosome number 2n=48 and other cytological features typical of *C. taenia*. Other morphologically similar species and hybrids commonly encountered in central and middle Europe appeared to be absent from British populations.

Generally, spined loach have low levels of genetic variation, probably because of their relatively recent re-colonisation of Europe following the last glacial period. At the European scale, however, there are at least three distinct evolutionary lineages of spined loach, which should be considered when planning a representative network of SACs for this species.

Genetic variation within spined loach populations in the five catchments in which they occur in England was found to be limited. Despite this, there was evidence of genetic population structure. Significant genetic differences were found between the Trent/Witham catchments and the Welland/Nene/Great Ouse catchments, reflecting the lack of connection between these two groups of catchments. The genetic variation within these groups was found to be mainly in inter-population differences in the Trent/Witham group. Both Witham populations (Metheringham Delph and River Witham) differed significantly from both Trent populations (River Mease and Stoke Bardolph). By contrast, the populations within the Welland/Nene/Great Ouse group showed little genetic differentiation.

Of all the spined loach examined in this study, those from the rivers Mease (Trent catchment) and Witham (Witham catchment) were the most genetically distinct. Fish from these rivers were found to harbour haplotypes that do not occur anywhere else in Europe, a discovery of some importance given the generally low genetic diversity of the species. This discovery suggests that the Witham and Trent catchments may be potentially important areas for spined loach conservation.

The genetic data collected in this study suggests that at least three of the seven UK populations should be managed in distinct units. These are the Witham, the Mease, and one of the rivers from the Welland/Nene/Great Ouse interconnected catchments. The latter two are already included in at least one SAC, but the Witham is not. We recommend that these units should be monitored and managed separately. It would also

be prudent to maintain the physical separation of the Trent/Witham and Welland/Nene/Great Ouse groups to prevent genetic homogenisation of the species. For example, specimen transfers between these roughly defined areas should be avoided, as should artificial connections between the Trent/Witham and Welland/Nene/Great Ouse, such as the proposed Fens Waterways Link that will connect the rivers Witham, Welland, Glen, Nene and Great Ouse.

Finally, the results of the habitat preference experiments carried out in this study suggest that the way populations are currently monitored may need to be rethought, along with the design of habitat management plans. Current understanding of the ecological requirements of spined loach has been largely derived from daytime habitat surveys. However, our results show that nocturnal habitat requirements are often different and perhaps more important, since food foraging occurs at night. Thus, management efforts directed at maintaining daytime habitats may not necessarily be beneficial to the species.

The spined loach (*Cobitis taenia L*)

Photograph reproduced by kind permission of Dr. Bardukh Gabrielyan

Contents

1. Introduction

The spined loach *Cobitis taenia*, first identified by Linnaeus in 1758, is a small, poorly-known bottom-dwelling freshwater fish. Erectile spines below the eyes give the species its common name. It is found in shallow, slow-flowing or stagnant water in all manner of water bodies such as rivers, streams, canals, ditches, drains and lakes (Robotham, 1978; Marconato and Rasotto, 1989). Optimal habitat is thought to be patchy cover of submerged (and possibly emergent) macrophytes - aquatic plants which are important for spawning - and fine-particle substrata such as mud, sand and sediments with high organic components (Robotham, 1977; Marconato and Rassoto,1989; Perrow and Jowitt 1997, 2000; Slavík et a 2000). The close association between spined loach and fine substrata undoubtedly comes from the fish's specialised feeding technique of sifting (Robotham 1982) and its reliance on small invertebrates, such as chydorids, copepods and rhizopods, which live in the surface layers of sediments (Robotham 1977).

Cobitis taenia was long believed to range widely across Europe and Asia, but recent studies have shown that the species is actually restricted to northern Europe (Bohlen and Ráb, 2001). In Britain, spined loach is found only in five east-flowing river systems: the Trent, Welland, Witham, Nene and Great Ouse (Figure 1.1).

The spined loach is threatened in Europe. It is listed on Appendix III of the Bern Convention and protected under Annex II (species given full protection) of the EC Habitats and Species Directive. The latter entails an obligation for member states to designate Special Areas of Conservation (SACs) in key areas where the spined loach occurs. In Britain, five sites within the core area of spined loach distribution have been selected as SACs. The sites are:

(1) *Baston Fen, Lincolnshire.* The counterdrain, a large drainage channel running alongside Baston Fen, has high numbers of spined loach and serves as an example of spined loach populations in the Welland catchment. The patchy cover from submerged plants provides excellent habitat for the species.

(2) *Nene Washes, Cambridgeshire.* Moreton's Leam, a large drainage channel running along the eastern flank of the Nene Washes, has the highest recorded density of spined loach in the UK. There may also be thriving populations in the smaller ditches of the Washes. The site represents spined loach populations in the Nene catchment.

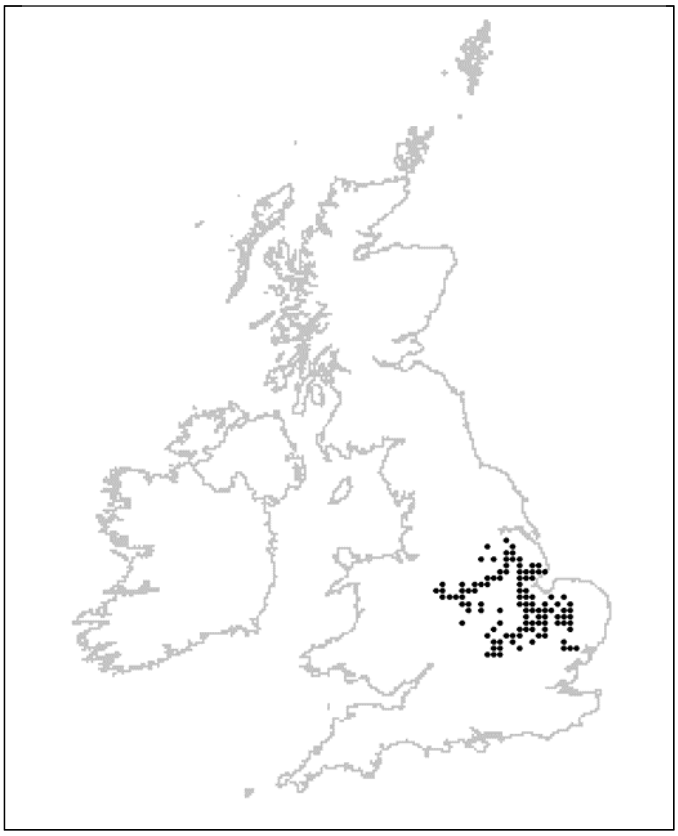


Figure 1.1

Current distribution of spined loach in the UK (modified from Perrow and Jowitt, 2000)

(3) *Ouse Washes, Cambridgeshire/Norfolk.* The Ouse Washes represent spined loach populations within the River Ouse catchment. The counterdrain, with its clear water and abundant macrophytes, is particularly important and a healthy population of spined loach is known to live there.

(4) *River Mease, Derbyshire/Leicestershire/Staffordshire.* The River Mease is a good example of a riverine population of spined loach. It is a small tributary of the River Trent and has retained a reasonable degree of channel diversity compared to other rivers containing spined loach populations. The Mease has extensive beds of submerged plants along much of its length which, together with its relatively sandy sediments, provides good habitat opportunities for the species.

(5) *Fenland, Cambridgeshire.* Fenland SAC was primarily designated because it contains Annex I habitats (*Molinia caerulea – Cirsium dissectum* fen-meadow and calcareous fens), but it also contains good-sized populations of spined loach.

While these SACs were selected to serve as examples of the range of habitats in which the species occurs (such as rivers and artificial drainage systems), these designations were made without knowledge of the species' underlying genetic diversity. The spined loach's bottom-dwelling habit (Sawada, 1982), combined with its tendency to burrow (Culling *et al.,* 2003) and a relatively poor swimming ability, clearly limits its potential for

long distance dispersal. Populations are thus likely to reproduce in isolation, with concomitant sparse genetic differentiation.

1.1 Project aims

Management strategies for the conservation of a species should, to some degree, be determined by its ecological requirements and the spatial distribution of its genetic diversity (Franklin, 1980; Soulé and Mills, 1998; Booy *et al*., 2000). The lack of information about the latter provided the main impetus for this study which had four aims, outlined below.

1.1.1 Confirm the species identity of spined loach in the UK

A considerable portion of the known distribution range of cobitids, diploid, triploid and tetraploid - almost all female - hybrids between *C. taenia* and *C. elongatoides*, as well as triploid and tetraploid all-female hybrids between *C. elongatoides* and *C. tanaitica*, have been found to co-occur sympatrically with the parent species (Slechtova *et al*., 2000; Bohlen and Ráb, 2001; Persat *et al*., 2002; Boroń, 2003). Not only are parental diploid species morphologically indistinguishable from their polyploid hybrids, but several distinct *Cobitis* species, for example *C. taenia* and *C. tanaitica* (Bacescu and Maier, 1969), are also very similar morphologically. To confirm the identity of English spined loach as pure *C. taenia*, we used cytogenetic methods to test whether individual fish from all five catchments showed the typical *C. taenia* karyotype, which is characterised by having 2n = 48 chromosomes (Vasil'ev *et al*., 1989; Boroń 1992, 1995, 1999, 2001; Ráb and Slavik, 1995; Ráb *et al*., 2000).

1.1.2 Establish the post-glaciation re-colonisation routes of spined loach

Quaternary glacial events have left a signature upon the genome of many species. These genetic imprints have been used in phylogeographic analyses to reconstruct the routes taken by various species to re-colonise Europe. These analyses are not only of theoretical interest, but also provide a broader geographic context against which smaller-scale patterns may be appreciated. For this reason, the genetic variability of spined loach at the European level was investigated to gain an understanding of the recent evolutionary history of the species.

1.1.3 Determine the extent of genetic differentiation among spined loach populations in Britain

The genetic structure of the English *Cobitis taenia* population was investigated to provide evidence for the presence (or absence) of evolutionarily significant units (ESUs) or management units (MUs) within the UK. A European-wide analysis was used to put the UK results into context. Any geographical partitioning of genetic diversity found for spined loach should influence the choice and design of conservation sites in the UK and in Europe.

1.1.4 Test intra- and inter-population variability in habitat preferences

The preference of spined loach for fine substrata appears to be well established. However, the distribution of spined loach within a site has been shown to vary seasonally (Robotham 1978), and there is evidence that individuals within a population can show different microhabitat preferences (Slavík et al., 2000). We therefore examined whether sediment preferences were consistent among individuals of different ages and sex groups and across populations.

2. Cytogenetic confirmation of the species identity of spined loach in England

The aim of this study was to establish the taxonomic status and presence or absence of polyploid forms of *Cobitis* in the natural distribution range in England. The study looked at key cytogenetic features such as the diploid chromosome number, karyotype, the number and location of silver-stained nucleolar organising regions (Ag-NORs) and DNA sites rich in GC pairs stained with chromomycin CMA_3, and the distribution of heterochromatin as C bands (Vasil'ev *et al*., 1989; Boroń 1992, 1995, 1999, 2001; Ráb and Slavik, 1995; Ráb *et al*., 2000).

2.1 Methods

Sixty-eight individual fish were collected from six populations in England: from the Inner River (ten females) and the Outer River (four females, one male, two juveniles) of the Ouse Washes, Baston Fen (fourteen females), Morton's Leam (eight females, five males), Washingborough on the River Witham (twelve females, three males) and from the river Mease, a tributary of the River Trent (four females, five males) (Table 2.1).

Table 2.1
Geographic origin and number of individuals of *Cobitis taenia* used in the cytogenetic analysis.

Catchment	Location	Individuals	Coordinates
Great Ouse	Ouse Washes	17	52:07:49N 00:08:42E
R. Nene	Morton's Leam	13	52:34:25N 00:03:10W
R. Welland	Baston Fen	14	52:44:43N 00:18:19W
R. Witham	Washingborough	15	53:13:35N 00:28:49W
R. Trent	R. Mease, Croxall	9	52:43:24N 01:43:02W

The chromosome preparations were made from kidney cells using an air-drying technique. C-banding was carried out according to Sumner (1972). The active nucleolar organising regions (NORs) were visualised with silver nitrate ($AgNO_3$) following the method of Howell and Black (1980). Some of the slides were stained with GC-specific chromomycin (CMA_3) according to Sola *et al*. (1992). After CMA_3 staining, some of the slides were destained and the same metaphases stained with $AgNO_3$. The chromosomes were classified according to the system of Levan *et al*. (1964). In total, 224 metaphases were analysed after giemsa staining, 88 metaphases after C-banding, 90 metaphases after $AgNO_3$ staining and 86 metaphases stained with CMA_3.

2.2 Results

No polyploid forms were detected. All individuals had 2n = 48 chromosomes and the following karyotype formulae: 10 metacentric (*m*), 18 submetacentric (*sm*), and 20 subtelo- and acro-centric chromosomes (*sta*), with 76 chromosome arms.

C-banding revealed the occurrence of the constitutive heterochromatin in the centromeric region in most of the chromosomes. Among the *m* chromosomes there were four marker chromosomes. The largest chromosome pair had the large heterochromatin block at a centromeric position. The chromosome pairs 2, 3 and 4 had the massive heterochromatin block located at the peri-centromeric site along one of the chromosome arms. Among the *sm* chromosomes, pair number 10 with deeply stained pericentromeric regions and pair numbers 12 and 13 with C-bands along the short arms were markers for the karyotype of *C*. *taenia*. The *sta* chromosomes characterised by C-bands in centromeres and chromosomes of pair 19 also had weak C-bands in the telomeric region.

In most of the metaphase plates, two pairs of NOR-bearing chromosomes were detected after $AgNO_3$ staining. The NOR sites were located on the short arms of one *sm* pair and one *st* chromosome pair.

The number of CMA_3 positive sites ranged from four to eight. They were located on the short arms of one *sm* and one *sta* chromosome pairs, near the centromere from the side of the long arm of one pair of *sm* chromosomes and on the telomeres of two *a* chromosomes.

In most metaphase plates (more than 80 per cent) six CMA_3-positives were observed. They were located on the short arms of one pair of *sm* chromosomes, on the short arms of one *sta* chromosomes, near the centromere from the side of the long arm of one *sm* chromosomes and at the telomeric position of the *sta* chromosome.

2.3 Conclusions

According to diagnostic cytogenetic features, all specimens could be assigned to the species *C. taenia*. The results thus confirm that *Cobitis taenia* is the only bisexually reproducing *Cobitis* species inhabiting England and that the polyploid forms typically encountered in central and middle Europe appear to be absent from all British populations.

3. Genetic differentiation of spined loach: post-glacial history of the species in Europe

In this section, phylogeographic patterns of *C. taenia* using mtDNA analyses are inferred to identify the probable location of its refuges during ice ages and postglacial colonisation routes. Analyses are based on DNA sequences of the mitochondrial gene cytochrome *b*.

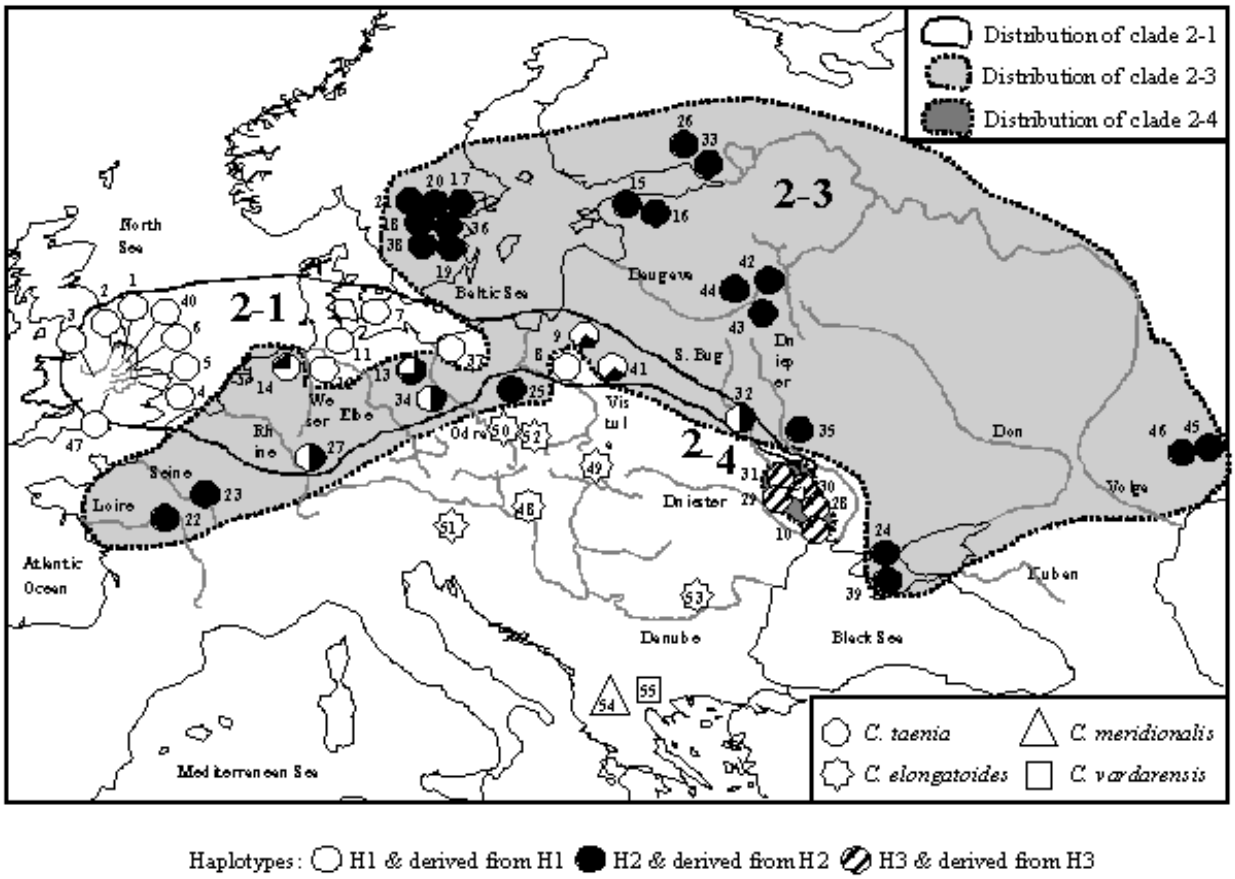
3.1 Methods

A total of 174 individual specimens of *Cobitis* were obtained from 47 locations across central and eastern Europe (Figure 3.1; Table 3.1). Eight samples of *C. elongatoides*, two of *C. vardarensis* and two of *C. meridionalis* were also used as outgroups in the analyses.

DNA was extracted from fin clips preserved in ethanol (99 per cent), either by phenol – chloroform extraction (Sambrook *et al*., 1989), or by salt extraction after Sunnucks and Hales (1996) and Aljanabi and Martinez (1997). The entire cytochrome *b* gene (1140-bp) was amplified and sequenced using the method described in Culling et al. (2006).

The phylogenetic relationships of all haplotypes recovered were inferred employing a neighbour-joining (NJ) algorithm and a maximum likelihood (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). A phylogeographical nested clade analysis (NCA) was performed to test for non-random geographical grouping of haplotypes and infer the demographic processes behind 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).

Details of these statistical methods are provided in Culling *et al*. (2006).



Haplotypes: H1 & derived from H1 H2 & derived from H2 H3 & derived from H3

Figure 3.1

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 3.1). The numbers correspond to location numbers listed in Table 3.1. The distribution of haplotype 1 also includes derivatives H8-24, H28-35, H46, H49; haplotype 2 includes derivatives H4-6, H38-H42, H50-51 and haplotype 3 includes derivatives H25-27, H36, H48. Clade 2-2 (not shown) occurred only in the River Elbe.

Table 3.1.
Geographic origin and number of individuals of *Cobitis taenia* (*C. tae.*), *C. elongatoides* (*C. el.*), *C. meriodionalis* (*C. mer.*) and *C. vardarensis* (*C. var.*) used in the phylogeographic analysis of the Cyt b DNA sequences.
Location codes are shown in Figure 3.1.

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

Table 3.1 (Continued)

Code	Locality	*Biotype*	*N*	Coordinates	Cyt-*b* haplotypes
49	Zierná Voda River, Danube, SK	*C. el*	1		E5
50	Polska Woda River, Odra, PL	*C. el*	1		E8
51	Mur River, Danube, A	*C. el*	1		E11
52	Polska Woda River, Odra, PL	*C. el*	2		502, 516
53	Timis/Albina River, Danube, RO	*C. el*	2		81, 82
54	Lake Prespa/Psarades, GR	*C. mer*	2		83, 84
55	Agiaki/Kastanies River, Vardar, GR	*C. var*	2		79, 80

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

3.2 Results

3.2.1 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% of total sites) were polymorphic. Of these, 175 positions (15% of total sites) were parsimony informative. Within the ingroup, 49 positions were polymorphic (4%), 14 of which were parsimony informative (1%). Low mean pair wise differences (MPD) and nucleotide diversity were found among all the *C. taenia* haplotypes (3.295 $\pm$ 1.703; 0.003 $\pm$ 0.0017).

3.2.2 Phylogenetic analysis

The NJ and ML tree topologies revealed four main groups.The first group contained the two outgroup haplotypes of *C. meridionalis*, the second contained two haplotypes of *C. vardarensis*, the third comprised the eight *C. elongatoides* haplotypes, and the fourth comprised all 51 *C. taenia* haplotypes, confirming the monophyly of *C. taenia* (Figure 3.2). There was over 98% (NJ) and 93% (ML) bootstrap support for these four groupings.

3.2.3 Nested clade phylogeographical analysis

The nested clade configuration of cytochrome b sequences of *C. taenia*, constructed from the haplotype network, consisted of four levels (Figure 3.3). Low mean pairwise differences and nucleotide diversity were found within all the major clades of *C. taenia (*clade 2-1: 1.74 $\pm$ 1.0203, 0.0015 $\pm$ 0.001; clade 2-3: 2.62 $\pm$ 1.43, 0.0023 $\pm$ 0.0014; clade 2-4: 4.56 $\pm$ 2.609, 0.0041 $\pm$ 0.0027).

Mapping the distribution of the major clades shown in Figure 3.3 (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 (Figure 3.4). A

minor clade (2-2) consisted of a single haplotype from the Elbe River that was one mutation outside of clade 2-1. The nested clade phylogeographical analyses revealed significant geographical associations for clades 1-1, 3-1, and the total cladogram, and nearly significant associations for clade 2-1. Interpretations of these results, 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. This was corroborated by a significant Mantel test for independence between geographical and genetic distances (*P* = 0.006), indicating a pattern of isolation-by-distance.

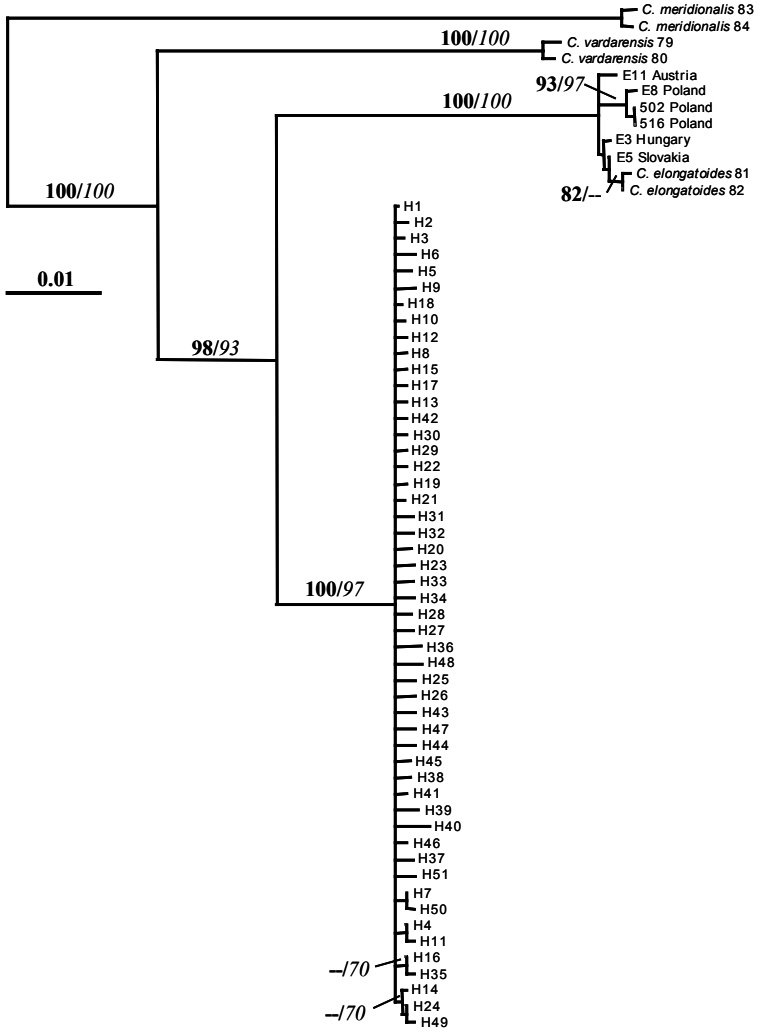


Figure 3.2

Phylogenetic relationships among *Cobitis taenia* haplotypes reconstructed using the neighbour-joining and maximum likelihood analyses. The tree is rooted with *C. meridionalis* and includes *C. vardarensis* and *C. elongatoides* haplotypes from Perdices and Doadrio (2001). Numbers along the branches indicate the percentage bootstrap support obtained in the neighbour-joining (**bold**) and maximum likelihood analyses (*italics*). The origin of samples showing the various haplotype numbers is given in Table 3.1.

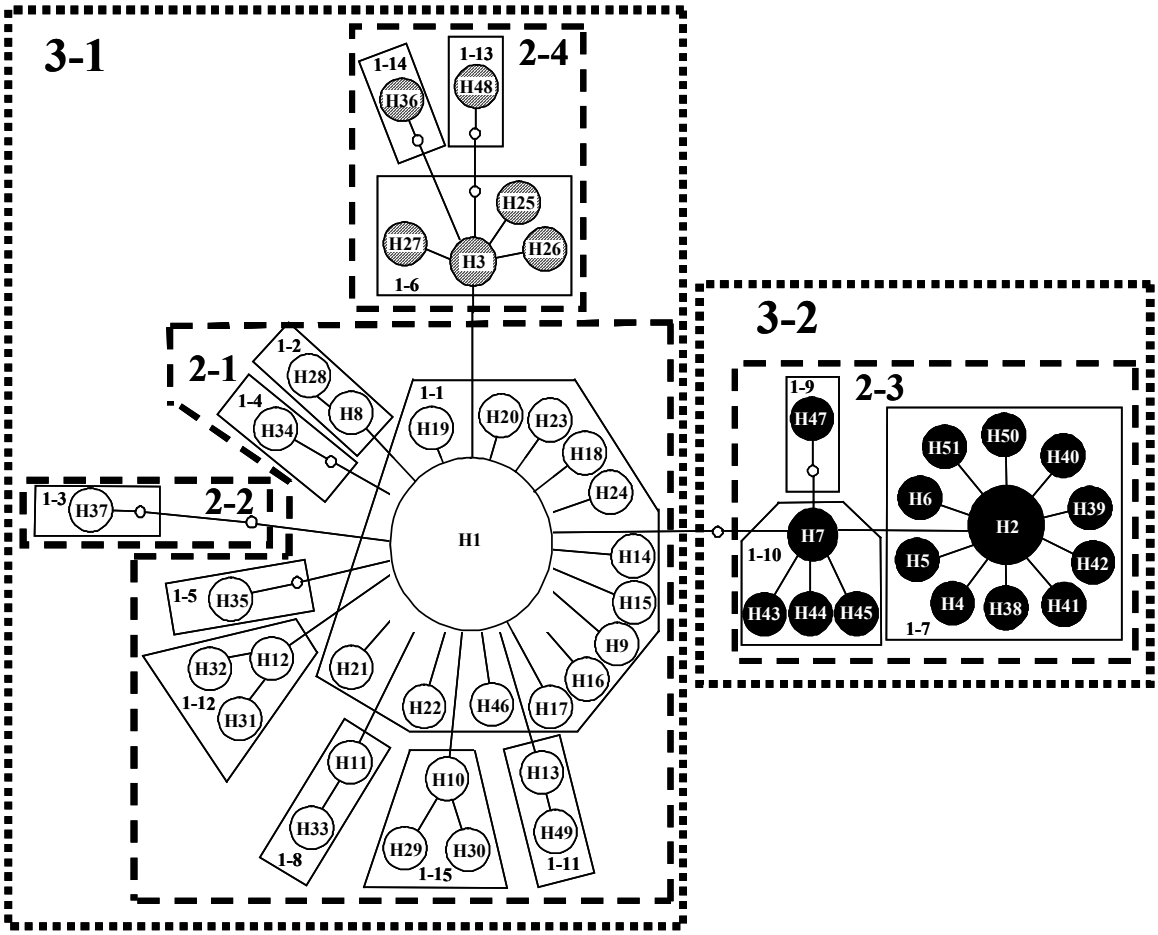


Figure 3.3

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. Empty small circles indicate intermediate haplotypes that are not present in the sample, but that 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 location map (Figure 3.1).

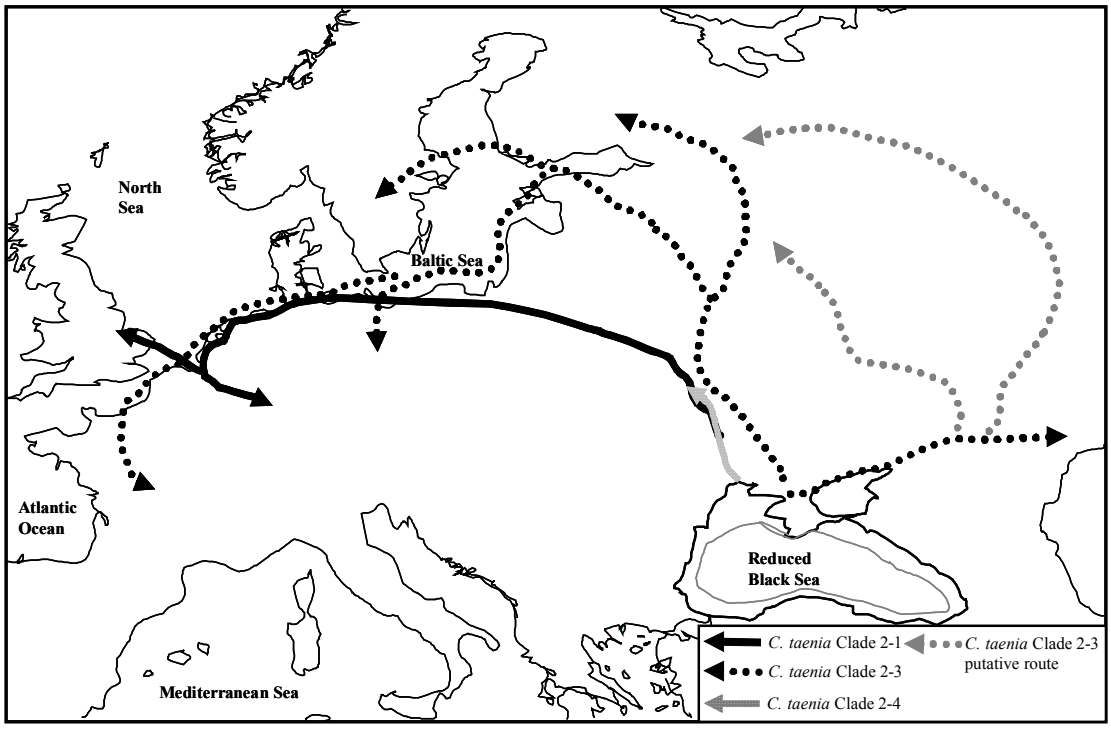


Figure 3.4

Possible re-colonisation routes of *Cobitis taenia*. Clade 2-1 (identified by the nested clade analysis shown in Figure 3.3) is represented by a black line, clade 2-3 by a black dotted line (grey dotted line indicates putative north east route), and clade 2-4 by a patterned line. Clade 2-2 (not shown) occurred only in the River Elbe.

3.2.4 Divergence times

C. taenia divergence was dated using the marine isotope stages (MIS) (Imbrie *et al*. 1984) because many glacial (and interglacial) periods have different names in the literature, with only the marine isotope stages being common to all. The time to the most recent common ancestor (MRCA) of clade 2-1, which includes all British spined loach, corresponds to the MIS 5c interstadial (87-99 ka) when estimated by the Saillard *et al*. (2000) method (93,527 ± 18,275 years BP) or even the MIS 6 glacial period (128-186 ka) when considering the mean pair wise within-clade divergence (183,899 ± 107,869 years BP). Both methods date the MRCA of clade 2-3 further back, to the MIS 8 glacial period (245-303 ka) (271,297 ± 141,948 or 277,432 ± 141,948 years BP). But the methods varied extensively in dating the MRCA for clade 2-4 which was solely located in the Southern Bug River, to 112,775 ± 131,570 years BP which corresponds to the MIS 5e interstadial (110-128 ka) or 482,111 ± 275,839 years BP that is in the MIS 13 interglacial period (478-524 ka). Overall, both methods showed some congruence in estimating the time to the MRCA from the MIS 8 glacial period (240-303 ka) to the MIS 10 glacial period (339-362 ka) for all the *C. taenia* clades (299,455 ± 50,155 or 348,353 ± 180,040 years BP).

3.3 Conclusions

C. taenia survived the last glacial maximum in at least three refuges in the Ponto-Caspian area of the Black Sea basin. These refuges gave rise to two major lineages that re-colonised Europe in separate directions: one westward to England and the other spreading north into Russia before moving west. A minor lineage that contributed little to the re-colonisation of Europe remained near its Black Sea refuge. The nested clade phylogeographical analysis indicates an overall pattern of restricted gene flow with isolation by distance, which is consistent with the poor dispersal ability of spined loach. 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 colonisation of Europe. The low genetic diversity within *C. taenia* suggests that its colonisation of Europe is relatively recent. British spined loaches are genetically allied to German loaches (both within clade 2-1), suggesting that the species, like other stenohaline fish, became established in England in the last part of the Upper Pleistocene (195-12 ka), having colonised from the Rhine system while the land bridge was present between Europe and England.

4. Genetic differentiation of UK spined loach populations

The aim of this study was to identify any geographical partitioning of the genetic diversity of spined loach within the UK, using mitochondrial control region DNA sequences. The control region has the highest substitution rate of all mitochondrial regions and is therefore a marker of choice for investigating phylogenetic relationships among closely related species and for studies at the population level. In particular, this study aimed to investigate population structure within the UK and provide genetic evidence for the establishment of ESUs or MUs within the UK. This report also provides a European analysis of genetic variability to put the UK analysis into context.

4.1 Methods

In total, 153 samples from 40 locations were identified as diploid 'true' *Cobitis taenia* and used in this study (Figure 4.1; Table 4.1). In addition, two samples of *C. elongatoides*, belonging to the same subgenus *Cobitis sensu stricto* were included as the outgroup. The DNA extraction, amplification, sequencing and analysis were as described previously and in Culling *et al*. (in prep).

4.2 Results

4.2.1 MtDNA genotypes

Nucleotide sequence was determined for 508 bp obtained from between the Phe-tRNA gene and the end of the central conserved region of the mtDNA control region for 153 *Cobitis taenia* individuals. Alignment of all control region sequences revealed a total of 22 different haplotypes, within which 21 positions (4% of total sites) were polymorphic. Of these, 11 positions (2% of total sites) were parsimony informative. Haplotype 1 was the most frequent (47%) haplotype recovered. A microsatellite (TA_7) was found in the control region of all *C. taenia* samples, but those from the Witham catchment, in England, contained a shorter repeat (TA_6). This microsatellite could be used as a marker in further analyses. Low average mean pairwise differences and nucleotide diversity were found among all the *C. taenia* control region haplotypes (2.33 $\pm$ 1.28; 0.0046 $\pm$ 0.0028).

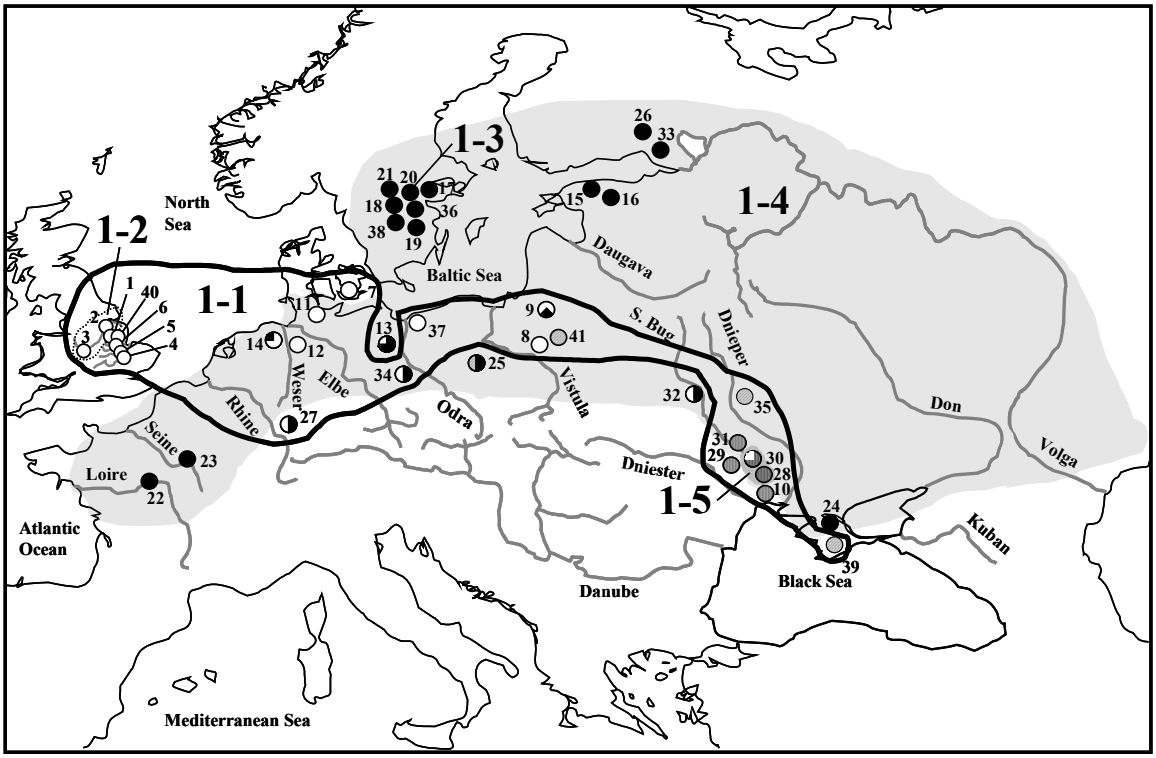


Figure 4.1

Geographic origin of *Cobitis taenia* samples in the analysis of the mitochondrial control region DNA. Sample number correspond to those in Table 4.1. Clade numbers (1-1 to 1-5) correspond to those identified in the nested clade analysis (Figure 4.3). Open circles = haplotype 1; black circles = haplotype 2; vertical hatched circles = haplotype 3; diagonally hatched circles = haplotype 7. Relative frequency of haplotypes at each site is indicated by relative amount of shading.

4.2.2 Phylogenetic analysis

The neighbour-joining and maximum likelihood trees revealed two main groups; the first group contained the two outgroup control region haplotypes of *C. elongatoides* and the second group comprised all 22 *C. taenia* control region haplotypes (Figure 4.2). There was 100 per cent bootstrap support for these groupings in both trees.

Table 4.1

Geographic origin and number of individuals of *Cobitis taenia* used in the phylogeographic analysis of the mitochondrial control region DNA.

Code	Locality	*N*	Coordinates	Control region haplotypes
1	R. Witham at Cannick, ENG	12	53:13:35N 00:28:49W	H3(12)
2	R. Trent at Stoke Bardolph, ENG	4	52:57:37N 01:02:04W	H1(3) H4(1)
3	R. Trent trib., R. Mease, ENG	8	52:43:24N 01:43:02W	H1(4) H4(4)
4	Great Ouse at Ouse Washes, ENG	17	52:07:49N 00:08:42E	H1(16) H10(1)
5	R. Nene at Mortons Leam, ENG	13	52:34:25N 00:03:10W	H1(13)
6	R. Welland at Baston Fen, ENG	13	52:44:43N 00:18:19W	H1(13)
7	R. Susaa, DEN	3	55:16:00N 11:43:00E	H1(2) H13(1)
8	R. Vistula at Zegrzynski Res., PL	3	52:27:00N 21:02:00E	H1(1) H2(2)
9	R. Vistula at Wigry Lake, PL	3	54:01:58N 23:06:29E	H1(1) H5(1) H2(1)
10	Southern Bug R., UKR	2	47:08:10N 31:25:22E	H1(2)
11	L. Ploen in the Baltic, D	3	54:10:00N 10:25:00E	H1(2) H5(1)
12	R. Elbe trib., R. Ilav, D	4	53:23:00N 10:25:00E	H1(1) H2(1) H6(1) H14(1)
13	R. Odra, D	4	52:34:07N 14:36:16E	H2(2) H5(1) H7(1)
14	R. Weser trib., Haaren Crk, D	3	53:05:00N 07:50:00E	H1(1) H2(2)
15	R. Jagala in the Gulf of Finland, EST	3	59:28:33N 25:09:04E	H2(1) H5(1) H21(1)
16	R. Loabu in the Gulf of Finland, EST	3	59:33:59N 25:48:00E	H2(3)
17	L. Malaren at Bockholmsundet, SV	2	59:16:32N 17:39:34E	H1(1) H2(1)
18	R. Soderkopingsan, SV	1	58:22:00N 16:27:00E	H2(1)
19	R. Vindan at L. Vindommen, SV	1	58:08:36N 16:24:28E	H2(1)
20	L. Karringfisket, SV	1	58:44:22N 15:49:33E	H15(1)
21	Kilaan Cr'k at Albergaan Cr'k, SV	1	58:43:36N 16:31:44E	H2(1)
22	R. Loire nr Orleans, F	2	47:16:00N 02:11:00W	H2(2)
23	R. Seine nr Troyes, F	4	48:18:00N 04:05:00E	H2(2) H16(1) H17(1)
24	R. Alma nr the Black Sea, CR	2	44:08:10N 31:51:00E	H2(1) H19(1)
25	R. Odra at Slesinski Channel, PL	2	52:23:00N 18:20:00E	H2(2)
26	R. Kangaskoski, FI	5	61:25:00N 29:25:00E	H2(5)
27	R. Rhine, D	2	49:38:08N 08:21:35E	H2(2)
28	Southern Bug R., UKR	3	47:30:34N 31:25:22E	H1(1) H8(1) H9(1)
29	Southern Bug R., Kodyma R., UKR	2	47:56:10N 30:45:53E	H1(1) H9(1)
30	Southern Bug R., Savranka R., UKR	3	48:07:34N 29:44:12E	H1(1) H8(1) H22(1)
31	Southern Bug R., UKR	4	48:57:33N 28:41:02E	H1(4)
32	R. Dnieper trib., Sluch R., UKR	1	50:39:29N 27:37:53E	H2(1)
33	R. Sahakoski, FI	3	61:50:00N 24:30:00E	H2(2) H20(1)
34	R. Ems trib., Hase R., D	3	51:09:00N 09:26:00E	H1(1) H7(1) H16(1)
35	R. Dnieper at Bicianka Cr'k, UKR	2	50:26:00N 30:31:00E	H1(1) H12(1)
36	R. Stangan, SV	1	57:39:00N 15:36:00E	H44(1)
37	R. Odra at Glebokie Lake, PL	2	53:40:00N 15:30:00E	H1(1) H11(1)
38	Kapellan Stream, SV	1	58:24:07N 15:28:58E	H2(1)
39	R. Cornaya Reka, CR	2	44:34:09N 33:38:23E	H1(1) H6(1)
40	R. Witham, Metheringham D'ph ENG	5	53:13:35N 00:18:19W	H3(5)

Location codes are shown in Figure 4.1. CR, Crimea; D, Germany; DEN; Denmark; ENG, England; EST, Estonia; F, France; FI, Finland; PL, Poland; SV, Sweden; UKR, Ukraine. Haplotype numbers refer to the NCA (Figure 4.3), with absolute sample frequencies given in parentheses.

4.2.3 Nested clade analysis (NCA)

The nested clade design of control region sequences for European *Cobitis taenia* constructed from the haplotype network consisted of two levels (Figure 4.3). Mapping the distribution of the five first level clades found in Figure 4.3 (1-1 to 1-5) revealed that two of these clades (1-1 and 1-4) were rooted in the Black Sea basin (Figure 4.1). Another first level clade (1-2) was rooted within two rivers in the UK (the Mease and the Witham). A minor clade of this first level (1-3) consisted of a single haplotype from L. Karringfisket in Sweden, which was a single mutation outside clade 1-1. Finally, a fifth clade at the first level (1-5) was solely located in the lower Southern Bug River in the Ukraine.

The nested clade analyses revealed significant geographical associations for clades 1-2, 2-1 and 2-2 and the total cladogram. Interpretations of these statistical results, employing the latest inference key from Templeton (2004), was of restricted gene flow with isolation by distance at all levels (clades 1-1, 1-2, 2-1, 2-2 and the total cladogram). The pattern of restricted gene flow is further corroborated by the significant Mantel test for independence between geographical and genetic distances (P = 0.001), which also indicates a pattern of isolation by distance.

4.2.4 Inter-population differences

In the analysis of all European populations there was significant population differentiation for just under two thirds of the population pairs (60% of the pair wise F_{ST} values were significant). All of the pairwise values that were non-significant were between clade 1-3, which contained a single haplotype, and all other clades. All the tests of differentiation at the different hierarchical levels (AMOVA) were significant. The populations grouped by the higher-level clades (2-1 and 2-2) showed less variance, -3.2%, among groups (F_{CT} = -0.015, P <0.0001), than variance among populations within groups, 70.9% (F_{SC} = 0.34, P <0.0001). Variance within populations was 32.3% (F_{ST} = 0.16, P <0.0001).

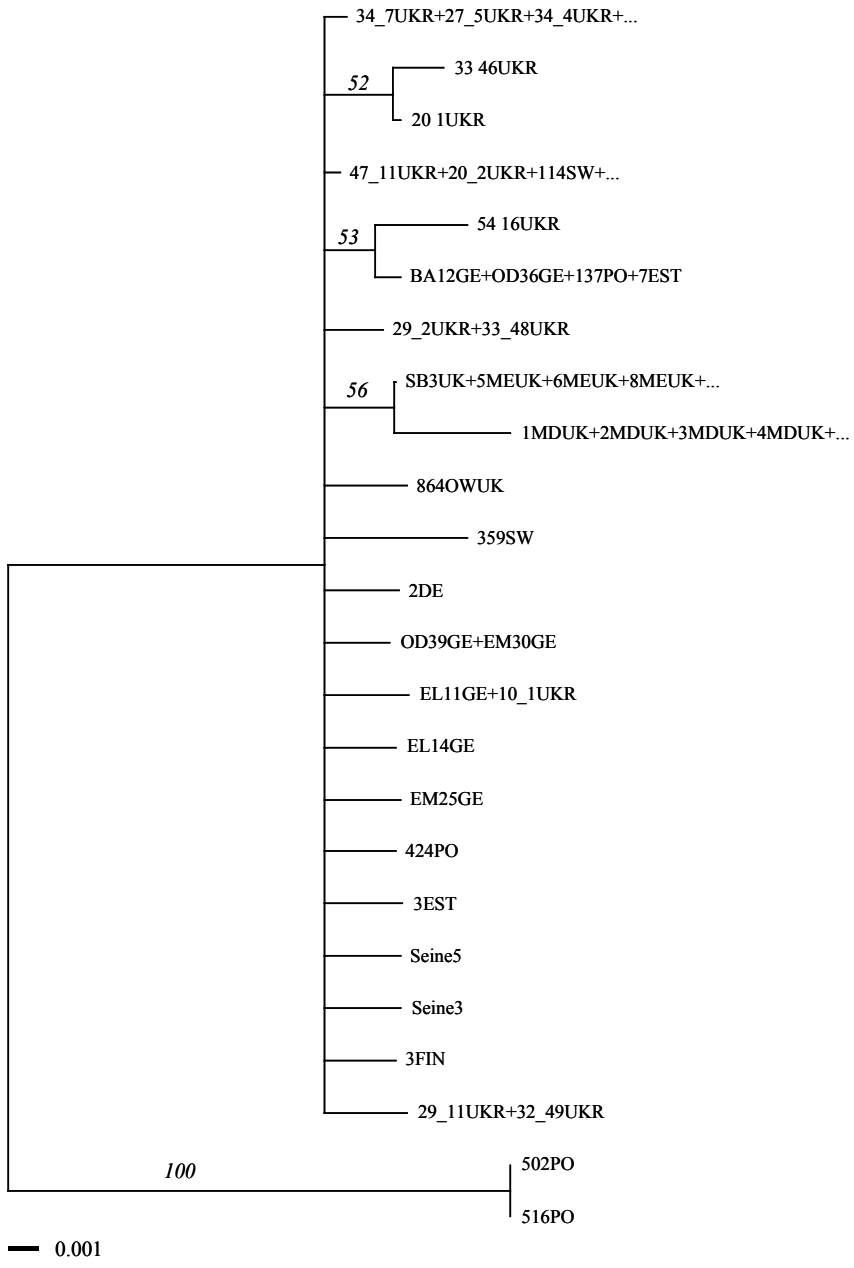


Figure 4.2

Phylogenetic relationships among *Cobitis taenia* haplotypes reconstructed using the neighbour-joining (NJ) and maximum likelihood (ML) analyses for all mitochondrial control region haplotypes. The tree is rooted with *Cobitis elongatoides* (502PO and 516PO). Numbers along the branches indicate the percentage bootstrap support obtained in the ML analyses.

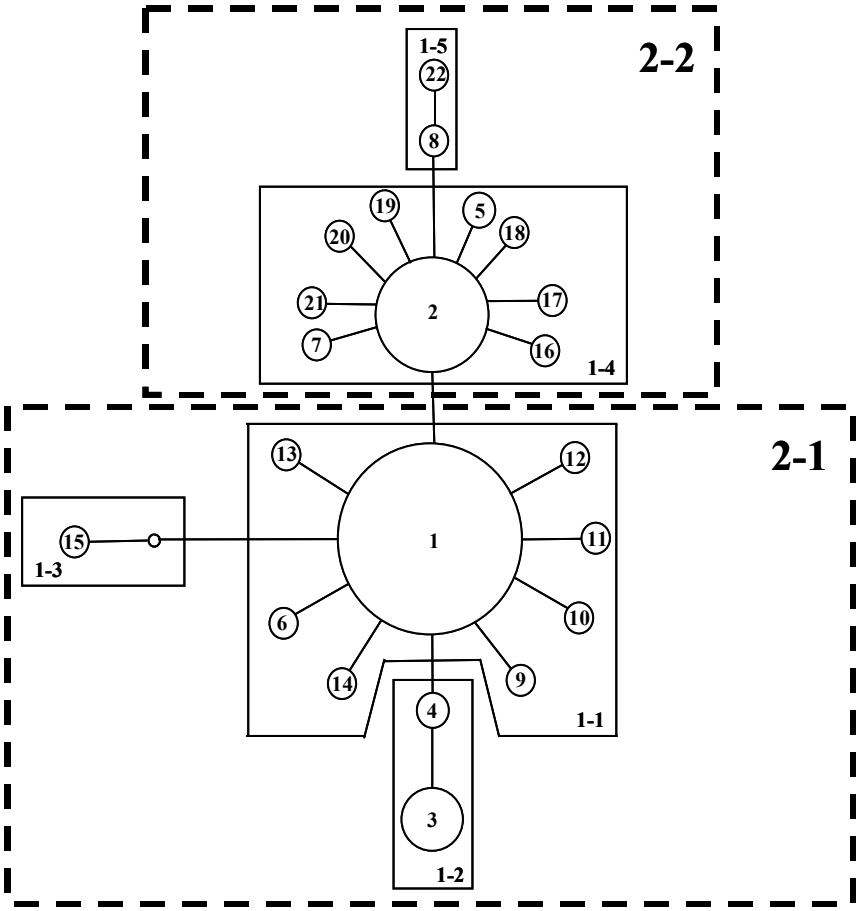


Figure 4.3

Cobitis taenia control region 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. Empty circles indicate intermediate haplotypes that are not present in the sample, but are necessary to link all observed haplotypes to the network. Clade 1-2 comprises fish from the Rivers Mease and Witham. Other UK fish are in clade 1-1.

In pairwise comparisons of UK populations only, there was significant population differentiation for nearly two-thirds of the population pairs (61.9% of the pair-wise F_{ST} values were significant) (Table 4.2). Most of the pairwise values that were non-significant were between populations from catchments that were connected. All the tests of differentiation at the different hierarchical levels (AMOVA) were significant. The UK populations grouped by catchment connections showed more variance, 54.0%, among groups (F_{CT} = 0.54, P <0.0001), than within group, 30.1% (F_{SC} = 0.65, P <0.0001). Variance within populations was 15.9% (F_{ST} = 0.84, P <0.0001).

Table 4.2

Pairwise population distance values (F_{ST} below the diagonal) and number of migrants per generation (*M* above the diagonal), using mitochondrial control region haplotypes in *C. taenia* populations from the UK. Inf = infinite number of migrants.

Populations	Metheringham	Witham	Mease	Stoke Bardolph	Welland	Ouse	Morton's Leam
Metheringham		inf	0.26	0.14	0.00	0.05	0.00
Witham	0.00		0.15	0.07	0.00	0.04	0.00
Mease	0.66**	0.77**		inf	0.46	0.64	0.46
Stoke Bardolph	0.78*	0.88**	0.08		1.08	3.75	1.08
Welland	1.00**	1.00**	0.52*	0.32		inf	inf
Ouse	0.91**	0.93**	0.44**	0.12	0.17		inf
Morton's Leam	1.00**	1.00**	0.52*	0.32	0.00	0.02	

*P < 0.05 **P < 0.001

4.3 Conclusions

The results shown in Section 4.2, using a different, faster-evolving molecular marker than in Part 4, confirm the presence of glacial refuges for spined loach located in the Ponto-Caspian area of the Black Sea and of two major lineages being responsible for the majority of the re-colonisation of Europe. Despite having low genetic variation, even at the mitochondrial control region site, spined loach show significant differentiation between populations in two of the five river catchments in which they occur in the UK. Both Witham populations (Metheringham Delph and River Witham) differed significantly from both Trent populations (River Mease and Stoke Bardolph). By contrast, the populations within the Welland/Nene/Great Ouse group showed little genetic differentiation, reflecting that fact that these catchments are interconnected. Most importantly, haplotypes that are not present elsewhere in Europe were found in fish of the rivers Mease and Witham. These haplotypes could have arisen from recent isolation and reduced population size, or from mutation prior to colonisation of the UK.

5. Habitat preferences of spined loach

The goals of this section were three-fold. Firstly, inter-population differences in sediment preferences from two contrasting habitats of spined loach were investigated in drainage ditches (four populations) and a river (one population). Secondly, the substratum preferences of males, females and juveniles from each habitat were compared. Finally, the day and night patterns of sediment preference and behaviour by different age and sex groups of fish from each habitat were considered.

5.1 Methods

The substratum preference of spined loach from five different locations in England was studied in laboratory experiments. Fish were collected from two main habitat types: drainage ditches and rivers. The drainage ditch sites were Baston Fen, Morton's Leam, the Ouse Washes and Wicken Fen, which are part of the rivers Glen (Lincolnshire), Nene and Great Ouse (Cambridgeshire) respectively. The sites are narrow, slow-flowing canals with fine, soft sediment bottoms and a high density of emergent marginal vegetation. The single river sampled was the River Twin, a tributary of the River Great Ouse. This site has abundant macrophytes, faster water flow and a greater variety of bottom sediment types than the drainage ditch sites. Thirty fish were collected at each location (10 males, 10 females and 10 juveniles) with a modified shrimp trawl.

Four sediment types were collected from each habitat (ditch and river) immediately prior to the experiments using a sampling bucket. Sediments were selected to represent the widest range of substrata available in each habitat. Ditch sediments were collected at sites with the highest spined loach abundance. Hence, two sediment types originated from Morton's Leam and two from Wicken Fen. All river sediments were collected from the River Twin in locations where spined loach were caught.

5.1.1 Substratum choice experiments

Two separate experiments were run. In the first, ditch fish were offered four ditch sediment types; in the second, river fish were offered four river sediment types. In each experiment, 30 aquarium tanks were each divided into four sections by 3-cm-high retaining partitions. The partitions allowed the four sediment types to be offered without mixing and yet did not impede fish swimming. The sediments were placed randomly into each section.

In Experiment 1, fish from drainage ditches were offered a choice between silt, dredged sediment, organic sediment and gravel. Silt and dredged sediment were obtained from Wicken Fen. Silt was a soft, fine (30 per cent by volume at 125 µm particle size) substratum generated by the accumulation of mud in areas with high vegetation density. Dredged sediment was finer than silt (43 per cent by volume at 125 µm particle size) and was collected where macrophytes were removed for waterway clearance by mechanical means. The organic sediment and gravel were obtained at Morton's Leam. Organic

sediment was taken from the bank of the ditch where vegetation accumulated and created a coarse, anoxic, black soil. Gravel was collected from the centre of the ditch and was composed of small pebbles (particle diameter 1-2 cm).

In Experiment 2, fish from river origin were offered a choice between gravel, organic sediment, sand and mud. The gravel (particle diameter 3-5 cm) was collected from the centre of the river where flow was fast and there was no accumulation of organic material. The other three substrata were collected near the river bank. Organic sediment contained larger particles of non-degraded vegetation than the equivalent sediment in Experiment 1. Sand and mud differed in texture, sand being looser and more mineral, and mud more compact and like clay.

All fish were photographed, measured, individually marked and sexed based on the presence or absence of the organ of Canestrini (Robotham,1981). Three sex/age groups were distinguished: females (80-110 mm), males (60-75 mm) and juveniles (< 40 mm).

A single fish was released in each aquarium. In Experiment 1, the position and activity (such as burying, swimming or feeding) of each fish was monitored for 1.5 minutes, five times a day and three times a night for ten days. In Experiment 2, monitoring was carried out three times a day (due to personnel constraints) and three times a night, also for ten days. Fish were fed in the holding tanks prior to the experiments but during the experiments, the fish fed exclusively on the sediment provided, to help replicate natural activity patterns.

5.2 Results

5.2.1 Substratum preference and activity patterns of drainage ditch fish

In most cases, spined loach from ditches preferred organic sediments (Table 5.1). This preference held for day and night for Morton's Leam fish, but was limited to daytime for fish from Baston and Wicken Fen and to night for fish from the Ouse Washes. At night, Baston and Wicken Fen fish showed no preference (Table 5.1). During the day, Ouse Washes fish preferred silt (Table 5.1).

Table 5.1

Summary of diurnal and nocturnal substratum preferences of spined loach from different origins, sexes and ages during laboratory choice experiments. *df* = 3 throughout. NS: *P* > 0.05.

Origin/Sex/Age	Day			Night		
	Preference	X^2	*P*	Preference	X^2	*P*
Ouse Washes	Silt	31.1	< 0.001	Organic	19.7	< 0.001
Baston Fen	Organic	19.2	< 0.001	Random	--	NS
Wicken Fen	Organic	36.6	< 0.001	Random	--	NS
Morton Leam	Organic	21.1	< 0.001	Organic	46.3	< 0.001
Drainage ditch						
Juvenile	Organic	8.6	< 0.05	Organic	52.4	< 0.001
Male	Organic	41.9	<0.001	Organic	15.9	< 0.001
Female	Organic	25.1	< 0.001	Random	--	NS
River						
Juvenile	Sand	8.7	< 0.05	Sand	14.1	< 0.05
Male	Organic	13.3	< 0.001	Organic	17.7	< 0.001
Female	Gravel	10.2	< 0.05	Sand	9.2	< 0.05

All spined loach from ditches, regardless of sex and age, preferred organic sediment at all times, with the exception of females which became randomly distributed at night (Table 5.1).

There was a clear pattern of nocturnal activity, measured as the number of substratum switches recorded per day (Figure 5.1a). Nocturnal activity was also witnessed in the smaller proportion of buried fish at night and the greater proportion of swimming and foraging fish (Figure 5.1b, c).

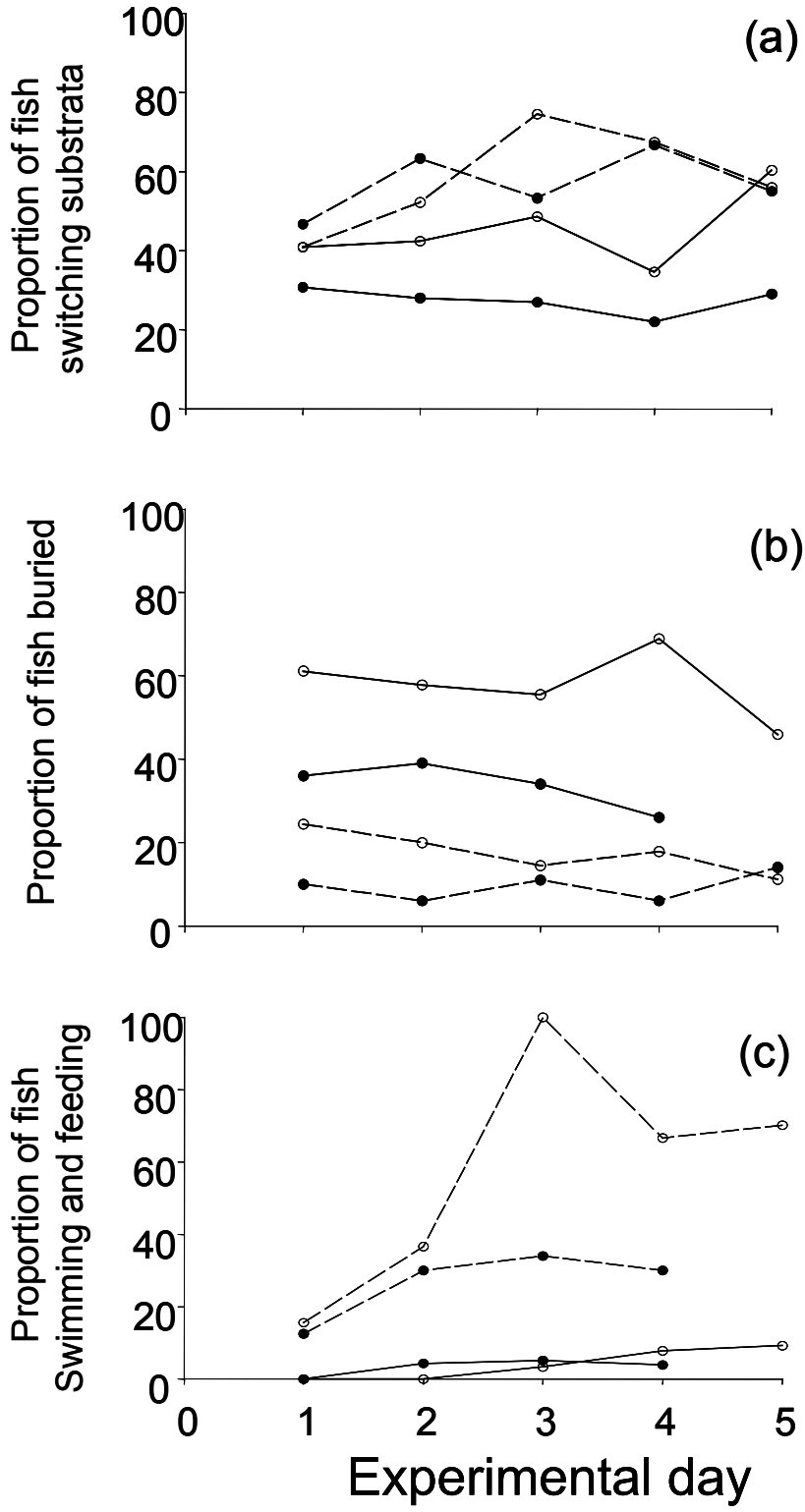


Figure 5.1

Proportion of spined loach from drainage ditches (filled circles) and river (open circles) (a) switching substrata between observation periods, (b) burying, and (c) swimming, in relation to experimental day. The solid lines represent daytime behaviour; the dashed lines represent nocturnal behaviour. The exact substratum upon which ditch spined loach foraged was recorded too few times to allow any analyses of feeding preferences. However, the pattern of burying was different on different sediments. Gravel was the

least used sediment for burying by all age/sex groups (3 per cent). Forty-eight per cent of ditch juveniles that did bury during the day used silt while only 16 per cent used organic sediment. Ditch females that chose to bury at night did so in silt (63 per cent) while juveniles and males appeared to express no preference at night.

5.2.2 Substratum preference and activity patterns of river fish

There were marked differences in sediment choice between juveniles, males and females, and evidence of shifting preferences at night in females from the River Twin (Table 5.1). River juveniles always preferred sand while females preferred sand during the night but switched to gravel during the day. River males preferred organic sediment during both day and night.

Regardless of age and sex, river fish were always more active at night than during the day (Figure 5.1a-c). River fish were more often buried during the day, and swimming and feeding during the night. Forty-eight per cent of river juveniles and 33 per cent of females chose sand for feeding while 35 per cent of males fed in organic sediment

Both river males and females preferred organic sediment for burying while only a small proportion (2.2 per cent) of the largest fish buried in gravel. Forty-one per cent of those river juveniles that buried did so in sand while gravel (4.6 per cent) was the least preferred burying substratum for all groups.

5.3 Conclusions

Spined loach from different habitats show different substratum preferences in the laboratory. Moreover, different populations from similar habitats also show variation in sediment choice. We found differences in substratum preference among sex/age groups in populations from both habitats, and shifts in preference between day and night in some groups. Spined loach are also clearly nocturnal, as evidenced by increased exploratory and feeding behaviours at night.

6. Implications for conservation and management of spined loach in England

The results of this study have a number of important implications for the management of spined loach in England and more broadly in Europe. The identity of English spined loach as *Cobitis taenia* has now been confirmed. Thus, there appears to be a single, diploid species in Britain, which should help management decisions, at least in the UK.

Generally, spined loach were found to have little genetic variation, probably because of their relatively recent re-colonisation of Europe following the last glacial period. At the European scale, there are at least three distinct evolutionary lineages of spined loach, which originated from the Ponto-Caspian region of the Black Sea in the Upper Pleistocene. One of the two main lineages is currently present exclusively in Denmark and all of the English catchments, and has likely spread from its refuge in an east-to-west manner, through Poland, Denmark, Germany and across to England. The second lineage is exclusively present in Estonia, France, Kazakhstan, Russia and Scandinavia. Its likely route of post-glacial colonisation was in an initially northerly direction from the refuge into Russia and Scandinavia, and then through the Baltic into Poland, Germany and France. Fish from these two lineages co-occur in the rivers Vistula, Odra, Elbe, Weser and Rhine. A third, more minor lineage which did not contribute to the re-colonisation of Europe was found in the lower Southern Bug River in the Ukraine. This clade is strikingly distinct, indicating very limited gene exchange with the other lineages. Thus, the results suggest the existence of at least three different evolutionarily significant units of spined loach within Europe, which should be taken into consideration when planning a representative network of SACs for this species.

Genetic variation within spined loach populations in England was found to be limited. Despite this, there was evidence of genetic population structure. The fact that the Trent and Witham catchments are connected via an ancient drainage system dating from Roman times (the Fosdyke), but disconnected from the other three inter-connected catchments (Welland/Nene/Great Ouse) was reflected in the significant genetic differences between these two groups of catchments. The genetic variation among populations within these groupings arose mainly from inter-population differences in the Trent/Witham group. Both Witham populations (Metheringham Delph and River Witham) differed significantly from both Trent populations (River Mease and Stoke Bardolph). By contrast, the populations within the Welland/Nene/Great Ouse group showed little genetic differentiation, possibly as a result of extensive past or present gene flow. Present-day gene flow could stem from the connection between the Great Ouse and the Welland via the Nene. The Great Ouse connects to the Nene via the 'Middle Level' Nene-Ouse Navigation Link from Peterborough to the Denver Sluice at Earith, and the Nene comes within four kilometres of the Welland via the South Holland Drain (the farm land in this area has a maze of smaller drains that may connect directly to the Welland). Furthermore, this area, which also includes the River Welland and straddles the three

counties of Cambridgeshire, Northamptonshire and Lincolnshire, is known as the Fens: a peat-based landscape drained by man-made dykes and drains that have left it extremely flat and prone to extensive flooding. This flooding could also boost gene flow and explain the genetic homogeneity of spined loach within the Welland/Nene/Great Ouse group.

Of all the spined loaches examined in this study, those from the Rivers Mease (Trent catchment) and Witham (Witham catchment) were the most genetically distinct. Fish from these rivers harboured haplotypes not found anywhere else in Europe, and which must have arisen either from recent isolation and reduced population size, or from mutation prior to colonisation of the UK. Given the generally low genetic diversity of the species, this important discovery suggests that the Witham and the Trent catchments are potentially crucial marginal areas for spined loach conservation. Populations such as these, which are at the edge of the species' range, are often genetically distinct because they are exposed to environmental conditions different to those experienced by populations at the centre of the range. From a genetic perspective, marginal populations may be particularly important in conservation because they could act as a reservoir of potential to adapt to changing environments and climate.

The results of this study suggest that at least three of the seven UK populations should be managed as distinct units. These are the Witham, the Mease, and one of the rivers from the Welland/Nene/Great Ouse interconnected catchments. The latter two are already included in at least one SAC, but the Witham is not.

Because of the inferred restricted gene flow, it is recommended that these units are monitored and managed separately. It may also be prudent to maintain physical separation of the Trent/Witham and Welland/Nene/Great Ouse groups to prevent genetic homogenisation of the species. For example, specimen transfers between these roughly defined areas should be avoided, as should artificial connections between the Trent/Witham and Welland/Nene/Great Ouse, such as the proposed Fens Waterways Link that will connect the rivers Witham, Welland, Glen, Nene and Gt. Ouse.

Finally, the results of the habitat preference experiments carried out in this study suggest that the way populations are currently monitored may need to be rethought, along with the design of habitat management plans. Current understanding of the ecological requirements of spined loach has been largely derived from daytime habitat surveys. However, our results show that nocturnal habitat requirements are often different and perhaps more important, since food foraging occurs at night. Thus, management efforts directed at maintaining daytime habitats may not necessarily be beneficial to the species.

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