An invasive lineage of sculpins, *Cottus* sp. (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups

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Fish abundance surveys in the Rhine system have shown in the past two decades that there is a rapid upriver invasion of a freshwater sculpin of the genus *Cottus*. These fish are found in habitats that are atypical for the known species *Cottus gobio*, which is confined to small cold streams within the Rhine drainage. Phylogeographic analysis based on mitochondrial haplotypes and diagnostic single nucleotide polymorphisms indicates that the invasive sculpins are hybrids between two old lineages from the River Scheldt drainage and the River Rhine drainage, although it is morphologically more similar to the Scheldt sculpins. Most importantly, however, the invasive population possesses a unique ecological potential that does not occur in either of the source populations from the Rhine or the Scheldt, which allows the colonization of new habitats that have previously been free of sculpins. Microsatellite analysis shows that the new lineage is genetically intermediate between the old lineages and that it forms a distinct genetic group across its whole expansion range. We conclude that hybridization between long separated groups has lead to the fast emergence of a new, adaptationally distinct sculpin lineage.

Keywords: hybridization; speciation; invasion; adaptation; range expansion

1. INTRODUCTION

In Central Europe, sculpins commonly known as *Cottus gobio* L. are stenoeicous inhabitants of well oxygenated cold streams and lakes (Voigt & Hofer 1909). They are generally absent from downstream habitats such as large rivers or artificial canals. Unexpectedly, less than 20 years ago, Cazemir (1988) and van den Brink *et al.* (1990) reported sculpins to be common in the Lower Rhine of the Netherlands, which is a typical summer warm potamal habitat. In the ecologically similar German stretch of the Lower Rhine, parallel new records of sculpins were made by Schleuter (1991) and by Lelek & Köhler (1993). A comparison between the distribution in 1960–1979 and the more recent situation in 1991–1995 in the Netherlands was published by De Nie (1997). While sculpins were identified only in a few lowland streams in the earlier surveys, the later ones showed them to be very abundant in the Rhine Delta, the River Maas and the IJsselmeer. Intriguingly, sculpins were now preferentially found in large bodies of water, which were not used as a habitat before (De Nie 1997). Again, this was paralleled by findings from the German Lower Rhine where the invading sculpins were also found in large stagnant water bodies connected to the Rhine, for instance, backwaters and harbours (own data). All of these observations indicate that the sculpins recently invading parts of the Lower Rhine drainage display a previously unknown tolerance to summer warm and turbid waters within the lower reaches of large rivers. The sudden appearance of

the invasive sculpins raises the question of their origins. Köhler *et al.* (1993) discussed two possible ways of colonization. Sculpins could have persisted in the Lower Rhine and recolonized the riverine habitats after the improvements in water quality, starting in 1980s. Alternatively, they could have colonized the main river downstream from source populations in its tributaries. The invasive sculpins could also represent introduced, non-native invaders since they share the conspicuous skin prickling with populations from elsewhere in Europe (Koli 1969). This includes the Scheldt system, which became connected to the Rhine system via canals in earlier centuries and would therefore be an obvious source for a non-native invasion. However, apart from the identification of the source populations for the invasion, the most intriguing question relates to the factors that have allowed this population expansion in the first place, since neither the surveys in the Scheldt nor in the Rhine basin (Vandelannoote *et al.* 1998; Köhler *et al.* 1993) have previously documented sculpins to be eudominant members of the fish communities of large lowland rivers.

Phylogeographic analyses of mitochondrial DNA of European sculpin populations, the so-called *Cottus gobio* complex, have revealed several clearly distinct groups across Central Europe (Englbrecht *et al.* 2000; Volckaert *et al.* 2002). This makes them one of the most deeply substructured European fish taxa studied so far. Sculpins have persisted during glacial cycles within separate refugia across much of their Central European range. The oldest lineages have been separated for up to 3 Myr, and the ones from the Rhine and the Scheldt have been separated for up to 1 Myr. Intriguingly, the Upper Rhine tributaries

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harbour a different lineage than the Lower Rhine tributaries. The two have not formed a homogenized gene pool although a river capture united them in a single basin about 1 Myr ago. Thus, riverine habitats that would connect subpopulations apparently acted as a prohibitive barrier. The only previously noted exception to the otherwise clear phylogeographic structure was related to sculpins from the Lower Rhine. In some populations from the area where the above described surveys have indicated a recent invasion, mtDNA haplotypes originating from adjacent phylogeographic lineages were discovered (Englbrecht *et al.* 2000; Volckaert *et al.* 2002).

Here, we show that the invasive sculpin from the Lower Rhine harbours indeed a hybrid gene pool, derived from its ancestral lineages from the Scheldt and the Lower Rhine system. Only the hybrid population has been successful in invading riverine habitats of the Lower Rhine. Representatives of the ancestral lineages have not expanded their ranges despite the absence of geographical barriers. We discuss these results in the context of models for hybrid speciation.

2. MATERIAL AND METHODS

(a) Sampling

Fish were collected using portable electroshockers. Fin clips were preserved in 99% ethanol. Specimens for morphological analysis were fixed in 4% formaldehyde and later transferred to 70% ethanol. The fish community of the River Sieg has been studied by Freyhof in a monitoring project since 1992. Sampling was done twice annually allowing to reconstruct population dynamics of sculpins in detail. The fish fauna of the River Mosel and the Middle and Northern Upper Rhine was studied from 1997 to 2001. Further details for all sampling sites are provided in supplementary table 1 in the electronic supplementary material.

(b) Morphology

Sculpins vary in the degree to which spinelike scales (prickling) cover the body (Koli 1969). Five classes were distinguished for this study: 0 = prickles absent; 1 = less than 10 prickles present beneath pectoral fin; 2 = more than 10 prickles but all covered by the pectoral fin; 3 = prickling extends beyond pectoral fin but ends anterior to the middle of the second dorsal fin; 4 = prickling extends back beyond the middle of the second dorsal fin. A subset of individuals was examined for differentiation in body shape. Well preserved Rhine sculpins from small streams (populations # 23, 24, 25, 26, 28, 29, 31, 34, 35), invasive sculpins (populations # 8, 10, 12, 13) and Scheldt sculpins (populations # 65, 66) were analysed using landmark based methods (Rohlf & Marcus 1993). A set of 14 anatomical landmarks was chosen to capture the shape from a lateral view. Data were analysed using the software packages Tps (Rohlf 2003) and Imp (Sheets 2002). All specimens were superimposed by procrustes methods. Partial warp scores were used for the morphometric analysis. Overall differentiation in shape was measured as bootstrapped full procrustes distance between group means. Canonical variates analysis (CVA) was used in order to identify those shape vectors serving to discriminate best between groups. The shape change implied by the discriminant axes was visualized as vectors on a deformation grid. Partial warp scores were regressed on centroid size to evaluate the confounding effects of allometry. This did not notably affect the outcome of the analysis

suggesting that growth contributes little to the observed differentiation.

(c) Life history data

Fully mature females were collected at the beginning of the spawning season (March 2003). Forty-three females from two invasive sculpin populations (# 13, 64) and 93 females from four Rhine sculpin populations (# 24, 25, 28, 63) were included. Premature females of Scheldt sculpin were collected in January 2004 from two streams (# 65, 66) in the River Scheldt drainage. Own laboratory observations showed that all sculpins studied are single clutch spawners. All specimens were measured (SL) before the gonads were removed. Age was determined by otolith analysis.

(d) Molecular analysis

The mitochondrial control region of sculpins was sequenced as previously described (Englbrecht *et al.* 2000). All mt-haplotypes could be unambiguously assigned to known groups (following Vockaert *et al.* 2002 and Knapen *et al.* 2003). SNP markers were developed from sequences of 12 random genomic clones. PCR primers were chosen to amplify fragments ranging from 500 to 700 bp, which were directly sequenced. The sequences were screened for polymorphisms in 20 specimens representing different phylogeographic lineages. Using *Cottus sibiricus* and *Cottus ricei* (River Olkha, southwest of Irkutsk, Siberia/Smoky River, Alberta, Canada; by courtesy of D. Neely, St Louis) as outgroups we could identify high frequency alleles that were derived states for a given lineage. Out of the 12 loci analysed in this way, the following loci yielded diagnostic SNP differences (primer details in suppl. table 2; genotype details in suppl. table 3 in the electronic supplementary material): CgoSNP1, CgoSNP2, CgoSNP3 (two separate SNPs, A and B), CgoSNP4 and CgoSNP5 (GenBank accession CL242132; CL242133; CL242134; CL242135; CL242136). Additional specimens were typed for these loci by pyrosequencing on a PSQ 96 MA (Pyrosequencing AB). The microsatellite loci Cgo18, Cgo33, Cgo56, Cgo42, Cgo114 and Cgo1033 (Englbrecht *et al.* 1999) were typed on a MegaBace 1000 sequencer (Amersham Biosciences) to assess the population substructure of 23 populations (populations # 6–9, 11–16, 22–26, 28–29, 31, 33–35, 65–66; total *n*: 950 individuals) within the Lower and Middle Rhine basin. An analysis of overall genetic distance of the invasive sculpin gene pool to representatives of the presumed ancestral lineages was based on fewer populations (invasive sculpin # 10, Rhine sculpin, # 24, 26; Scheldt sculpin, # 65, 66) but involved typing of 120 microsatellite loci (Nolte *et al.* 2005).

(e) Population genetic analyses

MSA 3.15 (Dieringer & Schlötterer 2003) was used to calculate pairwise genetic distances. We used Nei's standard genetic distance to make our data comparable to Knapen *et al.* (2003). Furthermore, deviations from a stepwise mutation model were detected for some loci, thus distances had to be based on an infinite allele model. The choice of alternative genetic distance measures did not significantly change the outcome of the analyses presented here (not shown). Exact tests for population differentiation in diagnostic SNP frequencies and for differential contribution of haplotype groups were conducted using Arlequin version 2001 (Schneider *et al.* 2000).

Figure 1. Colonization of rivers through invasive sculpins in the recent past. Data from regular fish abundance surveys in (a) the River Mosel and (b) the River Sieg are depicted for selected years. Open circles indicate the increasing range of invasive sculpins (as inferred from skin prickling) within main rivers, black circles represent the more or less static populations of non-prickled Rhine sculpins. The depicted length of the Mosel is approximately 200 km, the depicted length of the Sieg approx. 65 km, the double line depicts the Rhine.

3. RESULTS

(a) Invasion of a new sculpin into river habitats of the Rhine

Fish abundance surveys detected in 1992 sculpins with intense skin prickling (see below) in the lowest reaches of the Sieg. In the following 10 years, this sculpin expanded its range upriver with an average rate of approximately 4–8 km per year (figure 1). During this whole period, sculpins without skin prickling remained confined to their native streams and did not colonize riverine habitats despite the fact that many sculpin larvae are known to drift downstream after they hatch (Bless 1990). A similar pattern of colonization was observed for the German part of the River Mosel, another major tributary of the Rhine (figure 1). Previous surveys have documented numerous populations of sculpins in small tributaries to the Mosel but never in riverine habitats (Pelz 1985; Pelz & Brenner 2000). In 2000–2001, we found unprickled sculpins to be restricted to small tributaries, while prickled sculpins were abundant in the main channel of the Mosel (figure 1). Finally, the explosive spread of sculpins in the Netherlands described by De Nie (1997) was apparently due to the spread of prickled sculpins as well, since the specimens we examined from Lake IJsselmeer were prickled sculpins.

In order to resolve the origins of the invasive fish and to uncover its population structure we have obtained samples from the Lower Rhine drainage and from the western Scheldt drainage. In the following, we will distinguish three groups of animals: the ‘invasive sculpins’ that have recently colonized the main stream and the large tributaries of the Rhine, the ‘Scheldt sculpin’ from small upstream tributaries of the Scheldt drainage, and the ‘Rhine sculpin’ from the small upstream tributaries of the Lower Rhine drainage (note that the sculpins in the small upstream tributaries of the Upper Rhine drainage belong to the Danubian phylogeographic group—Engbrecht *et al.* 2000).

Morphological and ecological analysis of all samples shows a clear differentiation between the invasive sculpins and the Rhine sculpins and a less pronounced differentiation between the invasive sculpins and the Scheldt sculpins (figure 2). A particularly prominent diagnostic difference between invasive sculpins and Rhine sculpins is the occurrence of skin prickling (figure 2a) which is strong in the invasive sculpins and virtually absent in the Rhine sculpins (figure 2b). However, Scheldt sculpins show also skin prickling comparable to that of the invasive sculpins (figure 2b). A significant differentiation among the latter two groups can be observed in a geometric morphometric analysis (figure 2c). Invasive sculpins differ from adjacent Rhine sculpins by having a deeper trunk and caudal peduncle, a shorter body and a relatively larger head. Invasive sculpins differ from Scheldt sculpins in having a less deep anterior trunk (figure 2d). The anterior body depth of invasive sculpins is intermediate between Scheldt sculpins and Rhine sculpins. Still, the average shape of invasive sculpins is more similar to Scheldt sculpins as compared to Rhine sculpins (procrustes distance: 0.0217 vs 0.0321, respectively). The morphological characteristics of the invasive sculpins are retained in animals raised in aquaria and are thus not simply a plastic response to riverine habitats (A. W. Nolte, personal observation).

There are also life history differences between the groups. Female Rhine sculpins grow for at least 2 years before first reproduction. In contrast, Scheldt sculpins and invasive sculpins start to reproduce in their first year (figure 2e). Both, Scheldt sculpins and invasive sculpins compensate for the smaller size at reproduction by producing smaller eggs relative to their size than Rhine sculpins (figure 2f).

(b) Invasive sculpins are hybrids

Given the hints from phylogeographic analysis of mitochondrial haplotypes (Engbrecht *et al.* 2000; Volckaert *et al.* 2002) of recent admixture of sculpins within the Lower Rhine, we developed nuclear diagnostic markers to trace a possible hybridization within the nuclear genome. Primers were constructed for randomly cloned genomic fragments and sequences were determined from 280 animals from an expanded dataset covering the major phylogeographic lineages that occur adjacent to the Rhine drainage (see §2 for details). This allowed us to identify five single nucleotide polymorphisms (SNPs) that represent derived states and that were diagnostic in at least one of a known phylogeographic lineages (figure 3). These SNPs, as well as a further mitochondrial haplotype sequence analysis was then used to characterize the groups of sculpins described above (table 1).

Rhine sculpins from upstream tributaries in the Lower Rhine drainage carry specific mitochondrial haplotypes (group I and III; this and the following haplotype groups *sensu* Engbrecht *et al.* 2000) and derived alleles at SNP loci CgoSNP 1, 2 and 3a (table 1). Scheldt sculpins carry group IV haplotypes and a diagnostic fixed allele at locus CgoSNP4 (table 1). Sculpins from the upper Rhine drainage and from neighbouring drainages in the east of the Rhine show group I haplotypes and diagnostic fixed alleles at loci CgoSNP 3b and 5 (table 1). In contrast to all stream populations, invasive sculpins reveal a mixture of group I, III and IV haplotypes as well as a mixture of SNP

Figure 2. Morphological and ecological analysis of sculpin samples. (a) Depiction of the five categories of spinelike scales covering the body (see §2) and (b) frequencies for all groups (size of circle represents frequencies found). (c) Differentiation in body shape among the three lineages. Each comprises a distinct cluster that separates along two CVA axes (axis 1: $\lambda=0.13$, $\chi^2=941.6$, $df=48$, $p<0.01$; axis 2: $\lambda=0.62$, $\chi^2=215.3$, $df=23$, $p<0.01$). (d) The shape change captured by the CVA axes plotted as vectors at 14 anatomical landmarks (depicted in upper panel) on deformation grids (middle panel: invasive sculpins vs Rhine sculpins; lower panel: invasive sculpins vs Scheldt sculpins). (e, f) Life history characters (age, fecundity, size) from reproducing females of Rhine sculpins (black circles), invasive sculpins (open circles) and Scheldt sculpins (black triangles).

alleles otherwise diagnostic for Rhine or Scheldt sculpins (table 1). Thus, invasive sculpins harbour a hybrid genome that is derived from Scheldt sculpins and Rhine sculpins.

(c) *Invasive sculpins form a genetically distinct group*

To test for a possible population substructure within the lineages, we typed six microsatellite loci for 950 individuals from 23 sampling sites. We found that the three groups of sculpins form three corresponding genetic clusters (figure 4). Most notably, the invasive sculpins from the IJsselmeer, Mosel and Sieg cluster together, suggesting that they form a genetically homogeneous group. The genetic differentiation (figure 4a) among different populations within the Rhine sculpin cluster is greater than within the invasive sculpin cluster (average D : 0.47, range: 0.22–0.77 vs 0.12, range: 0.02–0.24), which indicates a shallower population substructure of the invasive sculpin. This is in line with the fact that Rhine sculpins occur in isolated subpopulations in separate streams whereas invasive sculpins are interconnected in their distribution (figure 3).

To assess the relative genomic contribution of the different lineages within the hybrid gene pool, we typed a smaller number of populations for a larger number of microsatellites. For this we used a population of invasive sculpins from the Sieg, two populations of Rhine sculpin and two populations of Scheldt sculpin (see §2) and typed them for 120 microsatellite loci. Genetic distance (D , as above) analyses revealed the same grouping as in figure 3

and more importantly, places the sample of the invasive sculpin similarly distant to both the Scheldt and the Rhine sculpins (figure 4b—average D to Rhine sculpins: 0.62; average D to Scheldt sculpins: 0.46). This result suggests an almost equal mixture of the source gene pools in the invasive sculpins on average. However, this statement needs to be cautioned, since the primary source populations that have been involved in the initial hybridization event are not necessarily those that we have sampled here.

4. DISCUSSION

Hybridization between distinct populations or between closely related species is well known to occur both in animals and plants (Arnold 1997). Usually the evidence stems from non-concordant phylogenies of nuclear and mitochondrial markers (Adams *et al.* 2003; Avise 2000; Rognon & Guyomard 2003). However, it has so far only been shown for plants that rapid adaptations to new habitats and corresponding new colonizations can be caused by hybridization (Rieseberg *et al.* 2003). Still, it is *a priori* likely that the same should also occur in animals. As a possible example, Schlieven & Klee (2004) provide evidence for a hybrid species with very distinct ecology among the sympatrically evolved cichlid species flock in the crater lake Barombi Mbo in Cameroon. Seehausen (2004) has recently presented a model where he considers invasion of new areas and habitats as a trigger for hybridization between lineages and species, which may result in new lineages with a new adaptive potential. This process is thought to contribute greatly to speciation in

Figure 3. Distribution of evolutionary lineages of sculpins around the River Rhine basin as inferred from a combined analysis of mt-haplotypes, nuclear SNPs and literature data. The numbers refer to the sampling sites of fish used in this study. Note that there are further populations of sculpins in the west, which represent different lineages but which are not relevant for this study and are therefore omitted for clarity. Several populations (marked yellow) in tributaries to the middle Rhine or River Main carry introgressed haplotypes of danubian origin but belong to the Rhine sculpin lineage according to nuclear data (see text).

those systems. Our observations on sculpins suggest also that a new lineage has emerged from hybridization of long separated groups and most importantly, that this new lineage indeed has a novel adaptive potential that is absent in its ancestors. This is apparently a very recent and still dynamic process that we observe directly, which makes it very different from cases where the event of hybridization lies back in time. It is also somewhat different from situations where artificial introductions have led to fast adaptations in the respective new environment (Hendry *et al.* 2000; Streelmann *et al.* 2004). In our case, hybridization and new adaptation not only occurred in the same lineage but also appear to have emerged jointly. In the following we evaluate the molecular phylogeographic context, the evidence for specific adaptations and propose a scenario for the course of the hybridization.

(a) Molecular phylogeographic context

The new phylogeographic data presented here including those from mitochondrial haplotypes as well as SNPs are largely in line with previous inferences on the pan-European population structure of sculpins (Englbrecht *et al.* 2000; Volckaert *et al.* 2002). However, there is an interesting deviation. Animals of the Upper River Main as well as some tributaries to the Middle Rhine (Pops. 35, 36, 37) harbour eastern (Danubian; group I) mitochondrial haplotypes, but belong to the Rhine sculpins based on SNP analysis. Such a grouping with populations from the

Lower Rhine drainage was already reported by Hänfling *et al.* (2002). It seems likely that a mitochondrial introgression has occurred in previous times in these populations, which has by now led to the fixation of typical eastern group I haplotypes in a nuclear genome background of the Rhine sculpins. For the context of the present study this is only important with respect to understanding the rare occurrence of the eastern group I haplotypes in the invasive sculpin (table 1), while no corresponding eastern SNP alleles were found in these animals, i.e. the occurrence of eastern mt-haplotypes in the invasive sculpin gene pool does not require a direct involvement of danubian ancestors.

Apart of this slight complication with the group I mitochondrial haplotypes, the pattern revealed in our study is remarkably clear. A mixture of haplotypes and diagnostic SNP alleles is only seen in animals from riverine habitats of the Rhine drainage, while populations representative of the parental lineages persist in small head-water streams throughout the study area. The hybridization that has formed the hybrid invasive gene pool must have occurred very recently, since we have not found private SNP alleles in these hybrids.

The group IV haplotypes in the invasive sculpin gene pool are identical to haplotypes H41 and H42 that otherwise occur in Scheldt sculpins within the River Nete (Volckaert *et al.* 2002; Knapen *et al.* 2003, our study). Intriguingly, exactly this tributary to the River

Table 1. Diagnostic SNP alleles and mt-haplotype groups for different sculpin lineages.

(SNP alleles were classified as ancestral (a) or derived (d), based on outgroup comparisons (see §2). The table provides frequencies for the respective populations, dashes indicate absence of character states. Invasive sculpins (bottom) show a combination of diagnostic alleles from Rhine sculpins (Lower Rhine streams) and western sculpins (including Scheldt sculpins and samples from Great Britain)) (loci SNP1, SNP2, SNP3a and SNP4), but none of the diagnostic alleles of the eastern animals (SNP 3b and SNP5). Note that the mt-haplotype group I sequences of Rhine sculpins are restricted to a few tributaries only (figure 3; see §4).)

population locations	N pop/N indiv typed	phylogeographic lineage	locus Cgo SNP1	locus Cgo SNP2	locus Cgo SNP3a	locus Cgo SNP3b	locus Cgo SNP4	locus Cgo SNP5	haplo type group
41–61	21/72	eastern Danubian sculpin	a: 1.0 d: —	a: 1.0 d: —	a: 1.0 d: —	a: — d: 1.0	a: 1.0 d: —	a: — d: 1.0	I: 1.0 III: — IV: —
1–5; 65; 66	7/45	western Scheldt sculpin	a: 1.0 d: —	a: 1.0 d: —	a: — d: 1.0	a: 1.0 d: —	a: — d: 1.0	a: 1.0 d: —	I: — III: — IV: 1.0
17–21; 24–32; 34–40	21/87	Lower Rhine Rhine sculpin	a: 0.14 d: 0.86	a: 0.07 d: 0.93	a: — d: 1.0	a: 1.0 d: —	a: 1.0 d: —	a: 1.0 d: —	I: 0.29 III: 0.71 IV: —
6; 10; 15	3/76	invasive sculpin	a: 0.67 d: 0.33	a: 0.81 d: 0.19	a: — d: 1.0	a: 1.0 d: —	a: 0.84 d: 0.16	a: 1.0 d: —	I: 0.13 III: 0.53 IV: 0.34

Scheldt is the closest to the Lower Rhine and was one of the first that has become connected to the Rhine system via manmade canals, starting about 200 years ago. In contrast to the group IV haplotypes, the group III haplotypes provide no hint for the origin of the source population of the hybrids since only broadly distributed common haplotypes are found.

The hybrids form a distinct and rather homogeneous lineage in comparison to representatives of the parental lineage according to the microsatellite data (figure 4). Furthermore, populations of the invasive sculpin are largely in Hardy–Weinberg equilibrium (data not shown) and represent a morphologically homogeneous group (figure 2). Thus, the invasive sculpin represents a homogeneous population and not simply a mixed pool of individuals from different origins.

(b) *Morphology and adaptations*

The animals invading the Lower Rhine drainage were morphologically identifiable by their strong prickling. Since we know now that this is characteristic for the hybrid lineage, we can conclude in retrospect, that the Rhine sculpins from streams flowing into major rivers remained confined to their native habitats and did not expand their range, which is in perfect agreement with our field data. Thus, despite their geographic proximity and direct waterway connection, only the invasive sculpin was able to colonize the vacant habitats. This pattern can only be explained by an autecological advantage of invasive sculpin over Rhine sculpins in large river habitats. While the exact nature of this advantage remains unknown, the most inclusive measure of overall fitness, namely thriving populations vs apparent inability to survive in large rivers provides the best general evidence for differential adaptation.

Comparable systematic survey data as for the Sieg and the Mosel do not exist for the main river of the Lower Rhine. Nevertheless, samples from the Mosel (# 16), the confluence of the Mosel and the Rhine at Koblenz (# 15), the Sieg and the Rhine at Bonn (# 8, 9 and 11–13), the confluence of the Düssel and the Rhine at Düsseldorf (# 14) and the IJsselmeer in the Netherlands (# 6, 7) all correspond to the invasive sculpin (figures 3 and 4). Moreover, sculpins from the Dutch lowlands show the mixture of mitochondrial haplotypes (Volckaert *et al.* 2002; this study) characteristic for the invasive sculpin and it is therefore likely that these are representatives of the invasive lineage, although the SNP data to confirm this are not available.

In contrast to the Rhine sculpin, only circumstantial evidence for an ecological differentiation of the Scheldt sculpin and the invasive sculpin is available. First, a population expansion or invasion of the main River Scheldt has not been described so far and past survey data suggest that Scheldt sculpins are usually confined to headwaters of small tributaries (Vandelannoote *et al.* 1998). Knapen *et al.* (2003) have analysed the population genetic structure of Belgian sculpin populations with a partially identical set of microsatellite markers that was used for this study. They found an equally or even more pronounced population substructure among Scheldt sculpins than we observe here for Rhine sculpins. This confirms that the overall population structure of Scheldt sculpins resembles that of Rhine sculpins in that isolated subpopulations are restricted to headwaters of small tributaries with no regular gene flow between separate streams. With respect to invasive sculpins this implies that Scheldt sculpins from small tributaries constitute the ancestral population that has no pronounced ability to colonise riverine habitats.

Figure 4. Neighbour-joining trees of genetic distances (Nei's standard distance) among sculpin populations based on analysis of six microsatellite loci. (a) Tree based on six loci from 23 sampling sites and 14–48 individuals per site. The clustering is congruent with the combined SNP/haplotype inference as depicted in figure 3. Populations of stream sculpins are genetically more subdivided than invasive sculpins. (b) Tree based on 120 loci from five sampling sites and 12–48 individuals per site, representing the ancestral lineages and invasive sculpins. This shows that invasive sculpins are not particularly similar to either one of the presumed ancestral lineages on the genome level.

(c) A hybridization scenario

All major European rivers like the Rhine and Scheldt as well as their large tributaries such as the Mosel have been channelized, dredged and dammed in past centuries to create waterways suitable for large ships. In addition, the waterways were fortified with rocks which act as microhabitats for sculpins (Knaepkens *et al.* 2002). Furthermore, the Rhine Delta and large bays (e.g. the IJsselmeer) were cut off from the open sea eliminating tide and saltwater as ecological key factors in large areas. In this way a novel interconnected system of new habitats has become available through human activities. The invasive sculpin has originated in the lowest reaches of the Rhine drainage and has then spread within the Netherlands and upstream the Rhine. The fact that there are no known records of the invasive sculpin in the Scheldt drainage yet, suggests that there might have been an initial unidirectional migration of Scheldt sculpins via the newly built canals towards the Rhine drainage, where the hybridization began. Because of the major changes to the river systems, one can only speculate of how these lineages would have originally met. An initial phase would require at least short-term success within restricted areas and could have been highly dependent on transient ecological conditions or chance. It is known that larvae of European sculpins can enter the open water (Wanzenböck *et al.* 2000) and can be washed downstream after they hatch (Bless 1990). Such drifting larvae might survive for some time in some parts of those riverine habitats, which were changed by human activities and could thus have contributed to mating with sculpins from other lineages.

This drift effect could contribute to homogenize the hybrid gene pool and would explain why the eastern mitochondrial haplotypes from middle rhine tributaries occur in the hybrid gene pool, even in the IJsselmeer, without a direct contact of the respective source populations. Thus, over time a population of hybrid animals and backcrosses would have become established somewhere in the Lower Rhine area which eventually obtained the capacity to spread into new habitats.

From plant studies it is known that such hybridization is a potential source of new genotypes from which selection could pick favourable heterospecific gene combinations to allow adaptation to new environments (Rieseberg *et al.* 2003). Barton (2001) has recently reviewed the role of hybridization in generating new adaptations. The respective models usually assume that hybrid genotypes are less fit than the parental genotypes, although some of the very large number of possible backcross hybrid genotypes may be fitter than either parent. This effect will be particularly pronounced under conditions where habitat changes occur for the parental lineages, as it would have happened in the Rhine delta in the past centuries. Under such conditions, a hybrid population exploiting a combined pool of traits could adapt to new habitats that are not available to either parental lineage. This process of hybridization, backcrossing and adaptation may have gone on for some time, before a distinct new lineage emerged, which constitutes now the invasive population.

An alternative scenario would posit that the Scheldt animals have started to spread along the Rhine before the

hybrid gene pool was established, but would have picked up alleles from Rhine animals at contact sites in the tributaries. Such contact sites do indeed exist (A. W. Nolte, personal observation), but this scenario is still difficult to reconcile with the data. Given that the spread of the invasive sculpins has occurred within two decades, there would not have been enough time to allow the degree of mixing of the gene pool along the Rhine that we observe. For example, we would not expect in this scenario to find such a close grouping of genotypes from the Middle Rhine and the IJsselmeer (compare figure 4), since the allelic influx would have come from different source populations. Also, this scenario cannot explain why the Scheldt animals should have spread in the first place, given that they have been confined to non-river habitats for a long time. The scenario of an initial formation of a hybrid gene pool with new adaptations, before the spread began, remains therefore more likely. Still, the possibility of further introgression of alleles from the stream populations remains. We have recently completed the draft of a genome map for *Cottus* (Stemshorn *et al.* 2005), which will allow the mapping of regions of the genome that have introgressed.

NOTE ADDED IN PROOF

According to Freyhof *et al.* (2005) the sculpins here referred to as Scheldt sculpins correspond to *Cottus perifretum*, Rhine sculpins to *Cottus rhenanus* and those from more eastern rivers to *Cottus gobio*. Invasive sculpins are formally retained in *Cottus perifretum*, which does not affect the conclusions presented here.

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