Population genetic aspects of a newly established parasite-host system between the nematode invader *Anguillicola crassus*, and the North Atlantic freshwater eels, *Anguilla sp.*

Dissertation zur Erlangung des akademischen Grades

des Doktors der Naturwissenschaften (Dr. rer. nat.)

an der

Universität Konstanz, Mathematisch-naturwissenschaftliche Sektion

Fachbereich Biologie

vorgelegt von

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Tag der mündlichen Prüfung: 02. Juni 2009

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DANKSAGUNG

Für die finanzielle und logistische Unterstützung meiner Promotionsarbeit danke ich Prof. Axel Meyer sehr herzlich. Die internationale Ausrichtung seines Lehrstuhls hat viele Gäste, Doktoranden und Postdoktoranden aus aller Welt an den Bodensee geführt, die mein Interesse an der Evolutionsbiologie nachhaltig entfacht und mitgeprägt haben.

Ich danke insbesondere Thierry Wirth für seine großartige Betreuung des populationsgenetischen Aspekts meiner Arbeit, und die große Geduld mit meiner Verkomplizierung ohnehin schon komplizierter Tatsachen ;-). Die *Anguillicola*-Studie war Teil seines DFG-Förderprojekts und ermöglichte mir die Teil-Finanzierung meiner Promotion.

Mein Dank gilt überdies der Landesgraduiertenförderung Baden-Württemberg und der Haushaltsmittelabteilung der Universität Konstanz für die Weiter- und Abschlussfinanzierung meiner Arbeit.

Vielen Dank allen Kooperatoren mit deren Hilfe ich über den Tellerrand hinaus viele neue Einblicke gewinnen und Erfahrungen sammeln durfte: vor allem Horst Taraschewski, unter anderem für seine Hilfe bei der Beschaffung wichtiger Proben aus Asien, und allen Mitarbeitern von FISHPASS in Rennes (FR).

Mein Dank gilt auch allen Studierenden und Bachelor-Anwärtern für ihre tatkräftige Unterstützung bei der Feld-Arbeit und im Labor: Florian Hollandt (BSc), Eva Dillenius, Oliver Podlech, Kathrin Jeltsch und Sonja Erath, sowie all den fleißigen TAs im MeyerLab für ihre Mithilfe, insbesondere Elke Hespeler, Ursula Topel und Christina Chang-Rudolph.

Im MeyerLab habe ich stets neue weltoffene und hilfsbereite Menschen getroffen und liebgewonnen. Ich danke speziell: Thierry Wirth, Shigehiro Kuraku und seiner Familie, Kathryn Elmer, Nils Offen, Matthias Sanetra, Kai Stölting und all meinen Zimmerkameraden: Jody Shields, Frederico Henning, Karl Radtke, Chiara Reggio, Ji Hyoun Kang und Shaohua Fan. Ihr wart echt klasse!

Mein innigster Dank gilt meiner Mutter Hildegard und meinen Geschwistern Susanne und Tobias (Familie inklusive!), die uns in den letzten Jahren sehr vermisst und großartig unterstützt haben. Ein spezielles Dankeschön geht an den Temmenhausern Hans, Heike und Thomas Neuburger, die uns ein zweites Zuahuse gaben, um abzuschalten und unsere Kräfte neu zusammeln. Danke auch allen meinen Freunden und Bekannten, insbesondere Peter Kinzel und Matthias Brachtel.

Unsere Zeit hier in Konstanz neigt sich dem Ende entgegen, aber der Bodensee hat es sehr gut mit uns gemeint. Ich möchte meiner Frau Anna für eine fabelhafte Zeit, ihre innige Liebe, und zwei gesunde, prächtige Burschen danken, Franz und Jonathan, die uns alles Glück auf Erden sind.

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GENERAL INTRODUCTION

The current PhD thesis includes several studies comprising two partners in a newly established parasite-host system under strong anthropogenic influence, namely the freshwater eels of the genus *Anguilla*, and the nematode *Anguillicola crassus*. In the following section, I briefly introduce the general interest in this system.

0.1 The freshwater eels (*Anguilla sp.*)

0.1.1 Life cycle and developmental stages

It was only in the beginning of the twentieth century that a Danish marine biologist, Johannes Schmidt, discovered the North Atlantic freshwater eel's spawning areas in the vast Sargasso Sea (Schmidt 1923). All 15 species and three subspecies of the genus *Anguilla* (Ege *et al.* 1939; Minegishi *et al.* 2005) display a remarkable catadromous "migration loop", that comprises two long-range migrations in the open ocean, a continental growing phase, and a marine pelagic spawning stage (Tsukamoto *et al.* 2002; **Fig. 0.1**). Based on catchment records in several species of eels (Schmidt 1923; Schoth & Tesch 1982; Kleckner & McCleave 1980, 1988; Aoyama *et al.* 2003; Tsukamoto 2006), spawning grounds are generally localized in tropical areas of the seas, and are subject to warm, highly saline subtropical water currents, which are both ideal for eel spawning activity (> 20 °C) and to keep eggs buoyant to reach the surface (Tsukamoto *et al.* 2002). After hatching larvae are dispersed toward the continents by passive oceanic currents. Both, spawning ground circumference and location vary between species. For example, spawning Japanese eels only occupy a narrow area close to the Mariana's Trench in the Pacific Ocean (Tsukamoto 2006), while both North Atlantic eel species apparently spawn over a large area in the Sargasso Sea with spawning grounds broadly overlapping (Schmidt 1923; Tesch & Schoth 1982; Kleckner & McCleave 1988). Anguillid eels undergo two metamorphoses during their life-time, which are accompanied by distinct changes in body-shape, coloration and physiology (Tesch 2003; **Fig. 0.1**). Smallest larvae are caught near putative spawning sites, less then 5 days after hatching (pre-leptocephali; ≤ 7 mm). The fully grown eel larva is called leptocephalus, appears completely transparent and develops a willow leaf-shaped body form (Larval stage I). Given their planktonic, migratory life-style, its body-shape clearly favours rapid drifting along oceanic currents, and the European eel may reach continental water systems within two years by passive transport alone (Kettle & Haines 2006). Data derived from otoliths suggests an even shorter migration time (Lecomte-Finiger 1994; Arai *et al.* 2000), and thus implies active swimming performance. The first metamorphosis takes place when the leptocephali reach the continental shelves and transform into the anguilliform glass eel stage (VA). Though eels may stay in the open ocean for their whole life (Tsukamoto &

Arai 2001), most glass eels gather in the tidal zone of estuaries and prepare their ascent into freshwater by alternating positive and negative rheotaxis with the turn of tides. This behaviour characterizes the glass eels' rhythmic response to constantly changing water currents (Bolliet *et al.* 2007).

Ascending small eels (elvers) start a predominantly benthic life-style in freshwater and are referred to as yellow eels when reaching lengths around 20 cm, owing to their mainly dorso-lateral yellow-green or olive coloration (Tesch 2003). After several years of continental residency, the second metamorphosis marks the onset of sexual maturation. Thereby eels'

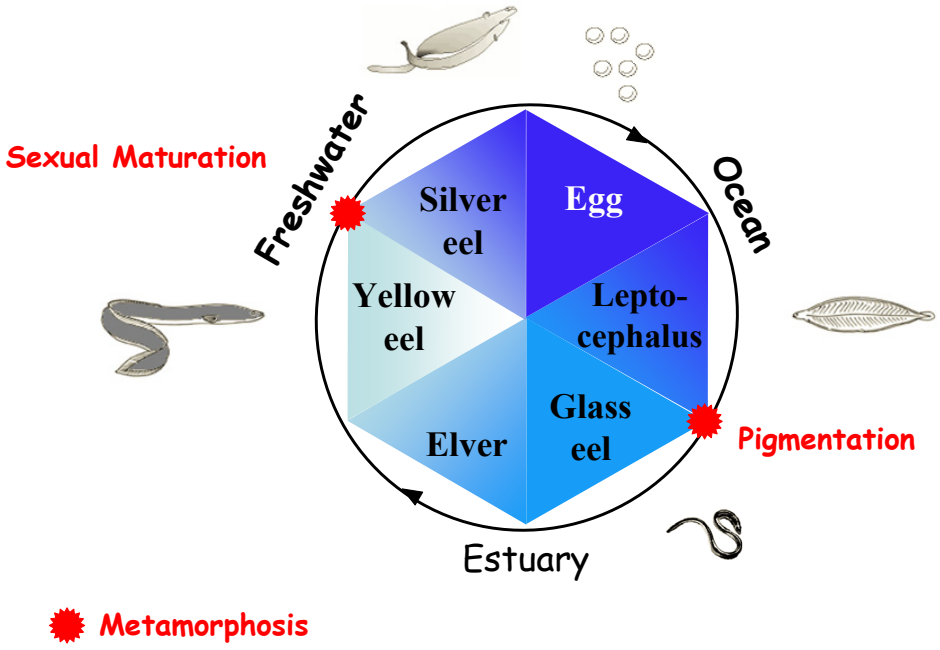

Figure 0.1 Schematic representation of the life cycle of catadromous freshwater eels of the genus *Anguilla*. Catadromy is characterized by a marine phase of reproduction (spawning) and a (facultative) growing phase in freshwater.

gonads become fully developed, their eyes' diameters and visual capabilities increase, and dorso-lateral blackening starts contrasting ventro-lateral silvering or bronze-staining (silver eel stage; Tesch 2003). Before entering sea water to complete their spawning migration in the open ocean, silver eels are capable of re-adapting to high salinities with help of their well developed epidermal mucosa. There is evidence that maturing migrants can hold a compass course during escapement of estuaries toward the open sea (Tesch 2003), however no single eel spawner has been caught in the oceans to this date, and the exact migration routes and the reproduction *in situ* remains a black box. Consequently, the field relies heavily on indirect observation.

0.1.2 Phylogenetics

While eel species are broadly dispersed and present on coastlines of the North Atlantic, the Indian Ocean, and the Eastern Pacific including Oceania, they are absent from the Arctic White Sea, the South Atlantic coastlines, as well as from the entire Pacific coast of the Americas (**Fig. 0.2**). Numerous molecular phylogenetic studies were conducted in the genus *Anguilla* (Aoyama *et al.* 1996, 2001; Aoyama & Tsukamoto 1997; Bastrop *et al.* 2000; Lehmann *et al.* 2000; Lin *et al.* 2001; Minegishi *et al.* 2005; Tagliavini *et al.* 1996). Based on

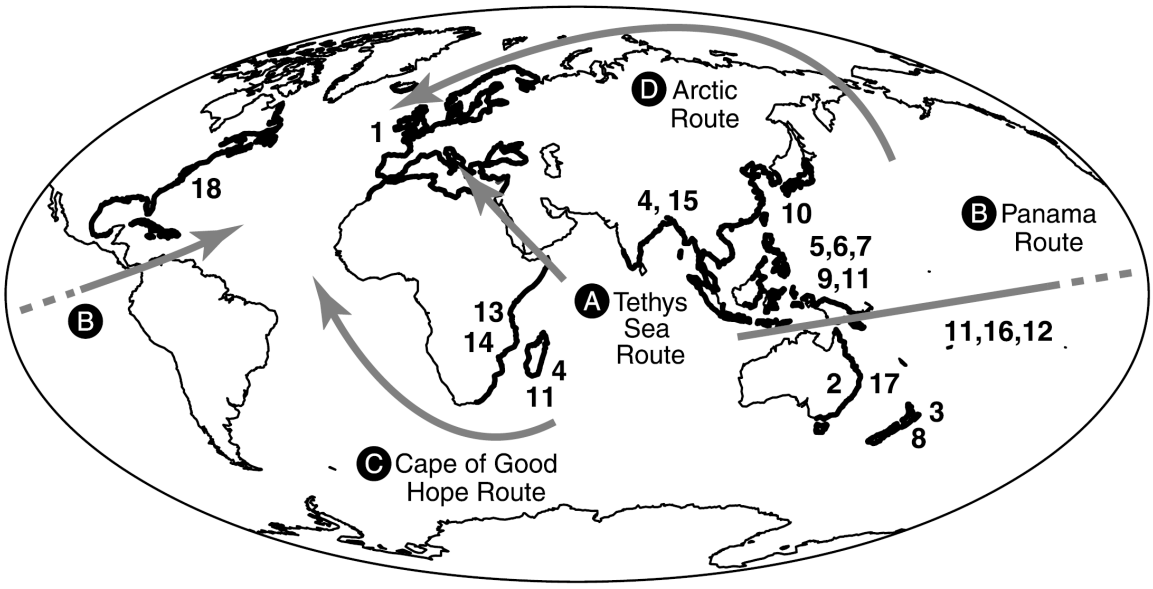

Figure 0.2 Schematic representation of geographic distribution of eel species word-wide. Grey arrows indicate proposed routes of dispersal of ancestors of the North Atlantic freshwater eels, which remain obscure to date. Numbers indicate each of the following species of eel, (1) *A. anguilla,* (2) *A. australis australis*, (3) *A. australis schmidtii*, (4) *A. bicolor bicolor*, (5) *A. bicolor pacifica*, (6) *A. borneensis*, (7) *A. celebesensis*, (8) *A. dieffenbachii*, (9) *A. interioris*, (10) *A. japonica*, (11) *A. marmorata*, (12) *A. megastoma*, (13) *A. mossambica*, (14) *A. nebulosa labiata*; (15) *A. nebulosa nebulosa*, (16) *A. obscura*, (17) *A. reinhardtii*, (18) *A. rostrata* (adapted from Minegishi *et al.* 2005).

these, the consensus message is that extant eel species have most likely originated from an Indo-Pacific ancestor 20-60 mya, with ongoing controversy over the most basal species, and the exact dispersal route of the Atlantic eels' ancestors. A range of different scenarios were suggested (**Fig. 0.2**), including a westwards route through the ancient Tethys Sea, separating Gondwana from Laurasia until 30 mya (Aoyama & Tsukamoto 1997), or following the opposite direction, through the Panama Isthums, which closed only some 2-3 mya (Lin *et al.* 2001). Interestingly, even the best supported phylogeny to date, including complete taxon sampling and data derived from the whole mitochondrial genome sequence (WGS), did not allow Minegishi *et al.* (2005) to infer a clear dispersal scenario. However, the authors find support for the monophyly of North Atlantic eels, which form a clade with the Oceanian

species *A. dieffenbachii* and *A. australis*, and the Borneo eel, *A. borneensis*, respectively. They also support the monophyly of the remaining eleven species, excluding the basal *A. mossambica*. To date, no phylogeny relying on nuclear markers is available.

0.1.3 Population genetic structure

The degree and pattern of population genetic structure varies among eel species and studies. Though there are temporally stable latitudinal differences in three allozyme markers (Koehn & Williams 1978), the American eel, *Anguilla rostrata*, is unstructured according to neutral markers (Wirth & Bernatchez 2003), which is evidence for selection at different life stages. On the other hand, the most wide-spread anguillid species, the giant mottled eel, *A. marmorata*, shows clear-cut population structure (Minegishi *et al.* 2008), probably due to the occupation of different spawning grounds in the Indian and Pacific Oceans (Ishikawa *et al.* 2004). Two independent studies reported isolation-by-distance (IBD) in *A. anguilla*, a result that speaks against the long-held paradigm of panmixia in this species (Wirth & Bernatchez 2001; Maes & Volckaert 2002). IBD characterizes a positive correlation of genetic and geographic distance, due to a smooth decline of geneflow over the whole distribution area of a species. Wirth & Bernatchez (2001) proposed several scenarios of larval homing to explain this pattern. First, a stable temporal delay of spawning migration in eels from northern habitats might cause the IBD, as distances are markedly extended compared to western European and southern habitats. Alternatively, more than one reproductive area is used and different currents carry the leptocephali back to their parent's original freshwater habitat. Thirdly, but more unlikely, assortative mating among regional groups might be held responsible. Importantly however, the IBD signal is temporally unstable in the European eels (Dannewitz *et al.* 2005), and Maes *et al.* (2006a) showed that isolation-by-time (IBT) in *A. anguilla* is more pronounced than IBD for certain years. In case of IBT, genetic differentiation is positively correlated with increasing temporal separation of samples. Maes *et al.* (2006a) interpret this as a consequence of the large variance in eel's spawning success and larval survival rates in the open ocean due to random oceanic processes, resembling the "sweepstake reproductive success" (Hedgecock 1994). On the other hand, the Japanese eel neither follows a pattern of IBD nor IBT (Ishikawa *et al.* 2001a; Tseng *et al.* 2003; Tseng *et al.* 2006; Chang *et al.* 2007), but rather shows a metapopulation structure, as localities cluster together according to latitude using UPGMA. The authors concluded that the member-vagrant-model of larval dispersal might apply to the recruitment of Japanese eels. This model states that despite panmixia physical oceanographic features restrict dispersal of eggs and early stage larva, consequently leading to population structure (Sinclair 1988).

0.1.4 Conservation status

Despite recent success in raising Japanese eels artificially to the glass eel stage (Tanaka *et al.* 2003), eels cannot be stably bred in captivity. Consequently, the global eel market relies on the international trade of livestock (Nielsen & Prouzet 2008). Of the total harvest of eels, 89-94% of the annual yield stems from aquaculture facilities, and 90% of the output derives from Asia (69% from China alone). The global trade of living eels is raising many problems concerning stock management and eel conservation measures (Feunteun 2002; Ringuet *et al.* 2002; Dekker 2003). Based on increasing commercial demand especially in China, wild stocks of traded American, European and Japanese freshwater eels, are subject to high fishing pressure at all life stages. Consequently, with the onset of systematic capture-based culturing techniques, glass eel recruitment declined by 80-99% in less than 50 years (Castonguay *et al.* 1994a,b; Haro *et al.* 2000; Dekker 2003; **Fig. 0.3**), and the European eel stock is now considered outside of safe biological limits (Stone 2003). All in all, the current decline in recruitment of European eels is probably driven by multiple factors, which also include barriers to up-stream migration, pollution, exotic parasitism and changes in oceanic currents (Behrmann-Godel *et al.* 2003; Castonguay *et al.* 1994a,b; Knights 2003; Kirk 2003; Wirth & Bernatchez 2003). Beside the direct consequences of over-exploitation and habitat destruction on standing fish stock size, the transfer of nonindigenous eel species might have increased the opportunities for introgression among the generally closely related anguillid eel species (Minegishi *et al.* 2005). The bones of contetion are the numerous European eels sampled in Japanese freshwaters and marine bays (Okamura *et al.* 2008), as well as the proven ability of hormone treated eels to interbreed (Lokman & Young 2000; Okamura *et al.* 2004). The direct consequences of interbreeding among domesticated and native species might be related to the break-up of co-adapted traits, thereby interfering with several especially adapted life history traits, which are considered most important for the successful closure of the eel's life-cycle (van Ginneken & Maes 2005).

0.2 The invasive eel parasite, *Anguillicola crassus*

0.2.1 Invasion history & Life cycle

In the light of many harmful environmental effects, diseases and parasites may seem to be of lower importance in eel conservation. However, recent experiments suggest that the invasive swimbladder nematode *Anguillicola crassus* is seriously affecting the fitness and viability of

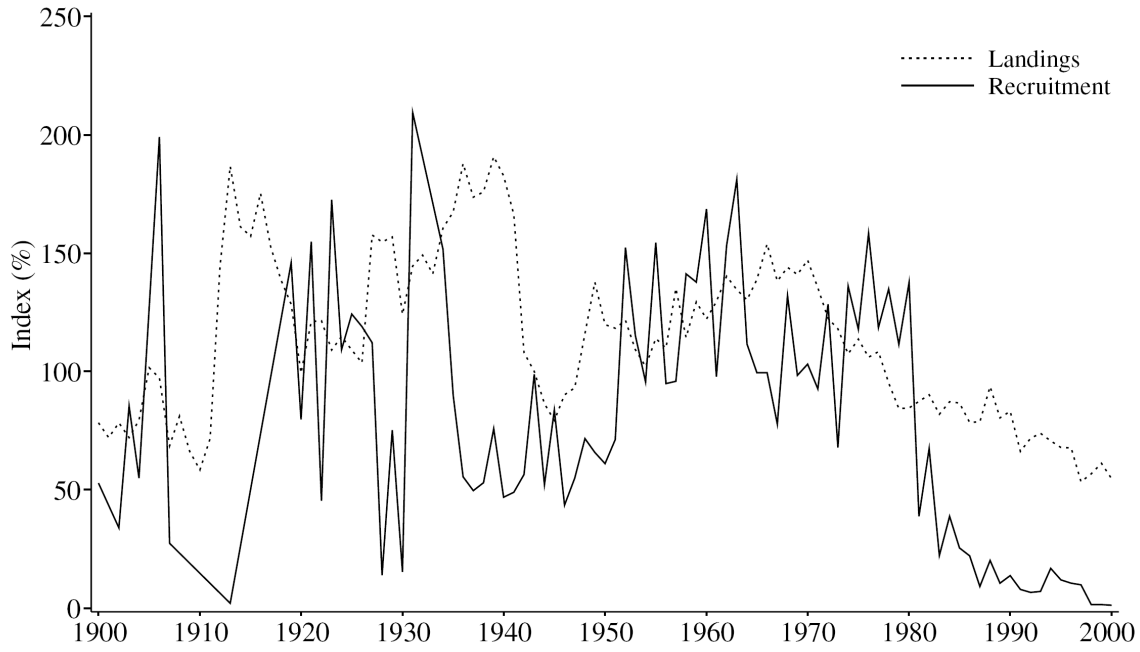

Figure 0.3 Time series of indexed total European eel landings (continental stocks) and glass eel recruitment. Recent drops in European eel recruits are significantly correlated with a preceeding reduction in continental eels, which coincides with the introduction of systematic eel farming world-wide (adapted from Dekker 2003).

parasitized eels (Gollock *et al.* 2005; Lefebvre *et al.* 2007). Of even greater concern are the results gathered from exhaustion swim tunnel experiments demonstrating that sexually mature silver eels reached lower cruising speeds and had higher costs of transport compared to healthy individuals, when swimbladders were either highly parasitized or otherwise damaged after infection (Palstra *et al.* 2007). If true, these fishes may fail to complete their reproductive migration back to their birthplace in the Sargasso Sea. Clearly, under such a peculiar and extreme stress situation paired with elevated susceptibility to infection, the impact of a blood parasite can be lethal in migrating, non-feeding silver eel stages (Lefebvre *et al.* 2007). Notwithstanding earlier evidence of parasitological threats promoted by global live eel trade (Egusa *et al.* 1979), the first infected European eels were detected in northern Germany soon thereafter (Neumann *et al.* 1985). Koops & Hartmann (1989) identified a transfer of 35 tonnes of live Taiwanese eels in 1980 as the most likely origin of the spread of *A. crassus* in Europe. Today, driven by eel trade, the prevalence rates of parasitized eels reaches more than 70% in most European countries (Kirk 2003), and parasites are only absent in cold regions with an average freshwater temperature below 4 °C (Kirk 2003), such as in Iceland (Kristmundsson & Helgason 2007). The parasite has also colonized populations of the American eel, *A. rostrata*, in North America (Johnson *et al.* 1995) and native and introduced eels on the Island of Reunion (Sasal *et al.* 2008). It is thus considered as a global invader (*sensu* Colautti & MacIsaac 2004; Taraschewski 2006). The

ease of sampling, the rapid determination of the infection status and the economic impact of this nematode has favoured extensive parasitological and epidemiological knowledge, and today, *A. crassus* is by far the best studied eel parasite (Kirk 2003; Taraschewski 2006). The specific steps constituting the nematode's life cycle are illustrated in **Fig. 0.4**:

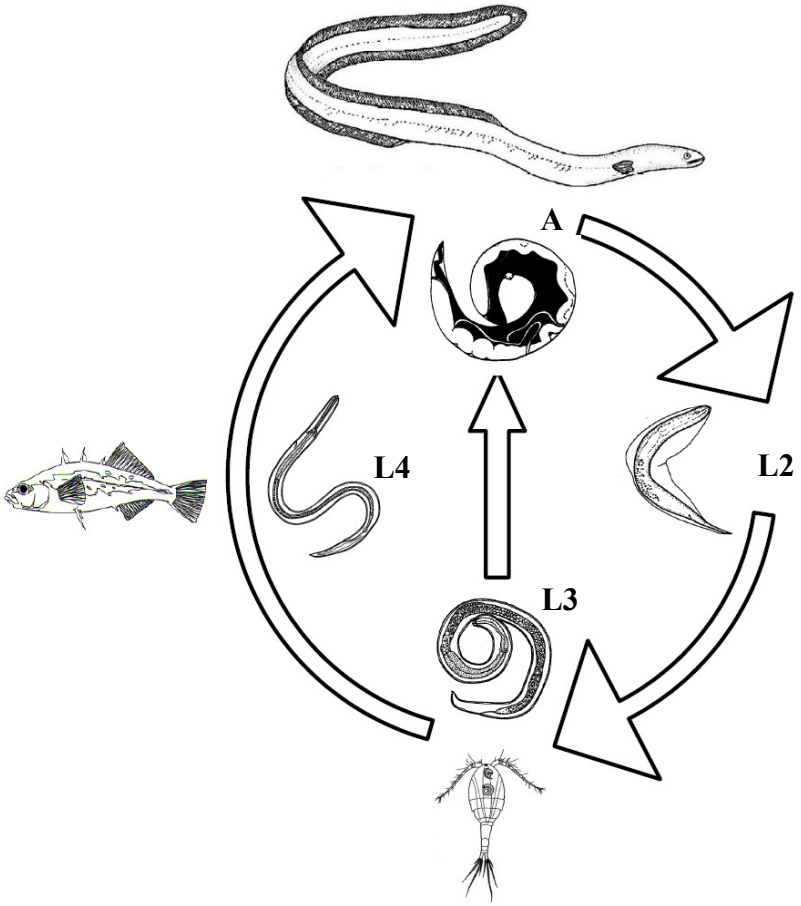

Figure 0.4 Schematic life cycle of the invasive nematode parasite *Anguillicola crassus*. The nematode reproduces sexually in the swimbladder lumen of its novel fish host, *Anguilla anguilla*, in Europe. See text for a description (adapted from Hollandt 2007).

In general, all nematodes are moulting four times during their life time (larval stages L1-L4), after which they become adults (stage A). The first moulting stage is already performed within the egg sheath in the eel host's swimbladder lumen. L2 larvae are extruded to the aqueous environment after escaping the host's swimbladder over the *ductus pneumaticus*, a connection of the swimbladder with the intestinal tract. In the open water, L2 larvae attach to substratum and attract zooplanktonic predators by undulating body movements. Upon ingestion, they subsequently infest in obligate copepod intermediate hosts (Thomas 1993), and moult to an L3 larva. These are infective for many freshwater fishes, in which they can moult to an L4 larva. However, the life-cycle is only completed in the final host. Thus, other fish hosts are paratenic hosts, and this part of the life cycle is facultative.

When inside the eel gut, larvae penetrate the mucosa and enter the swimbladder wall's connective tissue, in which they moult until they are pre-adult. Finally, individuals enter the organ's lumen after several weeks, and marked sexual dimorphism becomes apparent among males and females. After copulation, females constantly release eggs and internally hatched L2 larvae into the swimbladder lumen. Adults do not exit their hosts and decay inside the swimbladder lumen, causing fibrosis or inflammation of the organ.

0.2.2 Population structure in macroparasites

A key feature of macroparasite populations is that breeders are subdivided into within-host populations (infrapopulations; *sensu* Bush *et al.* 1997). These constitute a transient small subset of mating partners each generation (Criscione & Blouin 2005). For concealed organisms, such as parasites, the analysis of genetic variance considering several hierarchical levels remains, in most cases, the only way to investigate natural population parameters (Nadler 1995; Vilas *et al.* 2003). The first predictive concepts about the micro-evolutionary patterns affecting parasite populations were formulated relatively late by Price (1977, 1980). Thus, the field clearly lags behind those of free-living organisms, for which more predictive models are established (Criscione *et al.* 2005). The advent of molecular genetic tools has already strongly affected ecological parasitology, which is notoriously hampered by internal parasites' morphological simplicity, evolutionary parallelism and convergence (Nadler 1995; Criscione *et al.* 2005). Insights from empirical data suggest that early predictions for micro-evolutionary patterns, based mainly on plant parasitic arthropods (Price 1977, 1980), did not ubiquitously hold for macroscopic parasites (Nadler 1995; Criscione *et al.* 2005). Price (1977) predicted that host populations represent isolated parasite subpopulations (or demes), due to their strict dependence on "coarsely" distributed hosts in a given ecosystem. Thus, reduced host density would lead to increased levels of inbreeding and genetic drift, reducing local genetic diversity but increasing population genetic structure among hosts. However, contrary to many phytoparasites, parasites in animals usually show genetic diversities exceeding those of their free-living hosts because of accelerated host-borne dispersal (Blouin *et al.* 1995; Criscione & Blouin 2004). When host mobility is coupled with indirect life cycles, local populations mix rapidly by random back-recruitment into longer-lived individual hosts. Hence, population structure breaks down at the level of infrapopulations due to random effects (Nadler 1995). Thus, it remains controversial if infrapopulations (within a single host) or component populations (among all hosts in a habitat) are, in general, the relevant units of parasite evolution (Crisione *et al.* 2005).

THESIS OUTLINE

Part A: Isolation and characterization of microsatellite markers.

Because molecular markers are an important tool to answer specific questions on organismal biology and evolution (Avise 2004), it is imperative to get accustomed with the various markers and their strenghts and weaknesses before a study can be successful. With respect to the study system, I was interested in the shallow population structure of North Atlantic eels and the unresolved phylogeography of their recently introduced nematode parasite, *Anguillicola crassus*. To reach our goal, the use of highly powerful markers such as microsatellites was mandatory (Wirth & Bernatchez 2001, 2003).

Microsatellite loci are simple sequence repeats (SSR) of iterated 1-6nt motifs, thus also called short tandem repeats (STR), which are reminiscent of the much larger centromeric "satellite DNA" tandems. Most microsatellites apparently have no direct role in recombination or other genomic processes, and thus are assumed to evolve neutrally (Ellegren 2004; however see Kashi & Soller 1999). Both pure SSR and cryptically simple regions are found all over the genomes of eukaryotes at much higher rates than expected by chance. Combined with their extensive size polymorphism, they provide a significant amount of neutral genetic variance (Tautz *et al.* 1986). The rapid mutation process is caused by replication slippage of the DNA polymerase and a failure to repair mismatches, whenever a transiently disassociated DNA-strand misaligns before replication commences (Levinson & Gutman 1987; Schlötterer & Tautz 1992), adding or subtracting single repeat units one at a time. This process can be directly observed using pedigrees (Ellegren 2004). Therefore, deviations from neutral evolution possibly indicate tight linkage with loci under selection (Harr *et al.* 2002; Schlötterer 2003). However, since the bulk of empirical work has shown that a single stepwise mutation model (SMM; Ohta & Kimura 2007) is not the best fit for genotype data, it follows that the actual mutation processes must be more heterogeneous among loci, repeat types and organisms (Ellegren 2004). Microsatellite mutation rates range from 10^{-1} to 10^{-6} per generation, and are higher for longer repeats. Thus, rates are slower in organisms with shorter microsatellites, e.g., in *Drosophila* fruit flies (Ellegren 2004).

From a practitioner's point of view, microsatellites bear several advantages over previously used markers, such as RFLPs and RAPDs. Results are highly reproducible, and scoring is simple and unambiguous. Moreover, only small amounts of DNA are needed for PCR amplification, and these markers are co-dominant. These advantages promoted microsatellites' wide utilization in genome mapping, forensic DNA studies, paternity testing, population genetics and conservation/management of biological resources (Jarne & Lagoda 1996; Goldstein & Schlötterer 1999). The great popularity in the field of wildlife ecology and conservation is reflected in the extensive publication list of microsatellite-flanking primers in the journal "Molecular Ecology Resources" (formerly "Molecular Ecology Notes"); and the

wide range of isolation techniques that help extracting such loci from uncharacterized genomes within two weeks, excluding sequencing (Zane *et al.* 2002). However, it is necessary in most cases to extract them *de novo* for each new species (Zane *et al.* 2002).

As a prerequisite for inferring invasion history, phylogeography, and population genetic structure at the local and macrogeographic level, I describe the isolation and characterization of seven newly derived microsatellites for the nematode invader, *Anguillicola crassus* in **Chapter 1**. Novel microsatellite markers were also isolated for the European freshwater eel (**Chapter 2**) to assist future research in population genetics. The application of those markers in other anguillid species of eels demonstrates the high degree of conservation in flanking primer binding sites (see also Maes *et al.* 2006b).

Part B: Population structure and phylogeography in an invasive eel parasite.

Almost nothing is known about the population genetics and phylogeography of the parasitic invader of the North Atlantic eel stocks, *Anguillicola crassus*. The term phylogeography literally combines phylogeny with biogeography, and utilizes information from gene genealogies to explain contemporary and/ or historical spatial distribution of intra- and interspecific genetic variation (Avise 2000). The history of the field is closely entangled with the interest in mitochondrial DNA, on which basis the distinction of gene and species trees was originally based (see Avise *et al.* 1987 for a review), however, the advent of microsatellites was an important step in the field of phylogeography and molecular ecology (Goldstein & Schlötterer 1999), and the integration of allelic frequency data from multiple nuclear neutral genes offers additional lineage information and superior resolution power (Beaumont & Bruford 1999; Beaumont & Rannala 2004).

Recent advances in the isolation of highly polymorphic, co-dominant microsatellite markers facilitated the study of wild non-model organisms (Zane *et al.* 2002), and the inference of their population structure, migration rates and demographic history (Pritchard *et al.* 2000; Beerli & Felsenstein 2001; Nielsen & Wakeley 2001). Classically, the members of a population share a common geographic area and are often collected in a single temporal cohort. Such clusters are then evaluated by assigning pairwise genetic distance or maximum likelihood measures, and the identification of joint groups critically depends on both the *a priori* defined population and the choice of the graphical representation (a tree or scatter plot). However, as Pritchard *et al.* (2000) pointed out, genetic data require a genetic definition of a population. Therefore, these authors proposed a programme called STRUCTURE to study cryptic (unknown) population structure on the sole basis of mathematical formula and a Bayesian framework. It has been updated frequently since by Falush *et al.* (2003, 2007). This tool tackles disequilibrium of either linkage or Hardy-Weinberg assumptions by introducing population structure to ultimately find groupings of individuals that are in equilibrium given

their multi-locus microsatellite data. Additional information on geography or time can then be added to supplemement (or guide) the inference of the actual population membership in a second step (i.e., specifying informed priors).

Utilizing this Bayesian clustering approach, in **Chapter 3**, the genetic information in seven species-specific microsatellite markers (Wielgoss *et al.* 2007) was evaluated, and complemented by sequence data from a fragment of the mitochondrial cytochrome c oxidase subunit I (COI). The aims of this study were to (i) explore the population structure of *A. crassus* in its entire range, (ii) compare allelic and genotypic composition among Asian native and nonindigenous, invasive populations, and (iii) determine if biogeographic discontinuities appear between western Europe, northern Europe and the Mediterranean area. Understanding the epidemiology and the phylogeography of this invasive nematode will help to reconstruct the recent spread of this alien species and its secondary genetic differentiation. Moreover, the assessment of gene flow and genetic diversity, as well as number and origin of invasion events, is of prime importance for understanding this parasite species' invasion success. Anti-parasite measures, such as applying anthelminth drugs, rely on clear knowledge of population genetic and demographic patterns. Decades after its introduction to the North Atlantic, the nematode recently spread to the remote Island of Reunion in the Indian Ocean along with other anguillid parasites. Given Reunion's colonial history, and the knowledge about the European invasion history (Wielgoss *et al.* 2008a) a parasitological survey was conducted in three common eel species, to test the hypothesis that intensive trade with European countries affects the island's wildlife directly via the introduction of nonindigenous parasite species (**Chapter 4**). For *A. crassus*, both mitochondrial DNA and microsatellite genotype data from the previous chapter (Wielgoss *et al.* 2008a) are used as a baseline to assign sampled parasites from Reunion to their most likely population of origin in Europe and Asia.

The European Commission has recently proposed a Council Regulation to establish management actions for protection and restoration of the eel stocks (COM 2005, 472). Since freshwater eels are unevenly distributed over Europe and parts of North Africa (Dekker 2000), the cornerstone of this initiative is the development of regional and local restoration programs. One programme assessed different regional and global models aiding eel stock management (SLIME 2006), and its main conclusion highlighted that measures for securing the productiveness and stability of European eel stocks are undermined by the poorly understood population dynamics of eels. Thus, the short term impact of local stocking in freshwater habitats needs to be understood in order to devise fruitful management action plans. Yet, various localities differ greatly in the level of human interference (eel stocking).

In **Chapter 5**, using a population genetics approach, the usefulness of the omnipresent invasive eel parasite, *Anguillicola crassus*, as a biological tag for monitoring eel

stock management and eel migration behavior was assessed. Relying on microsatellite markers and using the genotype data from previously described locations across Europe (Wielgoss *et al.* 2008a), I contrast the infrapopulation samples of two European rivers differing in their eel recruitment management. First, special attention is paid to parasite clusters among host populations, by assessing Hardy Weinberg expectations (HWE) and population differentiation. Second, the roles of admixture and the presence of first generation migrants are weighed against increased levels of relatedness, which both can lead to deviations from HWE. The mixture of related and unrelated individuals in one sampling locality is discussed in the light of larval sib-cluster transmissions and differences in local intermediate host fauna.

Part C: Natural and anthropogenically driven hybridization in freshwater eels and its consequences on eel stock management.

Freshwater eels are often referred to as an excellent example of random mating in biology textbooks. However, two recent independent studies reported isolation-by-distance (IBD) in *A. anguilla*, which is clear evidence against the long-held paradigm of panmixia (Wirth & Bernatchez 2001; Maes & Volckaert 2002). Importantly however, although genetic differentiation remains significant over the whole distribution area and among years, the IBD signal is temporally unstable in the European eels (Dannewitz *et al.* 2005). IBD patterns might be caused by either stable temporal delay of spawning migration in geographically distinct stocks of European eels, the presence of separate spawning grounds, or assortative mating among spawning cohorts (Wirth & Bernatchez 2001). In contrast, Maes *et al.* (2006a) suggested that the random factors affecting spawning success in the open ocean might explain genetically patchy recruitment (Pujolar *et al.* 2006), and thus cause a strong isolation-by-time (IBT) signal among generations. On the other hand, IBD might be produced by inter-species hybridization. Since eels are hard to differentiate based on morphology, above all, the North Atlantic eels have been subject to debate considering their species status and potential for interbreeding. Though the utility of mitochondrial DNA provided strong evidence for the two-species-status (deLigny & Pantelouris 1973; Avise *et al.* 1986; Minegishi *et al.* 2005), as originally proposed by Schmidt (1925), the finding of a hybrid “population” in Iceland calls the two-species-model into question (Williams *et al.* 1984; Avise *et al.* 1990; Albert *et al.* 2006).

To date no study had explicitly tested the influence of hybridization among North Atlantic eels on the extent of genetic differentiation. In **Chapter 6** of this thesis, the occurrence of genotypic clines over the whole North Atlantic distribution area of the eel is investigated by combining and extending available microsatellite and mitochondrial genetic data (Wirth & Bernatchez 2001, 2003). This included a simulation approach to test explicitly if the original IBD in European eels (Wirth & Bernatchez 2001) could be explained by

admixture clines. Finally, the amount of gene flow that would be necessary to generate such a correlation between geographic and genetic data was quantified.

The steep increase of anthropogenically-driven translocations of nonindigenous species (NIS) depicts a great challenge for conservation biologists (Allendorf *et al.* 2001; Taraschewski 2006). Of significant importance are the surging imports of domesticated fish species, because natural hybridization among fishes is much more common than in other vertebrates (Campton 1987; Smith 1992). Salmonids are very well studied in this regard (e.g., Poteaux *et al.* 1998; Ruzzante *et al.* 2001; Hansen 2002; Roberge *et al.* 2008). In Iceland, there is evidence for naturally occurring hybridization, between European and American eels (Albert *et al.* 2006; Avise *et al.* 1990). Moreover, the presence of sexually mature European and American silver eels along the coasts and in estuaries of Japan and Taiwan (Han *et al.* 2002), raises concern about the potential for genetic pollution in native Eastern eel species. Moreover, earlier systematic introductions of exotic Australian, New Zealand and Japanese eels into Europe and North America might have also left its genetic trace, as successful production of artificial hybrids between *A. anguilla* and *A. japonica* (Okamura *et al.* 2004), and *A. australis* and *A. dieffenbachii* (Lokman & Young 2000), have been documented. Recently, two studies demonstrated a high conservation level of microsatellite binding sites in anguillid eels (Maes *et al.* 2006b; Wielgoss *et al.* 2008b), and assignment success using four species of eels reached levels > 90% if prior information on geographic sampling was used (Maes *et al.* 2006b). However, though the latter authors found evidence for admixture among geographically isolated eel species, they did not specifically screen for hybrid individuals in their dataset. Moreover, Maes *et al.* (2006b) chose loci according to maximal genetic differentiation, thereby possibly skewing the analysis toward high efficiencies in the pure category. Therefore, the levels of admixture among native and introduced eels remained unresolved. In **Chapter 7**, including a total of 704 eel specimens from five different species of commercially traded eels and using eight microsatellite markers, I assessed whether anthropogenic eel translocations may have promoted introgressive hybridization. Moreover, utilizing Bayesian clustering techniques, the influence of simulated inter-species hybridization on assignment efficiency and accuracy are evaluated for real and simulated datasets.

Part A: Isolation and characterization of microsatellite markers.

Chapter 1:

PRIMER NOTE

Isolation and characterization of short tandem repeats in an invasive swimbladder nematode, parasitic in Atlantic freshwater eels, *Anguillicola crassus*

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Published in

Molecular Ecology Notes (2007) **7**, 1051-1053

1.1 Abstract

We describe the isolation and characterization of seven polymorphic short tandem repeats (STR) for the eel parasite *Anguillicola crassus*. This invasive swimbladder nematode endemic in East Asia was recently introduced into Europe. The number of alleles for each STR ranged from 13 to 39 per locus with observed heterozygosities between 0.49 and 0.98. The Taiwanese population displayed higher genetic diversity compared to the Irish sample, an observation consistent with the Asian biogeographical origin of the nematode. Availability of the reported STR will facilitate the investigation of the population genetic structure with regard to multiple invasions.

1.2 Introduction

Translocation of organisms along with their parasites around the globe is of major relevance for the study of biological invasions and conservation genetics (Hochberg & Gotelli 2005). The swimbladder nematode *Anguillicola crassus* (Dracunculoidea; Anguillicolidae) was recently introduced to North Atlantic eel populations from its natural host, the Japanese eel (*Anguilla japonica*). The nematode rapidly expanded into European and North African eel populations within three decades (Kirk 2003), which is explained best by commercial trade of infected eels from Asia to Europe (Koops & Hartmann 1989). It is often the case that invasive parasites follow the main trading routes and switch from their natural reservoir to immunologically naïve hosts (Taraschewski 2006). The occurrence of the parasite in various thermohaline water regimes harbouring different intermediate and paratenic hosts ensures that eels in a broad range of habitats are constantly infected during their lifetime. The infection causes inflammatory reactions and fibrosis of the swimbladder wall, which may compromise the catadromous eels' spawning migration in the open ocean (Kirk 2003). Thus, the nematode is thought to be a serious threat to the already sharply declining freshwater eel stocks in Europe (Wirth & Bernatchez 2003). In order to determine the nematode's population structure and demography, and to examine the possibility of multiple independent invasions, we isolated and characterized highly variable short tandem repeats (STR).

1.3 Material & Methods

Extracting total genomic DNA (gDNA) free from host tissues is a crucial step. The nematode's intestine is filled with eel blood, which must be carefully removed to separate the tissues of the parasite from the host's. Forty eels from Lake Constance, Germany, were dissected and their swimbladders screened for adult nematodes. Twelve adult stages were found alive and female ovaries and uteri and the seminal ducts of males were dissected under a binocular microscope to rule out internal and external contaminations with eel tissue. Total gDNA was extracted following standard protocols of Proteinase K digestion (Sambrook *et al.* 1989). If required, hard-to-digest tissues (oviducts) were subsequently disintegrated by heating at 65 °C for 30 min in a Tris/EDTA-buffered cetyltrimethylammonium bromide (CTAB) solution at a final concentration of 1% m/v. Resuspended DNA was checked for contamination using both eel-specific mitochondrial (for the cytochrome b gene) and genomic DNA primer pairs (Wirth & Bernatchez 2001). Short tandem repeats were isolated and identified from partial genomic libraries enriched for CA or CT repeats with the help of a magnetic bead technique following the protocol of Tenzer *et al.* (1999), including modifications by Garner *et al.* (2000). Enriched DNA was ligated into the pCRII-TOPO

cloning vector and transformed into chemically competent Escherichia coli TOP10 cells supplied with the TOPO TA Cloning kit (Invitrogen), following the manufacturer's recommendations. After plating, the cells grew overnight on 1x LB agar, containing 50 µg/mL of ampicillin and 80 µg/mL of X-gal. Single colonies were picked and regrown for 14 h in a 96-well-plate-format in 150 µL liquid 1x LB medium, containing 50 µg/mL of ampicillin. Bacterial cells were disrupted using a 5-min heat shock at 94 °C and lysates were directly taken as polymerase chain reaction (PCR) templates. Inserts were identified and screened for STR using M13 forward and reverse primers. Sequencing reactions were performed with Perkin Elmer's recommended protocol for BigDye version 3.1 sequencing chemistry on a 3100 Genetic Analyser (ABI-Hitachi). Screening of 265 inserts of 91 CA and 174 CT clones revealed nine unique STR, of which seven could be successfully amplified later on (**Table 1.1**). These markers are the basis for inferring genetic structure within newly invaded eel populations and tracking down the source populations of the parasite. Specific primer pairs were designed using the primer 3 software (Rozen & Skaletsky 2000). The same PCR protocol was carried out on a GeneAmp PCR System 9700 (Perkin Elmer-ABI) for all loci as follows: a 15 µL total reaction contained 1x of Genaxxon's Reaction Buffer S (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100); 200 nM of each dNTP; 1 U of Red Taq (Genaxxon); 200 nM of each primer and 10–100 ng of gDNA. An initial 5-min hot start at 94 °C was followed by the 35 cycles of denaturation for 35 s at 94 °C, annealing for 35 s at 55 °C and elongation for 45 s at 72 °C, finished by a post-elongation step of 10 min at 72 °C. Forward primers with an attached fluorescent label at the 5′-end allowed multiplexing of differently coloured amplicons. Alleles were run against the internal size standard Genescan-500 ROX (ABI), analysed using genescan and scored in Genotyper (version 3.7NT). Single loci were tested for Hardy–Weinberg equilibrium (HWE) and pairs of loci for linkage disequilibrium (LD) in one European and one native Taiwanese population of *A. crassus* each, using genepop version 3.4 (Raymond & Rousset 1995) with 10 000 dememorization steps, 1000 batches and 1000 iterations. Sequential Bonferroni tests (Dunn-Sidák method) were conducted to correct for errors in multiple comparisons among means (Sokal & Rohlf 1995).

1.4 Results

None of the loci showed significant linkage disequilibrium after Bonferroni correction, and all loci but one, AcrCT53, agreed with Hardy–Weinberg expectations. A subsequent analysis run in MICROCHECKER (van Oosterhout *et al.* 2004) indicated the presence of null alleles for the deviating locus. The Taiwanese population displayed higher genetic diversity compared to the Irish sample, for similar sample sizes (**Table 1.1**), an observation consistent

with the Asian biogeographical origin of the nematode. Due to the fact that other species of the genus *Anguillicola* are difficult to obtain, we were only able to test one specimen of the closely related species, *Anguillicola globiceps* (Moravec & Taraschewski 1988) for cross-species amplification. We found that for all but two primer pairs, AcrCT53 and AcrCA102, amplicons could be obtained.

1.5 Acknowledgements

This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG) to T.W. and the University of Konstanz to A.M. We acknowledge Sonja Erath and Ursula Topel for technical assistance. Kieran McCarthy and Horst Taraschewski kindly supplied nematode and eel fin clip samples. Dave Gerrard gave valuable comments on the final version.

Table 1.1 Short tandem repeats of the nematode species *Anguillicola crassus* based on two populations, Rivers Shannon (Ireland) and Kao-Ping (Taiwan).

Locus	Primer sequences (5' - 3') (including label descriptors)	Ta (°C)	GenBank Accession No.	Core Motif	A	Size range (bp)	Null Genotypes (%)	Shannon (n = 37)		Kao-Ping (n = 44)	
								H_E	H_O	H_E	H_O
AcrCT04	**F**-CAGGGACATGGAAAGGTGT	58	EF216845	$(CT)_{56}$	39	100-260	0	0.91	0.95	0.95	0.98
	ACGACAGGCAGCATCTTTGT	61									
AcrCT27	**H**-TCCGATACCCGCATTATACAC	60	EF216846	$(CT)_{49}$	29	72-200	0	0.91	0.89	0.93	0.84
	TCCTTGGCCAATTGATTTAAC	59									
AcrCT29	**H**-CAAATGGCAATTTCGACCAG	61	EF216847	$(CT)_{36}$	15	168-228	0	0.77	0.62	0.83	0.69
	TGCGTTCGTTCAGTATAGCA	58									
AcrCT53	**F**-TCGTCCTTTTCCATTTGTCC	60	EF216848	$(CT)_{59}$	37	73-230	2.5	0.91	0.49*	0.95	0.76*
	GCGGAACAAAACAAATAAATG	57									
AcrCT54	**N**-AAACCCCATACTGTTCCTTGC	60	EF216849	$(CT)_{14}$ CC $(CT)_6$	18	168-236	1.25	0.80	0.78	0.82	0.87
	TCGAGAAGGCAAATATCTAGGC	60									
AcrCT103	**N**-CTGCCGATCCAACAAGACG	63	EF216850	$(CT)_{41}$	24	92-160	0	0.87	0.81	0.93	0.91
	GTTTCCCCTGAAAAAGTTCG	59									
AcrCA102	**H**-AAGTCTAACCCCGCTATTTTTG	59	EF216851	$(CA)_6$ TACATA $(CA)_5$	13	297-332	0	0.59	0.54	0.86	0.86
	GCGCATGTTTCTGTGTGTATAAG	60									

* Deviation from Hardy-Weinberg Equilibrium $P<0.0001$ (Bonferroni-corrected $\alpha = 0.00174$); **F-**, 6-FAM , **H-**, HEX, **N-**, NED;
n, sample size; A, number of alleles; Ta, exact amplification temperature; H_E, expected heterozygosity, H_O, observed heterozygosity.

Chapter 2:

PERMANENT GENETIC RESOURCES

Isolation and characterization of 12 dinucleotide microsatellites in the European eel, *Anguilla anguilla* L., and tests of amplification in other species of eels

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Published in

Molecular Ecology Resources (2008) **8**, 1382-1385

2.1 Abstract

Twelve polymorphic dinucelotide microsatellites in the freshwater eel *Anguilla anguilla* L. were isolated and characterized. Genetic diversity was assessed in eels from Lake Constance, Germany. Allele numbers ranged from five to 26 per locus with observed heterozygosities between 0.125 and 0.875. A portion of locus AangCT77 aligns with a transcribed region of the zebrafish gene crystallin beta B2. Cross-species amplification of most markers was possible for nine other *Anguilla* eel species. The newly developed primer pairs will facilitate population and conservation genetic studies in order to refine the understanding of the subtle population genetic structure typical of eels, and to identify interspecies admixture due to global trade.

2.2 Introduction

The European eel, *Anguilla anguilla* L., has been studied genetically for more than 20 years (e.g. Williams *et al.* 1984; Avise *et al.* 1986, 1990; Wirth & Bernatchez 2001, 2003). Using small sets of microsatellite markers (Daemen *et al.* 1997; Wirth & Bernatchez 2001), there is evidence for subtle population genetic structure in the European eel either following a statistically significant pattern of isolation by distance (Wirth & Bernatchez 2001) or isolation by time (Maes *et al.* 2006a). Moreover, in a recent effort, 12 microsatellite loci have been isolated and described for the Japanese freshwater eel (*Anguilla japonica,* Ishikawa *et al.* 2001b). Our objective was to increase the current set of oligonucleotide markers available for anguillid eels. A larger set of markers should facilitate examination of the subtle population genetic structure typical of eels, as well as identification of interspecies admixture due to global trade.

2.3 Material & Methods

Short tandem repeats (STR) were identified from partial genomic libraries enriched for CA or CT repeats and isolated with magnetic beads following the protocol of Tenzer *et al.* (1999), including modifications by Garner *et al.* (2000). Total gDNA was extracted from eel blood following standard protocols of proteinase K digestion (Sambrook *et al.* 1989). Enriched DNA was ligated into the pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10 cells supplied with the TOPO TA Cloning kit (Invitrogen), following the manufacturer's protocol. After plating, the cells were grown overnight on 1x Luria-Bertani agar, containing 50 µg/mL of ampicillin and 80 µg/mL of X-Gal. Single colonies were picked and re-grown for 14 h in a 96-well plate format in 150 µL liquid 1x Luria-Bertani medium, containing 50 µg/mL of ampicillin. Bacterial cells were disrupted using a 5-min heat shock at 94 °C and lysates were used directly as polymerase chain reaction (PCR) templates. Inserts were identified and screened for short tandem repeats using forward and reverse primers targeting the SP6 and T7 sites in the vector used, respectively. Sequencing reactions were performed with PerkinElmer's recommended protocol for BigDye version 3.1 sequencing chemistry on a 3100 Genetic Analyser (ABI-Hitachi). Screening of inserts revealed a total of 26 positive clones, two of which contained more than one repetitive region. However, only one STR per positive clone was considered for specific primer pair design using the primer 3 software (Rozen & Skaletsky 2000). Thirteen loci could be successfully amplified using the same PCR protocol for all loci on a GeneAmp PCR System 9700 (PerkinElmer-ABI): a 12.5-µL total reaction contained 1x of Genaxxon's Reaction Buffer S (10 mm Tris-HCl, pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$, 0.1%

Triton X-100); 200 nm of each dNTP; 0.8 U of Red*Taq* (Genaxxon); 200 nm of each primer and 20 ng of gDNA. An initial 5-min hot start at 94 °C was followed by 35 cycles of denaturation for 35 s at 94 °C, annealing for 35 s at 55 °C and elongation for 45 s at 72 °C. PCR amplification was terminated with a post-elongation step of 10 min at 72 °C. Forward primers with an attached fluorescent label at the 5′-end permitted multiplexing of differently coloured amplicons. Alleles were run against the internal size standard Genescan-500 ROX (ABI), analysed with genescan and scored in genotyper (ABI software version 3.7 NT). One marker (AangCA75, not listed) could not be scored consistently due to elongated stutter peaks and was removed from the submitted set of markers (**Table 2.1**). Single loci were tested for Hardy–Weinberg equilibrium and pairs of loci for linkage disequilibrium in a sample (n = 24) of an eel stock from Lake Constance, Germany, using genepop version 3.4 (Raymond & Rousset 1995) with 10 000 dememorization steps, 10 000 batches and 1000 iterations. Sequential Bonferroni tests (Dunn–Sidák method) were conducted to correct for errors in multiple comparisons among means (Sokal & Rohlf 1995).

2.4 Results

None of the loci showed significant linkage disequilibrium after Bonferroni correction. Three of the 12 loci violated Hardy–Weinberg expectations (**Table 2.1**). According to the MICRO-CHECKER program (van Oosterhout *et al.* 2004), two of those markers, AangCT67 and AangCT77, are expected to bear null alleles with a frequency of 0.144 and 0.134, respectively, when compared to 10 000 randomly generated genotypes. Blasting locus AangCT77 revealed a 132-bp long partial overlap (e-value = $7.0e^{-42}$) with the reversed sequence of crystallin beta B2 transcripts from several teleost fish species (*Danio rerio* and *Tetraodon* spp.). Thus, because of its tight linkage to a gene, the influence of selection is one probable explanation for the violation of Hardy–Weinberg expectations at this locus (**Table 2.1**). However, this marker may be useful in future studies on expressed sequence tags (EST)-linked microsatellites in the eel. Cross-species amplification was tested with nine different anguillid eel species (**Table 2.2**). Species status was verified by sequencing a portion of the 16S rRNA gene (Aoyama *et al.* 2001). Six of the 12 loci were successfully amplified for all specimens. Five of the remaining markers failed for one or the other eel species, mainly including native species of New Zealand and Australia. Only one locus, AangCA55, failed for the majority of eel specimens, and appeared monomorphic in species other than *A. anguilla*. These results suggest that the novel microsatellite markers can be useful for population and conservation genetics studies in other anguillid species as well.

2.5 Acknowledgements

This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG) to T.W. and the University of Konstanz to A.M. We thank Sonja Erath, Matthias Sanetra and Ursula Topel for technical assistance.

Table 2.1 Characterization of polymorphic microsatellite loci from European freshwater eel, *Anguilla anguilla* L., tested for an eel stock from Lake Constance, Germany (n = 24).

Locus	Core Motif	GenBank Accession no.	Primer sequences (5' - 3') (including label descriptors)	Tm/Ta (°C)	A	Size range (bp)	Null Genotypes (%)	H_E	H_O	F_{IS}	*P*	Micro-Checker performance
AangCT53	$(CT)_{17}$	EU310488	**H**-AGGTGACACACAGTCTCTTTGG ACAATGCATGTGCCTGAATG	59/ 55 60/ 55	8	74-94	2.5	0.72	0.63	0.15	0.100	+
AangCT59	$(CT)_{18}$	EU310487	**H**-GCAACCCTTTCTCACTCCAC CTCACTGCGCAAACAAGAAG	60/ 55 60/ 55	12	70-91	4.0	0.81	0.74	0.11	0.477	+
AangCT67	$(TG)_6N_8(TG)_5TA$ $(TG)_4(AG)_2(TG)_7$	EU310489	**H**-GACAGACGGACAGACAATGC GGTGGTGAATTTTGGTCCTG	59/ 55 60/ 55	19	124-236	4.0	0.91	0.65	0.31	0.001	*null*
AangCT68	$(AG)_{22}$	EU310490	**F**-CCAGGCAATTGCTTTCTCAC TCATTGTGTTTGGCACTTCC	61/ 55 60/ 55	11	169-195	0	0.86	0.83	0.06	0.873	+
AangCT76	$(TC)_{17}(AC)_{13}$	EU310480	**F**-CTTCAGCTTGGAGGTGTTCC CTGTGCAGGAGTCACGTTTC	60/ 55 59/ 55	10	196-232	4.0	0.80	0.74	0.09	0.389	+
AangCT77	$(TC)_{17}(AC)_{13}$	EU310481	**F**-CCTGATGTTTTCAGCGTTTG GAAAGTGGGCTCAGTTCTGG	60/ 55 59/ 55	10	101-117	0	0.70	0.50	0.31	0.000	*null*
AangCT82	$(GA)_{17}$	EU310479	**N**-CCACTCTAGCGACACAACACTC GCATTTTAACCTTGTCCCTGTC	60/ 55 60/ 55	12	188-214	0	0.87	0.83	0.06	0.312	+
AangCT87	$(GA)_{11}$ $GG(GA)_2$ $GG(GA)_7$	EU310483	**N**-CGATGAAGCCGAAAATTAGC TGGCTTTAAAGTGGCGATG	60/ 55 60/ 55	12	104-166	0	0.83	0.71	0.17	0.003*	+

F-, 6-FAM , **H-**, HEX, **N-**, NED; A, number of alleles; Tm, melting temperature; Ta, amplification temperature; H_E, expected heterozygosity, H_O, observed heterozygosity; F_{IS}, inbreeding coefficient; *P*, value of probability for Hardy-Weinberg equilibrium exact test (α = 0.05); +, flawless allele scoring; null, null alleles expected; Nx, deviating dinucleotide repeat within the core motif of length x; underlined P-values indicate significant violation from Hardy-Weinberg expectations (α = 0.05); * violation from HWE in the absence of allele-bound typing defects

Table 2.1 (continued): Characterization of polymorphic microsatellite loci from European freshwater eel, *Anguilla anguilla* L., tested for an eel stock from Lake Constance, Germany (n = 24).

Locus	Core Motif	GenBank Accession no.	Primer sequences (5' - 3') (including label descriptors)	Tm/Ta (°C)	A	Size range (bp)	Null Genotypes (%)	H_E	H_O	F_{IS}	P	Micro-Checker performance
AangCT89	$(CT)_{15}(TC)_3(CT)_4$	EU310484	**H**-AACCAGCGAGATGATGATTG AGAGCGTGAAGCCTTTTGAC	59/ 55 60/ 55	11	198-220	16.6	0.86	0.85	0.04	0.326	+
AangCA55	$(TG)_5$ AG $(TG)_3$ TA $(TG)_5$	EU310486	**N**-TCTGTACGGCGCTTCAGAC CAGGTGCTTTAGTCCAGTTACATC	60/ 55 59/ 55	5	114-132	0	0.16	0.13	0.23	0.126	+
AangCA58	$(CA)_8$ TA $(CA)_8N_{32}$ $(CA)_9$	EU310485	**N**-CAGTCAGACGTCAGCCACTG GAGGTCTCTCTCACTGCGAAC	61/ 55 59/ 55	26	164-286	0	0.95	0.88	0.10	0.127	+
AangCA80	$(TG)_4$ CG $(TG)_{14}$	EU310482	**F**-TTCCTCTGGTCTTTCACACG AGCTGGAGGACACGGATG	59/ 55 60/ 55	13	74-110	0	0.89	0.79	0.13	0.193	+

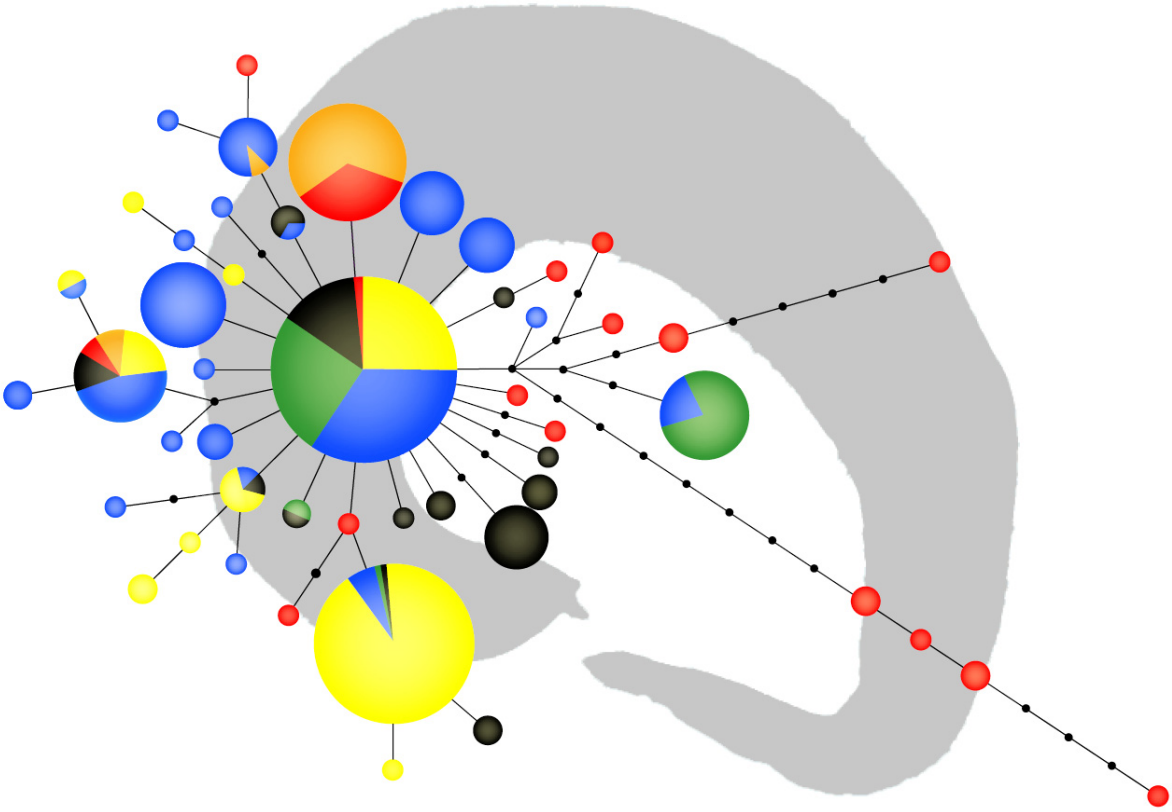
F-, 6-FAM , **H-**, HEX, **N-**, NED; A, number of alleles; Tm, melting temperature; Ta, amplification temperature; H_E, expected heterozygosity, H_O, observed heterozygosity; F_{IS}, inbreeding coefficient; P, value of probability for Hardy-Weinberg equilibrium exact test (α = 0.05); +, flawless allele scoring; null, null alleles expected; Nx, deviating dinucleotide repeat within the core motif of length x; underlined P-values indicate significant violation from Hardy-Weinberg expectations (α = 0.05); * violation from HWE in the absence of allele-bound typing defects

Table 2.2 Cross-species amplification with microsatellite primers developed for *Anguilla anguilla* using PCR conditions optimized for this species.

Taxon (no.)	*AangCT53*	*AangCT59*	*AangCT67*	*AangCT68*	*AangCT76*	*AangCT77*	*AangCT82*	*AangCT87*	*AangCT89*	*AangCA55*	*AangCA58*	*AangCA80*
A. australis (1)	72,192	70,74	97,97	169,171	X	127,145	192,192	X	208,212	X	X	78,84
A. australis (2)	72,190	66,74	97,97	173,191	X	109,111	190,192	X	210,212	X	261,261	78,78
A. australis (3)	74,78	68,68	97,97	179,191	X	109,111	192,192	X	206,214	X	164,164	78,80
A. bicolor bicolor (1)	72,72	72,72	97,97	157,169	202,202	113,121	196,200	100,160	204,204	X	192,232	74,80
A. bicolor bicolor (2)	72,72	72,72	97,97	161,169	202,202	113,119	198,200	138,138	204,204	X	202,230	72,80
A. dieffenbachi (1)	74,74	X	X	175,175	200,208	143,145	194,196	94,140	208,214	X	X	76,82
A. dieffenbachi (2)	74,74	X	X	175,177	206,241	141,143	196,196	94,94	208,208	X	X	82,82
A. japonica (1)	72,90	176,176	97,97	175,177	206,212	129,145	226,232	122,164	204,206	124,124	218,218	74,80
A. japonica (2)	70,72	172,176	227,227	171,175	204,204	101,121	206,208	154,154	208,208	124,124	236,236	80,80
A. mossambica	72,72	168,176	X	167,175	212,220	101,101	190,206	90,100	204,206	X	X	82,82
A. malgumora	74,74	168,172	97,97	169,173	214,214	101,101	208,214	132,132	204,214	X	178,178	72,78
A. marmorata	72,72	172,172	97,97	173,173	216,218	131,139	210,214	124,130	204,204	X	182,198	80,80
A. reinhardtii (1)	74,80	X	X	163,195	214,230	91,101	202,206	92,136	210,214	X	202,202	80,88
A. reinhardtii (2)	78,112	64,64	182,186	175,181	218,222	105,115	196,198	128,142	204,212	124,124	180,180	82,84
A. rostrata (1)	78,78	64,64	180,190	167,171	208,218	101,123	196,200	122,138	202,206	124,124	174,202	78,92
A. rostrata (2)	78,78	80,84	174,190	173,185	200,206	109,165	196,198	114,162	204,212	124,124	196,214	96,96

X, no amplifcation

Part B: Population structure and phylogeography in an invasive eel parasite.



Chapter 3:

Population structure of the parasitic nematode *Anguillicola crassus*, an invader of declining North-Atlantic eel stocks

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Published in

Molecular Ecology (2008) **17**, 3478–3495

3.1 Abstract

Probably half of all animal species exhibit a parasitic lifestyle and numerous parasites have recently expanded their distribution and host ranges due to anthropogenic activities. Here, we report on the population genetic structure of the invasive nematode *Anguillicola crassus,* a parasite in freshwater eels, which recently spread from Asia to Europe and North America. Samples were collected from the newly colonized naïve host species *Anguilla anguilla* (Europe) and *Anguilla rostrata* (North America), and from indigenous *Anguilla japonica* in Taiwan and Japan. Using seven microsatellite loci and one mitochondrial marker, we show that the parasite's population structure in Europe mirrors the zoogeographic Boreal–Lusitanian break along the English Channel. Both the north-to-south decline of nuclear allelic diversity and the loss of private alleles in the same direction are consistent with a significant isolation-by-distance pattern based on ρ_{ST} values. In combination with the specific topology of the distance tree among nematode populations, our data suggest that Europe was invaded only once from Taiwan, and that subsequently, genetic diversity was lost due to random drift. On the contrary, the North American sample shares distinct nuclear and mitochondrial signatures with Japanese specimens. We propose that the genetic structure in Europe was shaped by long-range anthropogenic eel host transfers in the north and a single dispersal event into the southwest. The genetically distinct Brittany sample at the edge of the Boreal–Lusitanian boundary is indicative of natural dispersal of fish hosts since recruitment occurs naturally there and invertebrate host dissemination is interrupted due to oceanic currents.

3.2 Introduction

Human-mediated global transfer of organisms, either shipped intentionally as live stock, or as blind passengers inside a carrying vector, has resulted in an unprecedented number of translocations, approaching half a million species (Pimentel *et al.* 2001). The subsequent establishment of alien species has resulted in far-reaching economic and ecological impacts on the affected biocoenoses (Sakai *et al.* 2001; Pimentel 2002; Cox 2004). Studies on the evolutionary aspects of invasions (see Lee 2002 for a review) are needed for both a comprehensive understanding of invasion biology and the elaboration of adequate management strategies against introduction and dispersal of economically important alien species. According to Colautti & MacIsaac (2004), an invasion should be regarded as the final of five stages leading to a widespread occurrence of a species following a human-mediated range-jump, in which the newly established population must become a dominant part of the ecosystem. In this sense, not all successful colonizations can be regarded as (completed) invasions.

More than 50% of all animal species are assumed to be parasitic in lifestyle (Bush *et al.* 2001). Despite their huge diversity, parasites are under-represented in the evolutionary biology literature (Criscione *et al.* 2005) and studied systems comprising parasitic invaders remain scarce, although numerous parasites have rapidly expanded their ranges (Taraschewski 2006). For parasites, it has been suggested that host movement is one of the most decisive forces shaping population structure, acting by accelerated gene flow over large distances (Blouin *et al.* 1995; McCoy *et al.* 2003; Criscione & Blouin 2004). However, in comparing the informativeness of neutral markers of both symbionts of a host–parasite system, Criscione *et al.* 2006 showed that population genetic structure was more pronounced in the trematode compared to its fish host. If this specific case was typical, then one might expect that with particular barriers to gene flow or differential selection regimes, parasite population structure might be established very rapidly after a successful invasion and within a few generations. Nuclear allele and genotype frequencies, as well as mitochondrial haplotype composition in the founding population(s) diverge from those of the donor environment, due to the action of genetic drift during the process of colonization (for a review refer to Sakai *et al.* 2001; Lee 2002; Cox 2004). Phenotypic changes as a response to differing biotic and abiotic stressors in their new habitat coincide with rapid genetic changes. Furthermore, within decades and in response to selection, newly established alien species have rapidly evolved divergent life-history traits as known from introduced sockeye salmon (Hendry *et al.* 2000) and have strong impacts on the communities due to predation and parasitism. The selective effects of the invasive green crab on *Mytilus edulis* populations

(Freeman & Byers 2006) and the mite *Varroa destructor* on stocks of the European honey bee *Apis mellifera* (Licek *et al.* 2004) are well- documented examples.

In this study, we investigated population genetic aspects of a parasite–host system, in which the nematode parasite *Anguillicola crassus* was accidentally translocated from its Asian sources to Europe in the early 1980s. Despite earlier management warnings (Egusa 1979), an import of 35 tons of live Japanese eels (*Anguilla japonica*) to European aquaculture presumably facilitated the nematode's rapid range-jump (Koops & Hartmann 1989). Neither the native final host, *A. japonica*, nor its East Asian intermediate hosts (copepods, ostracods) succeeded in establishing themselves in Europe (Taraschewski 2006). Today, the nematode is also found in eels on the North American East Coast (*Anguilla rostrata*; Barse *et al.* 2001) and in several imported and indigenous eel species on the remote Island of Reunion. The latter nematode population most likely stems from Europe (Sasal *et al.* 2008). *A. crassus* belongs to the family Anguillicolidae (Nematoda, Dracunculoidea) and parasitizes the swimbladder of fishes that belong to the genus *Anguilla* (Moravec & Taraschewski 1988). Growing parasitological and epidemiological knowledge has fostered its consideration as a model organism for invasion biology (Taraschewski 2006). Interestingly, in the invasive populations of the new hosts, both the prevalence and the intensities of the parasite are much higher than in those of the naturally affected host (*A. japonica;* Münderle *et al.* 2006). This coincides with elevated survival, longevity and reproductive output of the parasite in the immunologically naïve novel eel hosts (Knopf 2006; Taraschewski 2006). *A. crassus* has an indirect life cycle that starts and ends in its anguillid final host's swimbladder lumen, where sexual reproduction takes place (see Kirk 2003 and references therein for a more detailed overview). Subsequently, whereas adult nematodes die, both eggs and hatched L2 larvae escape by extrusion into the gastrointestinal tract. After anal release, the hatched free-living larval stage of the parasite attaches to benthic substratum and undulates to attract predatory crustacean intermediate hosts of the benthic zone (Thomas & Ollevier 1993). Upon ingestion, infected crustaceans display sluggish movement (Kirk *et al.* 2000a) and are assumingly preyed on by eel and other benthic fish predators, such as gobids and ruffe, in which the parasite cannot finish its life cycle but remains infective (Thomas & Ollevier 1992). Larger piscivorous eels (> 20 cm; Tesch 2003) may acquire larval parasites mainly through these infected prey fish that serve as so-called paratenic hosts and which thus potentially serve as an important natural link among vast ranges as compared to the less-mobile intermediate hosts. In European freshwaters many calanoid and cyclopoid copepods as well as ostracods are accessible for *A. crassus* (Kirk 2003). However, the ubiquitously distributed marine copepod *Eurytemora affinis* has been identified as a potential key intermediate host in estuarine and brackish waters (Kirk *et al.*

2000b). The only currently known direct limitations to parasite dispersal are temperatures below 4 °C and lack of suitable intermediate hosts (Kirk 2003).

Driven by the continental eel trade, *A. crassus* became established as one of the most dominant parasites in the European freshwater eel due to its rapid spatial and demographic expansion (Kirk 2003; Taraschewski 2006) and only Icelandic eels are currently unaffected. Transmission takes place in fresh- and brackish water, and a vertical transfer within host families is highly unlikely (Kirk 2003) due to the European eel host's peculiar life cycle. In fact, the European coasts and rivers are the locations where the susceptible eel stages are infected after an initial 5000 km passive larval migration from the Sargasso Sea. Eels have long generation times, reversing their initial larval migration back to their spawning area after 10 to 20 years (Tesch 2003). This catadromous life cycle results in a nearly panmictic population with a weak but significant population genetic structure (Wirth & Bernatchez 2001; Maes & Volckaert 2002; Maes *et al.* 2006a). Although the current decline in recruitment of European eels coincides with the arrival of the parasite, multiple factors other than diseases and parasites are implicated and include intensified fishing, barriers to both upstream and downstream migrations, pollution and changes in oceanic currents (Castonguay *et al.* 1994; Behrmann-Godel & Eckmann 2003; Knights 2003; Wirth & Bernatchez 2003; Kettle & Haines 2006). However, evidence for virulence and fatalities in European eels are numerous and indicate potential threats due to swimbladder infestations (e.g. Gollock *et al.* 2005; Knopf 2006; Lefebvre *et al.* 2007). Of even greater concern are the results gathered from exhaustion swim tunnel experiments demonstrating that sexually mature silver eels reached lower cruising speeds and had higher costs of transport compared to healthy individuals, when swimbladders were either highly parasitized or otherwise damaged after infection (Palstra *et al.* 2007). If true, these fishes may fail to complete their reproductive migration back to their birthplace in the Sargasso Sea. Clearly, under such a peculiar and extreme stress situation paired with elevated susceptibility to infection (Palstra *et al.* 2007), the impact of a blood parasite can be lethal in migrating, nonfeeding silver eel stages (Lefebvre *et al.* 2007).

A previous study on *A. crassus* that relied on random amplified polymorphic DNA (RAPD) markers (Rahhou *et al.* 2005) suggested that populations from the Mediterranean Sea differed from those of the Atlantic and the North Sea. Moreover, the absence of a significant correlation between genetic and geographic distances supported a multiple introduction scenario for this parasite. However, this type of genetic marker has its caveats. It is sometimes lacking reproducibility (Penner *et al.* 1993), and owing to its non-discriminatory characteristics, is highly sensitive to DNA contamination in this system (eel blood in the nematodes' intestines; larvae in the females' uteri). Even after careful cleaning and dissection, one cannot entirely eliminate incidental host DNA, and therefore, co-amplification

during the RAPD polymerase chain reaction (PCR) might blur the real signal, or even worse, generate a wrong signature.

Here, in order to overcome these caveats, we combined the genetic information contained in seven species-specific microsatellite markers (Wielgoss *et al.* 2007) with sequence data derived from a fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI). The aims of this study were to (i) explore the population structure of *A. crassus* in its entire range, (ii) compare allelic and genotypic composition among Asian native and nonindigenous, invasive populations, and (iii) determine if biogeographic discontinuities appear between western Europe, northern Europe and the Mediterranean area. Understanding the epidemiology and the phylogeography of this invasive nematode will help us to reconstruct the recent spread of this alien species and its secondary genetic differentiation.

3.3 Material & Methods

3.3.1 Sample collection

A total of 512 adult individuals of the parasitic nematode *Anguillicola crassus* were collected from 15 different localities in Europe (11), at the North American East Coast (1) and in East Asia (3) between October 2003 and October 2006, with the exception of samples from the River Tiber which were sampled in 1996 (**Table 3.1**; **Figs 3.1** and **3.2**) and preserved in 75% ethanol. All specimens were identified as *A. crassus* by applying taxonomic tools (Moravec & Taraschewski 1988), and in addition, two parasitological measures were examined (parasite prevalence and mean intensities; *sensu* Bush *et al.* 1997). We randomly chose and analyzed only one nematode per eel for all invasive populations, to avoid samples composed of siblings. In contrast, due to much lower parasite abundance in the sampled Japanese eels (**Table 3.1**), all specimens of Asian origin were genotyped. One individual Japanese eel sampled in Mikawa Bay contained an infrapopulation (*sensu* Bush *et al.* 1997) of 17 nematodes, which we considered as an additional population, MIK-2, thus increasing the number of populations to 16.

3.3.2 DNA extraction

Extraction of DNA in nematodes is prone to intra- and interspecific DNA contamination (Anderson *et al.* 2003; Wielgoss *et al.* 2007). To avoid foreign DNA, tissue was exclusively derived from the apical parts of the nematode bodies and L2-larvae which are sporadically attached to the exterior were removed along with the nematode's epidermis. Total DNA was extracted (Bruford *et al.* 1992), additionally treated with 30 µg of RNase A prior to precipitation, and resuspended in 25 µL of 0.1x Tris-EDTA buffer (pH 8.0). DNA yield

and quality was roughly gauged on 1.0% TAE-agarose gels and compared to *Hind*III-restricted λ-DNA (Pharmacia).

3.3.3 Microsatellite analyses

A total of 490 individuals were successfully genotyped at seven microsatellites loci as previously described (Wielgoss *et al.* 2007). The PCR products were diluted 1:20 in fully deionized water. For genotyping, amplicons were poolplexed in two groups: group 1 (*AcrCT04 + 29 + 103*) and group 2 (*AcrCT27 + 53 + 54 + AcrCA102*). Then, 1.2 µL of the bulk dilution was added to a sequencing plate containing 0.2 µL of ABI's standard GeneScan 500 ROX and 10.8 µL of HiDi-Formamide. Reactions were genotyped on a 3100 Genetic Analyzer (ABI-Hitachi) and scored in genotyper version 4.0 for Windows NT, after visual inspection. Scoring of loci was reliable, and repeating a subset of individuals for each locus did not challenge the initial allele calls.

3.3.4 Mitochondrial DNA sequencing

The mitochondrial locus cytochrome *c* oxidase subunit I (COI) was partly sequenced (552 bp) for a total of 419 individuals. In general, a subset of 30 individuals per population was picked randomly prior to sequencing, except for populations for which fewer specimens were available, in which case all specimens were sequenced. A 25-µL reaction contained 200 nm of each of the universal invertebrate primers HCO2198 and LCO1490 (Folmer *et al.* 1994), 1x of Genaxxon's Reaction Buffer S (10 mm Tris-HCl, pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$, 0.1% Triton X-100); 200 nm of each dNTP; 1 U of Red*Taq* (Genaxxon); and 10–100 ng of gDNA. An initial 3-min hot start at 94 °C was followed by 35 cycles of denaturation for 35 s at 94 °C, annealing for 1 min at 40 °C and elongation for 1 min 30 s at 72 °C, ending with a final elongation step of 15 min at 72 °C. The PCR fragments were purified using silica-based spin columns (PeqLab) and directly sequenced on an ABI 3100 using BigDye terminator chemistry (Perkin Elmer). Haplotypes were deposited in GenBank (Accession nos. EU376536–EU376954).

3.3.5 Data analysis and statistical evaluation

Microsatellites. To circumvent the problem of undetected population structure when checking for allele-size effects on heterozygote-dependent measures, a locus-by-locus analysis of molecular variance (amova) was performed using arlequin version 3.11 (Excoffier *et al.* 2005), which included intra-individual level variance related to F_{IS}. Subsequent analyses using microchecker (van Oosterhout *et al.* 2004) revealed the presence of null alleles at two loci, and their frequency was estimated using the Brookfield estimation. Both observed and expected heterozygosities, as well as F_{IS} (Weir & Cockerham 1984) values

were calculated using genetix version 4.05 (Dawson & Belkhir 2001). Deviations from both Hardy–Weinberg equilibrium (HWE) for single loci, and linkage disequilibrium (LD) among pairs of loci were determined using Fisher's exact tests in genepop version 3.4 (Raymond & Rousset 1995). Markov chain analyses were applied to estimate significance (10 000 dememorization steps, 1000 batches and 10 000 iterations per batch), and corrected for combined type I errors using sequential Bonferroni tests (Sokal & Rohlf 1995). Locus-wise total and private allelic counts were conducted using hprare (Kalinowski 2005). The calculations were performed for n = 24 genes per sample. Pairwise θ_{ST} estimates (Weir & Cockerham 1984) between pairs of populations were calculated and evaluated for deviations from the null hypothesis of panmixia after 10 000 permutation steps in arlequin version 3.11 (Excoffier *et al.* 2005). In addition, genetic differentiation for both F_{ST} and ρ_{ST}, an unbiased version of R_{ST}, based on the stepwise mutation model (Slatkin 1995; Rousset 1996), were calculated in genepop version 3.4 (Raymond & Rousset 1995) and compared to absolute and straight line geographic distances, which were derived from longitudinal and latitudinal positions (**Table 3.1**). Significance of the correlation was assessed applying a Mantel test (Mantel 1967) implemented in passage version 2 (Rosenberg 2008). In order to exclude possible effects of nonstandardized values on the outcome of the Mantel tests, we also considered a standardized measure of F_{ST}. The standardization was performed as suggested by Meirmans (2006) and indexed as θ'_{ST}. Based on the sampling coordinates in Europe (**Table 3.1**), we further assessed phylogeographic patterns by testing for the presence of genetic barriers using Monmonier's (1973) algorithm of maximum differences implemented in the program barriers version 2.2 (Manni *et al.* 2004). The nematode population structure was inferred based on a Bayesian clustering approach implemented in structure version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). We chose the admixture model, which best fits the nematode's purely sexual mode of reproduction and no prior information about the population geographic origin was used. To assess the most likely number of populations (K), we ran several tests varying the number of populations for K = 1–10. The data set was iterated 10 times for 200 000 Markov chain Monte Carlo (MCMC) repeats and a burn-in period of 100 000 steps to assess both the arithmetic means of the likelihoods and standard deviations at different K values. A final run of 1 million MCMC repeat chain was conducted after a burn-in length of 100 000 steps. The number of contributing populations was statistically tested using two different approaches. First, the ad-hoc statistic ΔK proposed by Evanno *et al.* (2005) was applied. This procedure is sensitive to pronounced changes in mean log-likelihood values between successive Ks and the degree of variance of any given mean. Second, the related point estimate E (K|X) for actual number of K populations contributing to the structure within the data set was inferred using structurama version 1.0 (http://www.structurama.org), which implements the Gibbs and split–merge sampler

described by Pella & Masuda (2006). This program allows for an independent estimation of the Dirichlet parameter α (Huelsenbeck & Andolfatto 2007). The settings were as follows: five chains were run in parallel and samples were taken every 10th step from 300,000 MCMC repeats, resulting in a total data set of 30,000 steps. Both the number of assumed POPULATIONS *K* and the Dirichlet parameter α were estimated independently without priors (the shape parameter of the γ-distribution was fixed at α = 1). Burn-ins of 100, 1000, and 10 000 steps were applied. The graphic display of the structure results was generated using distruct (Rosenberg 2004). Cavalli-Sforza & Edwards' (1967) chord distance was used to construct a phylogenetic tree using a neighbour-joining algorithm (Saitou & Nei 1987) implemented in POPULATIONS version 1.2.30b (http://bioinformatics.org). Support for the tree nodes was assessed by bootstrapping over individuals (100 iterations). A factorial component analysis (FCA) implemented in GENETIX version 4.05 (Dawson & Belkhir 2001) extracted a set of orthogonal axes of variation ranked by informativeness. The 3D-scatter plot based on the output matrix of eigenvalues was recalculated in STATISTICA version 6.0.

Mitochondrial DNA. Haplotypic diversity *h* (Nei & Tajima 1981), nucleotide diversity *π* (Nei 1987), Fu's *F*s (Fu 1997) and population genetics estimators (Weir & Cockerham 1984) were calculated in ARLEQUIN version 3.11 (Excoffier *et al.* 2005). An AMOVA was also performed (Excoffier *et al.* 1992) to compare geographic partitioning based on haplotype frequencies. A minimum-spanning haplotype network was constructed using the TCS version 1.20 software (Clement *et al.* 2000). This network was compared to a maximumlikelihood tree to correct for ambiguous, multiple connections. All redundant sequences were removed from the data set using COLLAPSE version 1.2 (http://darwin.uvigo.es) and the best-fit model of sequence evolution (HKY + G + I) was determined using MODELTEST version 3.4 (Posada & Crandall 1998), which estimated the shape parameter of the γ-distribution, α = 0.8848, and the proportion of invariable sites, P_{invar} = 0.6788. Finally, a phylogenetic tree based on maximum-likelihood criteria under the given model was inferred in PAUP* version 4.0b10 (Swofford 2003) and compared to the haplotype network derived by the parsimony approach in TCS.

3.4 Results

3.4.1 Microsatellites

Intrapopulation diversity, Hardy–Weinberg and linkage equilibrium, and the neutrality of loci. All microsatellite loci examined were highly variable ranging from 15 (*AcrCA102*) to 65 (*AcrCT04*) alleles per locus (*n* = 490; **Table S3.1**; Appendix 2). Exact tests for linkage disequilibrium (LD) only showed two instances of linked loci (α < 0.0002), restricted to the MIK-2 infrapopulation, i.e. *AcrCT04-AcrCT103* and *AcrCT04-AcrCT53*, respectively. Native

Asian populations were genetically more diverse than recently established invasive colonies. Expected heterozygosities (H_E; Nei 1978) are summarized in **Table S3.2**; Appendix 2. Averaged across loci, H_E were always lower in European samples (0.755–0.874) when compared to native populations like KAO (0.906) or MIK-1 (0.907), although Baltic samples display marginally higher values than KAO in three instances. Averaged values for observed heterozygosities (H_O) were consistently lower than expected values in all locations, and the Taiwanese sample showed the highest overall value of 0.834. Using a locus-by-locus amova including intra-individual level variance three loci displayed low but significant inbreeding coefficients, *AcrCT04* (F_{IS} = 0.054; P < 0.001), *AcrCT27* (F_{IS} = 0.046; P = 0.001), *AcrCT54* (F_{IS} = 0.018; P = 0.05), whereas *AcrCT29* (F_{IS} = 0.327; P < 0.001), *AcrCT53* (F_{IS} = 0.229; P < 0.001) and *AcrCA102* (F_{IS} = 0.195; P < 0.001) showed high and significant values, with *AcrCT103* (0.123; P < 0.001) being intermediate. Such an uneven pattern cannot be explained by population-level effects and is expected to be either due to technical or locusspecific effects. After excluding technical problems due to the reliability upon re-amplification trials of already scored individuals, we scrutinized the presence of allele size effects using the MICRO-CHECKER software (van Oosterhout *et al.* 2004). Whereas allelic drop-out could be excluded for any locus and population (112 comparisons), the software revealed a significant population-wide presence of null alleles at loci *AcrCT53* (9 populations) and *AcrCT29* (11 populations), with average null allele frequencies of $f_{NULL,53}$ = 0.09 and $f_{NULL,29}$ = 0.10, respectively. All remaining loci showed sporadic nulls with no observable trend. Although it is reasonable to assume that the bias introduced by each marker will be different, the presence of unobserved (recessive) alleles can influence the reliability of the data and may lead to overestimation of differentiation (Chapuis & Estoup 2007). Thus, it is important to consider their influence carefully. We repeated all analyses by (i) allowing for (unobserved) null alleles, if possible, and (ii) testing fractions of markers separately by deleting either the lowest or the highest F_{IS} value markers. Despite the presence of null alleles at some microsatellite loci, our results suggest that the amount of information gathered by the sum of markers outperformed the single-marker defects.

Genetic differentiation and relationships among populations. Unstandardized differentiation indices indicate a weak but significant global population structure for European populations. The global θ_{ST} (0.057; 95% CI ± 0.0090) and ρ_{ST} (0.059; 95% CI ± 0.012) are almost identical. Correction for null alleles using freena (Chapuis & Estoup 2007) only marginally decreased absolute values, which indicates that null alleles were not strongly affecting the differentiation indices (**Table 3.3**). However, standardization had a large effect on the average θ_{ST} value as it is increased significantly to 0.32 (95% CI ± 0.040) in both uncorrected and null-corrected data sets, identical to the average θ_{ST} of 0.32 (95% CI ± 0.044) among European populations based on the mitochondrial DNA marker. This is a

convincing value as it makes both measures comparable to one another. Pairwise comparisons of θ_{ST} and ρ_{ST} assessed among all populations behaved in parallel to overall differentiation (only pairwise θ_{ST} shown). In brief, the northern localities with the Irish (SHA–NEA), and the Baltic samples (OER–ALA) were least differentiated from one another. Similarly, the southwestern populations appeared least differentiated based on pairwise indexes. Bonferroni corrections performed on θ_{ST} values mainly indicated insignificant pairwise differences between southern populations and the Loire River (LOI). Most notably, among all Asian indigenous nematodes, Japanese MIK-1 and the Taiwanese sample are least differentiated from one another, despite their geographic distance. In fact, these localities share a similar level of differentiation to either of the invasive populations in Europe. Among Asian localities, YAM and MIK-2 are dissimilar to the MIK-1 and KAO samples and are both highly differentiated from European locations. Finally, the St Jones River population from North America is least differentiated from KAO and the northeastern European population of ALA.

Population structure and genetic admixture analysis. Given our microsatellite data for 16 populations, the estimated likelihood measures ln*P(D)* for the number of assumed populations (*K*) increased from *K* = 1 to *K* = 6, at which point the curve reaches a plateau (**Fig. S3.1a**; Appendix 1). When accounting for null alleles (Falush *et al.* 2007), the same pattern became apparent (**Fig. S3.1c**; Appendix 1). A sensitivity analysis which excluded either non-HWE or HWE markers confirmed the stability of the overall pattern, in which we inferred a minimum value of *K* = 2, separating the southwestern populations from the remainder of the sampling locations (**Fig. S3.2a–h**; Appendix 1). This is supported using Evanno *et al.*'s (2005) ad hoc statistic *ΔK*. The two highest rankings were obtained for *K* = 2 and *K* = 4, respectively, both times with and without correction for null alleles, **Fig. S3.1b** and **d**; Appendix 1). However, a scenario with *K* = 4 might best explain the data (**Fig. 3.3**) which is substantiated by both the shape of the likelihood saturation curves (**Fig. S3.1a** and **c**; Appendix 1), and the Dirichlet process in structurama (Huelsenbeck & Andolfatto 2007) that estimated the most probable number of populations E (*K*|X) = 4.03; with Var (*K*|X) = 0.725 (α-ESTIMATE = 0.591; Var (α|X) = 0.106). Since structurama did not allow for the corrections of null alleles, caution must be taken here. While the burn-in length variation between 100 and 10 000 steps had no measurable effect, sensitivity analysis revealed a lower number of *K* for the low- F_{IS}-marker-set (*K* = 2.70) compared to the complementary set (*K* = 5.02). This slight overestimation is expected, when null alleles are present (Chapuis & Estoup 2007). In sum, two major population signatures are apparent in Europe: a northeastern cluster (ALA, OER, SLP, NEA, SHA), and a southwestern group (LOI, ORI, RHO, TIB), while under the four-population scenario, an additional northwestern French population is proposed with Brittany (FRE, VIL). Interestingly, this region is located at an intermediate geographic

position relative to the other two groupings. Surprisingly, only the northeastern European (towards both KAO and MIK-1) and the North American sample (towards both YAM and MIK-2) show affinities to Asian populations under the four-population scenario. However, one has to bear in mind that native populations have not been sampled exhaustively and that the data presented here may not conform to the model assumptions in an ideal manner, since HWE and linkage equilibrium might not have been reached in the introduced populations within such a short time span. Both the southwestern and the northeastern European locations indicate a considerable amount of gene flow in either direction; with several individuals being completely displaced according to their proposed population origins. The northeastern European population signature resembles the Taiwanese one, whereas only weak Taiwanese signatures can be detected in Brittany and the southwestern European samples. One part of the sample collected in Japan (MIK-1) cannot be distinguished from the Taiwanese population, whereas the MIK-2 infrapopulation derived from one single eel swimbladder, shares a completely different signature with samples from the Japanese River Fushino (YAM) and the North American St Jones River (STJ). From such a pattern it is clear, that even among Asian nematode populations there will be considerable amount of gene flow despite significant distances among regions. To test for the influence of MIK-2 (putative sibship) and YAM (smallest sampling size) on our results we excluded them both. All previously detected groupings remained stable and the North American sample is always separated from the remainder of the data set at $K = 4$ (data not shown).

While no prior information was used in the Bayesian clustering approach, the midpoint-rooted neighbour-joining tree incorporated the geographic origin of individuals. Chapuis & Estoup (2007) suggested that correction for null-alleles is reliable for D_{CE} distances. Upon correction of the data set for the presence of null alleles, the topology of the tree slightly changed (**Fig. 3.4**), by placing MIK-1 into the Japanese–US clade compared to the uncorrected phenogram (**Fig. S3.4**; Appendix 1). In essence, it can be inferred that the North American (STJ) and the Japanese populations markedly differed from the remaining data set, conforming to the findings using structure and pairwise indices of differentiation. Moreover, there is strong statistical bootstrap support for biogeographically relevant clades such as Brittany (VIL, FRE), Ireland (SHA, NEA), the Baltic Sea (OER, ALA) and the southwestern (LOI, ORI, RHO, TIB) populations. The weak support of deep splits reflects the very recent expansion of the nematode in Europe and shared ancestry with the Taiwanese population, which is best reflected in **Fig. 3.4**. Moreover, whereas the Brittany and southwestern samples are both monophyletic groups, they both cluster within the northeastern samples, suggesting a common invasion history of all European samples.

FCA and AMOVA. Based on the microsatellite genotypes, we conducted an FCA. Three individual genotypes, all from Japanese MIK-1 (MIK08, 29 and 30) contained most of

the variability found in the data set, which rendered the remaining data set uninformative. After removal of these outliers, the first three axes represented 4.6% of the total variance. The southwestern European samples separate from the northeastern ones along the first axis, while most of the Brittany samples are distinguishable from the remaining individuals along the second axis (**Fig. S3.3**; Appendix 1). The Japanese samples are separated along the third axis. Interestingly, the Taiwanese population clustered approximately within the northeastern European 'data cloud' central to all other clusters, whereas about two-thirds of the Japanese samples scatter yet in a different area of the factorial space. The analysis does not consider null alleles, however, a sensitivity analysis excluding either null allele suspect or nonsuspect markers yielded comparable results, respectively (data not shown). When populations were grouped to demes according to structure and FCA analyses (northeastern, Brittany, southwest), an analysis of molecular variance revealed that most of the genetic variance was confined within the populations (92.84%). The remaining molecular variance was found among groups (3.98%) and among populations within groups (3.18%); Φ_{ST} = 0.0716; Φ_{SC} = 0.0331; Φ_{CT} = 0.0398.

Allelic richness and private alleles. After rarefaction, the number of alleles per locus was higher in the native Taiwanese (KAO) and Japanese (MIK-1) populations when compared to the European and North American populations (**Table S3.1**; Appendix 2), with the exception of locus *AcrCT103* in MIK-1. The MIK-2 infrapopulation, collected from only one swimbladder, and the modestly sized YAM sample (N = 9) exhibited low numbers of alleles per locus, which likely is a result of lumping the closely related individuals from single swimbladders. This result surely does not reflect the real genetic divergence of the parasite in these areas, as suggested by the aforementioned MIK-1 and KAO samples. We also calculated the allelic richness for the different clusters detected within Europe with the structure algorithm. While single-population data are less informative (**Fig. S3.5**; Appendix 1), an apparent cline can be observed when locations are grouped according the fourpopulation scenario (**Fig. 3.5a**). The highest allelic richness is, as expected, found in East Asia, followed by the north European, Brittany and finally the most depauperate southwestern European samples. The number of alleles per locus among European geographic groups was not significantly different at the 5% level (two-tailed *t*-tests and unequal variance between groups). Only the northeast vs. southwest comparison was almost significant (P = 0.077). However, differences between the average values for Taiwanese and southwestern European, as well as the Taiwanese and the Brittany samples, respectively, were significant according to the two-tailed *t*-tests (0.0117 and 0.0243, respectively), whereas a comparison between Taiwan and northern samples was not (P = 0.0637). Averaged over all markers, no single European or North American location topped one private allele per locus (**Table S3.1**; Appendix 2) and the frequencies observed for private

alleles mirrors the trend observed in allelic richness. Again, the northeastern European samples had the highest proportion of unique alleles, followed by the Brittany and finally the southwestern European samples (**Fig. 3.5b**). One informative microsatellite allele was '231' at locus *AcrCT54*, which shows relatively high frequencies in southern locations LOI (12.0%), ORI (5.88%), RHO (11.3%) and TIB (12.9%), but is not found in Taiwan and in the other European locations, except the Irish population SHA (2.78%). Since correction for null alleles cannot be performed in these tests (Chapuis & Estoup 2007), we did a sensitivity analysis using the two different sets of null-allele-affected markers (**Fig. S3.6a** and **b**; Appendix 1). The unique alleles declined from northern to southern European sampling localities in both sets, although some of the populations followed slightly different trends. In sum, null alleles do not appear to have changed the overall pattern.

Isolation-by-distance and genetic barriers. Neither standardized nor unstandardized θ_{ST} values correlated significantly with geographic distances (**Table 3.3**). However, using the ρ_{ST} estimator (Rousset 1996), a Mantel test revealed a highly significant isolation-by-distance (IBD) pattern in the European *Anguillicola crassus* populations (**Table 3.3**). This finding was robust against the influences of null alleles. As the estimator for population differentiation is based on the stepwise mutation model (SMM), it appears to be most suitable given our microsatellite data (Ellegren 2004). To explicitly test if the IBD pattern is caused by phylogeographic breaks in the data, the pairwise null-corrected $\rho_{ST(ENA)}$ estimates were compared to the triangulated geographic data using Monmonier's (1973) algorithm in barriers version 2.2 (Manni *et al.* 2004). Importantly, the same major phylogeographic breaks became apparent that are suggested when combining results from structure, the clines in allelic richness and distinctiveness and the population distance tree. Placing barriers one-by-one, geographic groupings were separated from the remaining locations in the following order: (i) Mediterranean Sea (RHO, TIB); (ii) Baltic Sea (OER, ALA); (iii) Bay of Biscay (ORI, LOI); (iv) Ireland (SHA, NEA); and (v) the Brittany samples (FRE, VIL) which left Great Britain (SLP) ungrouped. This last step suggests that there might be a migratory link connecting these historically and geographically closely related areas.

3.4.2 Mitochondrial DNA

Nucleotidic and haplotypic diversity. Fifty COI haplotypes were found among the 419 individuals typed. The data set contained 55 segregating sites (and 62 mutational changes) for a sequence length of 552 bp, of which 35 were parsimony-informative. Most of the substitutions were in the third codon position but nine out of 62 resulted in amino acid changes. One replacement took place at site 32 (A to G), a first codon position, resulting in a change from methionine to valine in a branch leading to five remote Mikawa Bay haplotypes (**Fig. 3.6**). This group included MIK08, MIK29 and MIK30 which also appeared as outliers in

the FCA. Another replacement occurred at position 164 (C to T) and induced a lysine to phenylalanine change; this mutational step is shared by 25% of the individuals from the Shannon River and Lake Neagh, emphasizing their common ancestry. Haplotype diversity (*h*) varied considerably among locations (**Table S3.3**; Appendix 2), but only one native and two invasive geographic populations were far below 0.5 (MIK-2, 0.228; STJ, 0.232; RHO, 0.384). The three highest absolute values were all found in indigenous populations (MIK-1, 0.970; YAM, 0.905 and KAO, 0.814). Moreover, estimated values for nucleotide diversities were below 0.005 for all locations, except the native Asian MIK-1. In combination, values of $h > 0.5$ and $\pi < 0.005$, respectively, are interpreted as signs of recent population expansion after a bottleneck restrained population sizes (Grant & Bowen 1998), because large populations sizes support the maintenance of (newly arisen) mutations of low frequencies. Based on Fu's *F*s (Fu 1997), however, only the Taiwanese sample of KAO showed a significantly negative value of –7.8.

Population differentiation. θ_{ST} values inferred from mitochondrial DNA sequences were 3.9 times higher than estimates for nuclear-derived microsatellites, as is expected due to the fourfold higher effective size of nuclear markers compared to mitochondrial loci (**Table 3.2**). A positive correlation between both estimates was observed ($r = 0.69$). Again, southern populations were genetically more similar to one another than to other populations, and no single pairwise comparison was significantly different after Bonferroni correction. As for the microsatellite data, the local northern clusters became apparent (SHA–NEA, $\theta_{ST} = 0.00$, not significant (n.s.); OER-ALA, $\theta_{ST} = 0.055$, n.s.). The θ_{ST} value between SLP and NEA of 0.29 indicated high differentiation and may reflect drift effects for SLP. The differences between north and south were more pronounced with θ_{ST} values up to 0.62 between ALA and RHO. Based on θ_{ST} values, the Taiwanese population KAO is not distinguishable from the Baltic population ALA $\theta_{ST} = 0.011$, n.s.), but more differentiated from OER, VIL, SHA and NEA (θ_{ST} = 0.093–0.098). Among Asian populations, Japanese samples from YAM and MIK-1 are the least differentiated native populations ($\theta_{ST} = 0.125$, n.s.).

Haplotype partition. The haplotype network shows an overall star-like pattern hinting towards a recent, common expansion of the native Taiwanese population, while Japanese individuals from MIK and YAM are more evenly spread (**Fig. 3.6**), favouring an older age of these populations. Excluding singleton haplotypes, there are three unique northern haplotypes, compared to one southern and no unique Brittany haplotype. In addition, northern samples contained twice as many haplotypes (22 out of 127) when compared to either the southern (10 out of 126) or the Brittany samples (five out of 61). All major southern haplotypes are present in northeastern Europe, Brittany and Taiwan, but not the other way around. A striking feature is that 29 out of 32 North American (STJ) individuals share the

unique MIK-2 haplotype (θ_{ST} = 0.025, n.s.) indicating that the US samples most likely originated directly from a Japanese introduction rather than from a primary colonization from Taiwan or a secondary jump event from Europe.

Analysis of molecular variance. When populations were grouped according to structure and fca (northeast, Brittany, southwest), most of the genetic diversity was found within populations (65.36%). In contrast to microsatellite data, a larger diversity was found among groups (26.50%; P = 0.003) and less among populations within groups (8.14%). Among groups genetic structure became apparent: Φ_{ST} = 0.346; Φ_{SC} = 0.111; and Φ_{CT} = 0.265.

3.5 Discussion

3.5.1 Inferring the number and origin of invasions

Anguillicola crassus shows a mild macrogeographic population structure in the European eel and there is a moderate genetic separation between southwestern and northeastern European samples. Although we detected the presence of null alleles in at least two out of the seven markers, correction did not alter the initial results upon close scrutiny. Moreover, using subsets of markers, the same trend was revealed and thus fostered the robustness of our inference. Three lines of support indicate that random genetic drift in the nematode's new ranges in Europe, rather than multiple independent invasions from Asia have generated this nuclear genetic structure. First, mixing of distinct imports upon or shortly after arrival would have artificially enlarged the gene pool (Hartl & Clark 1997). This would have increased the diversity in Europe relative to single Asian populations. Yet, this is not the case, since locus-wide microsatellite diversity for any colonizer population is below the values observed in the native Taiwanese (KAO) and Japanese (MIK-1) populations. Second, in a nuclear phylogenetic context, local samples in the southwest and Brittany form distinct monophyletic groups with high bootstrap support and both are nested within the northeastern group, suggesting a common origin of all European samples. Third, assuming a one-dimensional stepping-stone model of migration (Hartl & Clark 1997), we identified a decline in both nuclear rarefacted (private) allelic diversity, and mitochondrial haplotype diversity from the putative source of invasion in northern Europe (Neumann 1985) to the extant southern distribution areas of the Mediterranean Sea. The Brittany populations in the centre of our sampling range are always intermediate in that respect. Consequently, an isolation-by-distance pattern was detected for the microsatellite data. This overall pattern of decreasing diversity might have been accelerated by the north-to-south increase of generation times from approximately one to two generations per year.

Our results further suggest that the source populations of the invader differed between the New and the Old World. Based on the nuclear markers, the Bayesian likelihood estimation groups North American individuals together with two out of three Japanese samples. Furthermore, this grouping is supported by a population tree, based on chord distances, with high bootstrap support. Finally, the majority of North American *A. crassus* specimens (29 out of 32) share the most common Japanese haplotype found exclusively in MIK-2, which is not present either in Europe or Taiwan. Although we found a considerable nuclear genetic overlap among the Taiwanese (KAO) and northwestern European samples on the one hand, and the Japanese sample MIK-1 on the other hand, we can assume a common Taiwanese origin for European populations as has been previously proposed based on eel import data (Koops & Hartmann 1989). Whereas there is a high degree of nuclear genetic overlap among Japanese MIK-1 and Taiwanese KAO samples, the opposite is true for mitochondrial haplotype data. This somewhat contradictory finding appears plausible when recent admixture among Asian regions is assumed. Intensive eel trading activity around Mikawa Bay is reported in the literature (Usui 1991) and our assumption is further supported by the sampling of one silver eel carrying different population of nematodes (MIK-2) as compared to the other nine infected eels caught in Mikawa Bay (MIK-1). MIK-2 shared most affinities with a second southern Japanese sample from Yamaguchi (YAM). Importantly, the indigenous Taiwanese population shows consistent signatures of recent expansion based on mitochondrial haplotype partition, which is in contrast to Japanese samples. This is supported by a significantly negative F_S test for KAO (Fu 1997) and a combination of high haplotypic ($h > 0.5$) and low nucleotidic ($\pi > 0.005$) diversities. When rooting the mitochondrial haplotype tree with the putative sister species *Anguillicola globiceps* (data not shown) five MIK-1 samples appear basal to the remainder of the *A. crassus* data set, which suggests that the Taiwanese population was derived from an indigenous Japanese one, predating recent eel trading activities.

3.5.2 Aspects of European biogeography

Surprisingly, in the context of eel host trades, the population structure of *A. crassus* in Europe reflects trends already observed in marine invertebrates (Wilke & Pfenninger 2002; Luttikhuizen *et al.* 2003; Roman & Palumbi 2004). The three geographic clusters identified with the Bayesian tool (the northeast, the Brittany and southwest) are reminiscent of the Boreal–Lusitanian break between northern and western Europe along the English Channel, as described by Briggs (1970, 1974) for marine benthic zoogeographic regions. We interpret our finding in terms of the important influence of host movement and dispersal on the parasite population structure (Blouin *et al.* 1995; Blouin *et al.* 1999; Hawdon *et al.* 2001). Since the main eel trading countries were affected first, the change and expansion of trading

routes can be held responsible for the rapid spread of *Anguillicola* in the 1980s (Kirk 2003). While England was already affected through intensive eel trade by 1987 (Kennedy & Fitch 1990), it took another 11 years for the Irish eel stocks to be infected, after eels from England were stocked there (Evans & Matthews 1999). This event is apparent from our data, as Ireland and England share nuclear genetic affinities. Given the vast distribution area of extant populations both in the northern and southern parts of Europe, the maintenance of high levels of gene flow seems to be kept by recurrent long-range eel host transport within regions. In the northeast, this is consistent with reports on intensive restocking activities (Dekker 2003). This in turn contributes to keep a considerable amount of allelic diversity within these regions.

The presence of a local population of *A. crassus* in Brittany, which is genetically intermediate, suggests that occasional natural migration or passive dispersal of fish hosts have influenced the genetic make-up at this edge. Two lines of argument support this hypothesis. First, eel recruitment occurs naturally in Brittany (FISHPASS, Rennes (F), personal communication), and thus, introduction of the parasite by eel stocking seems improbable. Second, natural barriers to intermediate copepod hosts clearly separate Boreal and Lusitanian zones due to oceanic currents and strict temperature and salinity clines (Briggs 1974). This leaves natural fish host migrations as source of dispersal. The pronounced natural barriers in Europe are highlighted by the barriers tool, which groups major geographic units that are separated from other such groups, namely the Mediterranean from the Baltic Sea, and farther the Bay of Biscay from Ireland and the Brittany region. Interestingly, Brittany and Great Britain display the weakest genetic break among all geographic neighbours (Manni *et al.* 2004).

Alternatively, the biogeographic partitioning of *A. crassus* would mirror that of its marine benthic invertebrate hosts due to certain selective influences which cannot be measured with neutral genetic markers. Since the main isolating forces underlying Briggs's (1974) break are oceanic currents and temperature gradients along the European coastlines, the nematode's dependence on and acquisition of local invertebrate hosts, which follow Briggs's biogeographic break (Briggs 1974), could have maintained population genetic structure of *A. crassus* in Europe due to differential survival depending on the predominant copepod host communities. Although this scenario is highly speculative, it may serve as a working hypothesis for future studies. Importantly, it has been previously suggested that parasite population genetic structure could serve as proxy for inference of its host's structure (Wirth *et al.* 2005; Nieberding & Olivieri 2007). Thus, we can assume that population structure in parasites with indirect life cycles reflects a combination of intermediate, paratenic and final host migrations, which can be strongly influenced by humandriven host movement.

3.5.3 Perspectives

As Strayer *et al.* (2006) pointed out, a study on invasive species, which 'randomly' picks a given period in time, clearly delivers only a glimpse of an ongoing process. It is apparent from our data that many local populations of the nematode have not yet reached migration–drift equilibrium due to the relatively short time span between first observation and establishment in the new host. The observed swimbladder infestations are suggested to compromise successful completion of the eel host's spawning migration (Palstra *et al.* 2007; Lefebvre *et al.* 2007); thus, adaptations on the host's side will evolve rather rapidly. Rapid evolution due to parasite invasion has been previously shown for a vast range of phyla (Cox 2004). In the North Atlantic eel's case, the MHC class II B loci involved in the recognition of extra-cellular parasites and other genes related to acquired immunity are likely candidates for further investigations in this ongoing arms race. Loci with immunological function are expected to vary among species inhabiting different habitats with different parasite and pathogen pressures (displayed in benthic and pelagic sticklebacks, e.g. in lakes from the Plöner Seenplatte in northern Germany; Wegner *et al.* 2003). That is to say, the eels might be quite homogeneous in terms of neutral markers, although genetic diversity at loci under balancing selection could reveal higher heterogeneity and thus the potential to acquire resistance to the nematode. Evidence gathered from well-studied sites in Europe (Audenaert *et al.* 2003; Lefebvre & Crivelli 2004) shows stabilization and even a slight decline in nematode abundance and intensities in recent years, possibly reflecting increased resistance towards these parasites in the long term.

3.6 Acknowledgements

We owe thanks to all our collaborators who directly contributed specimens or otherwise helped with sampling (**Table 3.1**), including Mathias Wegner, and Ahmed Yahyaoui. We thank Pascale Chesselet, Helen Gunter, Kathryn Elmer, Jody Shields and three reviewers for stimulating comments on the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft to T.W. and A.M., and from the University Konstanz to A.M., as well as a studentship by the Landesgraduiertenförderung Baden-Württemberg to S.W.

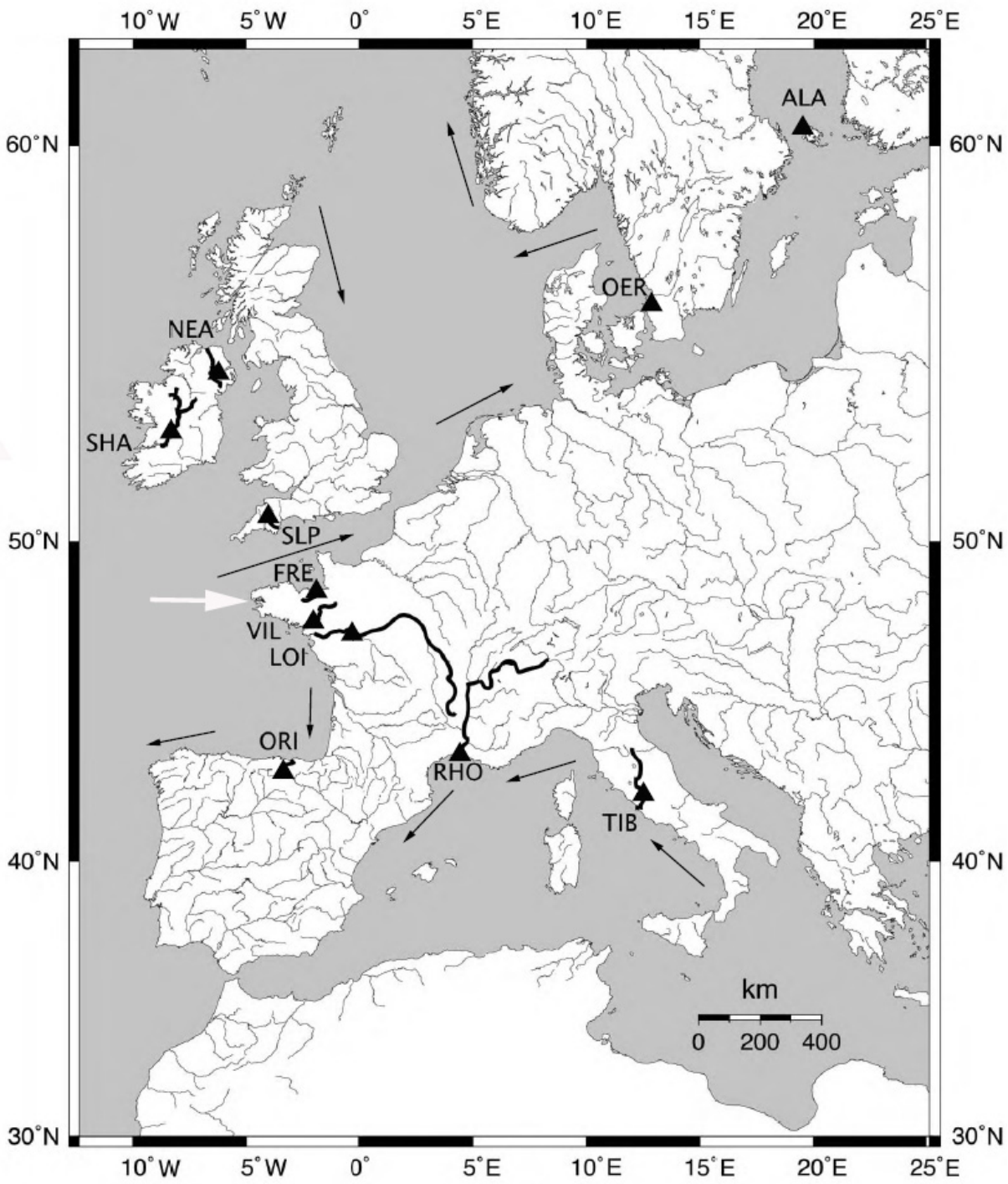

Figure 3.1 Sampling locations of the swimbladder parasite *Anguillicola crassus* (indicated by black triangles) covering most of the distributional range of its host *Anguilla anguilla* in Europe. The course of each sampled river system is highlighted in thick black lines. General marine circulation patterns have been indicated by black arrows, and Briggs' (1974) major biogeographic break-point of marine benthic zones within Europe is displayed by a single white arrow pointing at Brittany. Sample sizes are as follows; ALA, Åland Islands (n = 16); OER, Øresund (n = 30); SLP, Slapton Ley (n = 15); NEA, Lake Neagh (n = 40); SHA, River Shannon (n = 37); FRE, River Frémur (n = 39); VIL, River Vilaine (n = 44); LOI, River Loire (n = 50); ORI, River Oria (n = 30); RHO, River Rhône (n = 42); TIB, River Tiber (n = 40).

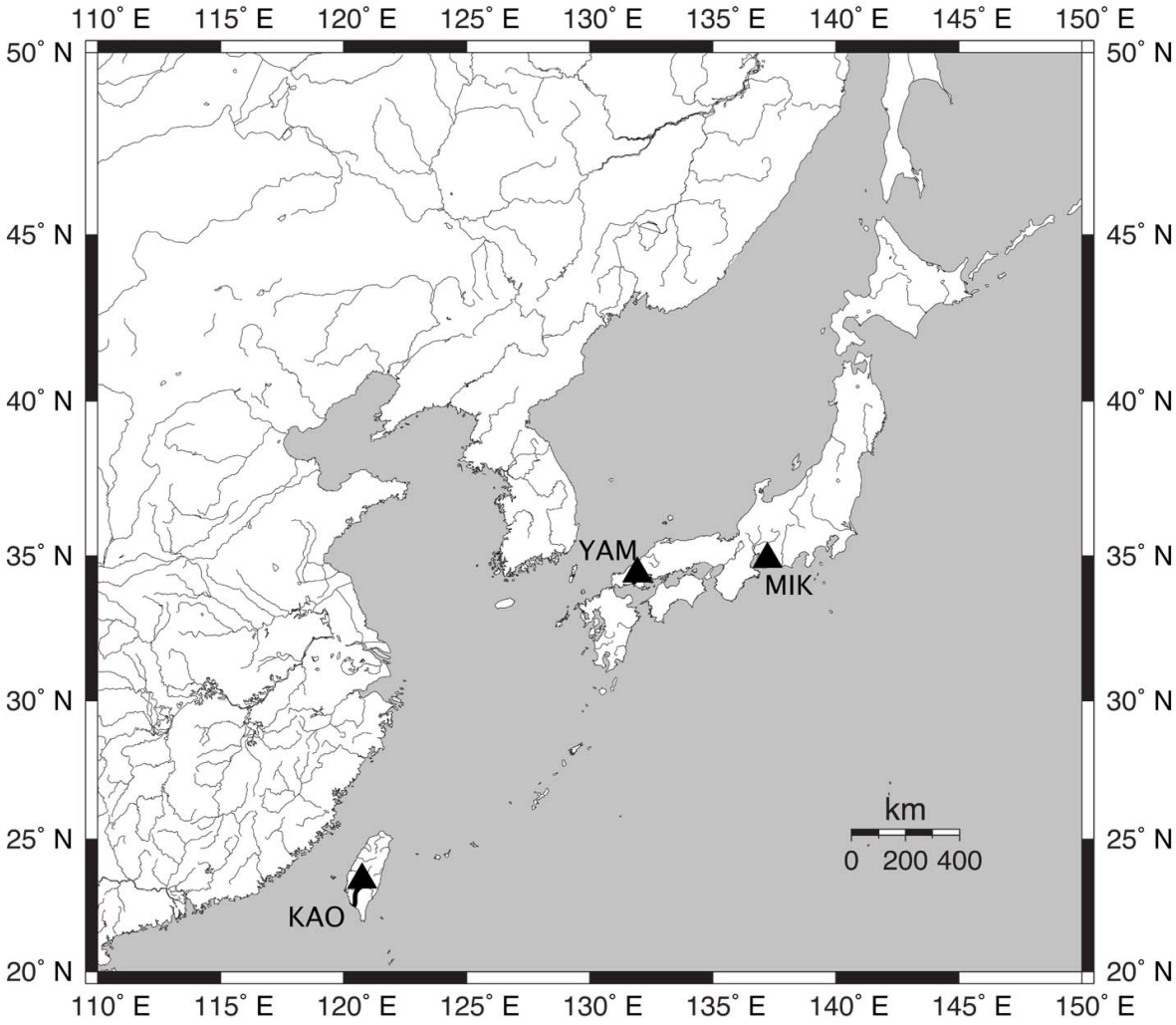

Figure 3.2 Sampling locations of *Anguillicola crassus* from native habitats in South-East Asia. Sample sizes are as follows: KAO, River Kao-Ping, Taiwan (n = 46); MIK, Mikawa Bay, Japan (n = 29); YAM, River Fushino, Prefecture of Yamaguchi, Japan (n = 9).

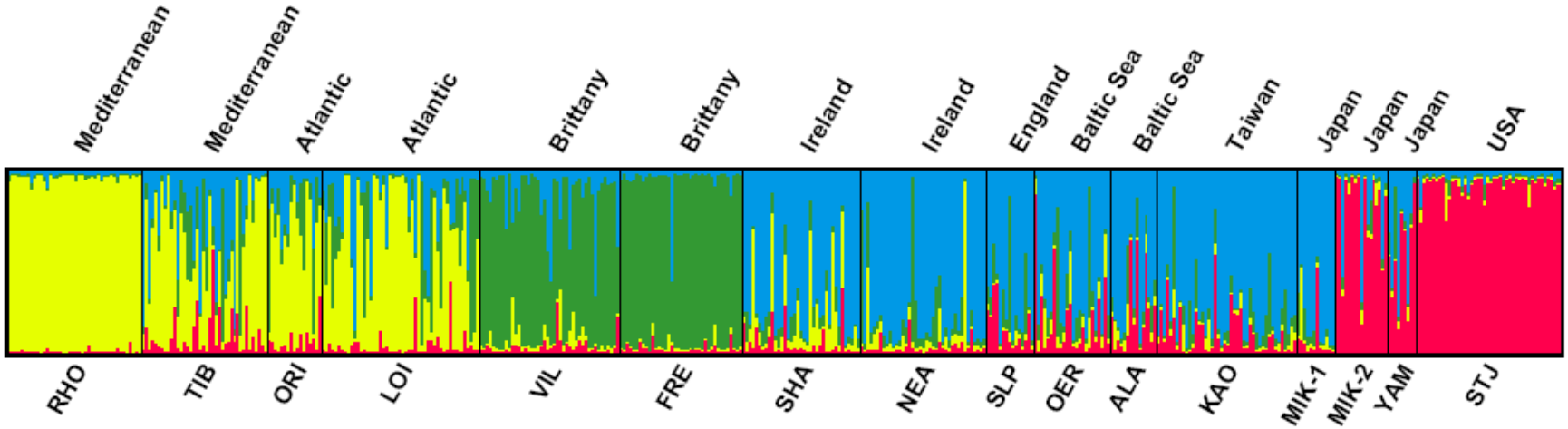

Figure 3.3 Individual-based cluster representation based on Bayesian inference of population structure (K = 4; ln$P(D)$ = -16,732.8; Burn-in period = 100,000; MCMC repeat length = 1,000,000; Pritchard *et al.* 2000). Each color represents one assumed population cluster K. Multiple colored bars display an individual's estimated membership proportion in more than one population (q), i.e. admixture. Sampling locations are ordered from Southern to North-Eastern Europe from left to right, followed by Asian and North American samples. The labels indicate sampling location (below; for abbreviations refer to **Table 3.1**) and the region of origin (above).

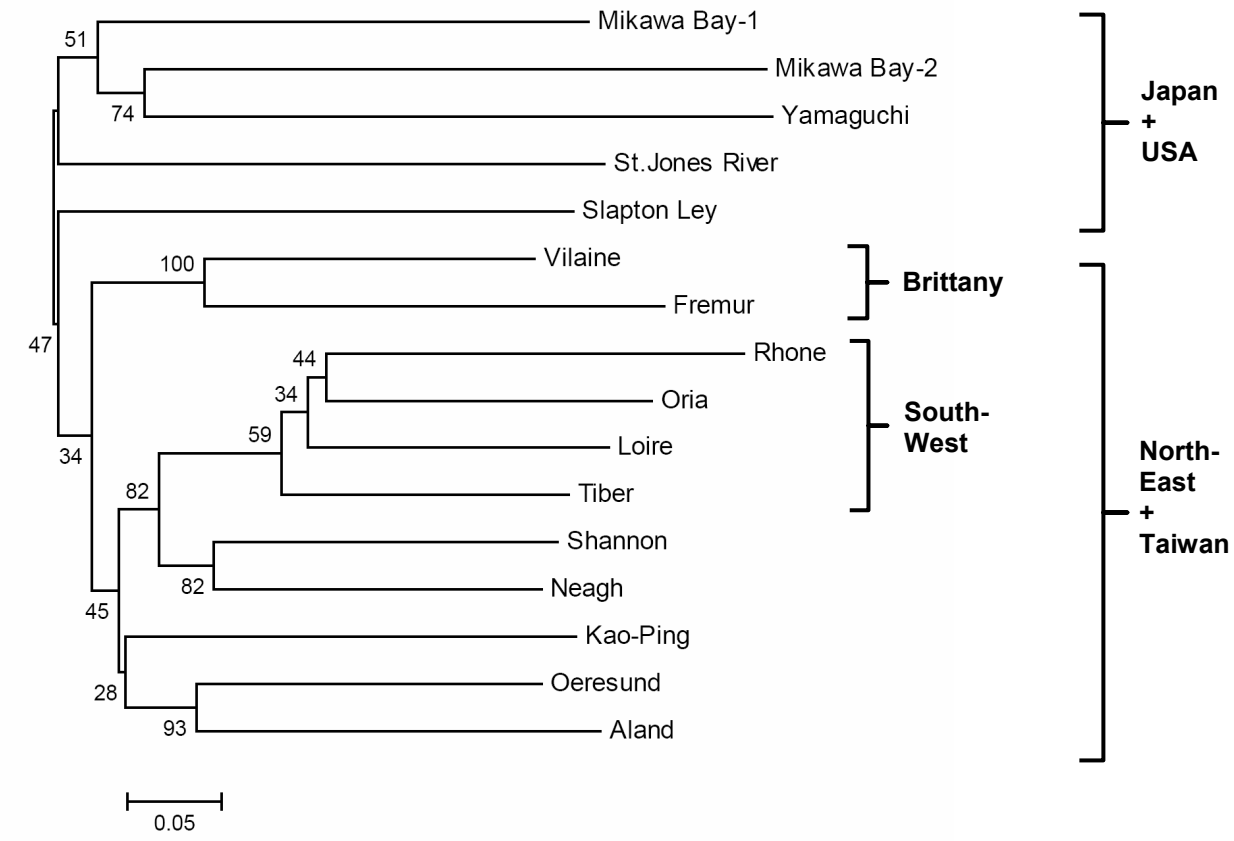

Figure 3.4 *Neighbor-joining* (NJ) phenogram summarizing Cavalli-Sforza & Edwards' (1967) D_{CE} chord distances corrected for null-alleles (Chapuis & Estoup 2007) among 12 invasive and three native populations. European groupings are highlighted by brackets based on the STRUCTURE tool. The out-group was defined according to the tree's mid-point. Values on the nodes represent the percentage of bootstrap replicates over loci (n = 100). Branch lengths are proportional to the genetic distance between the taxa. The scale bar represents a distance D_{CE} of 0.05.

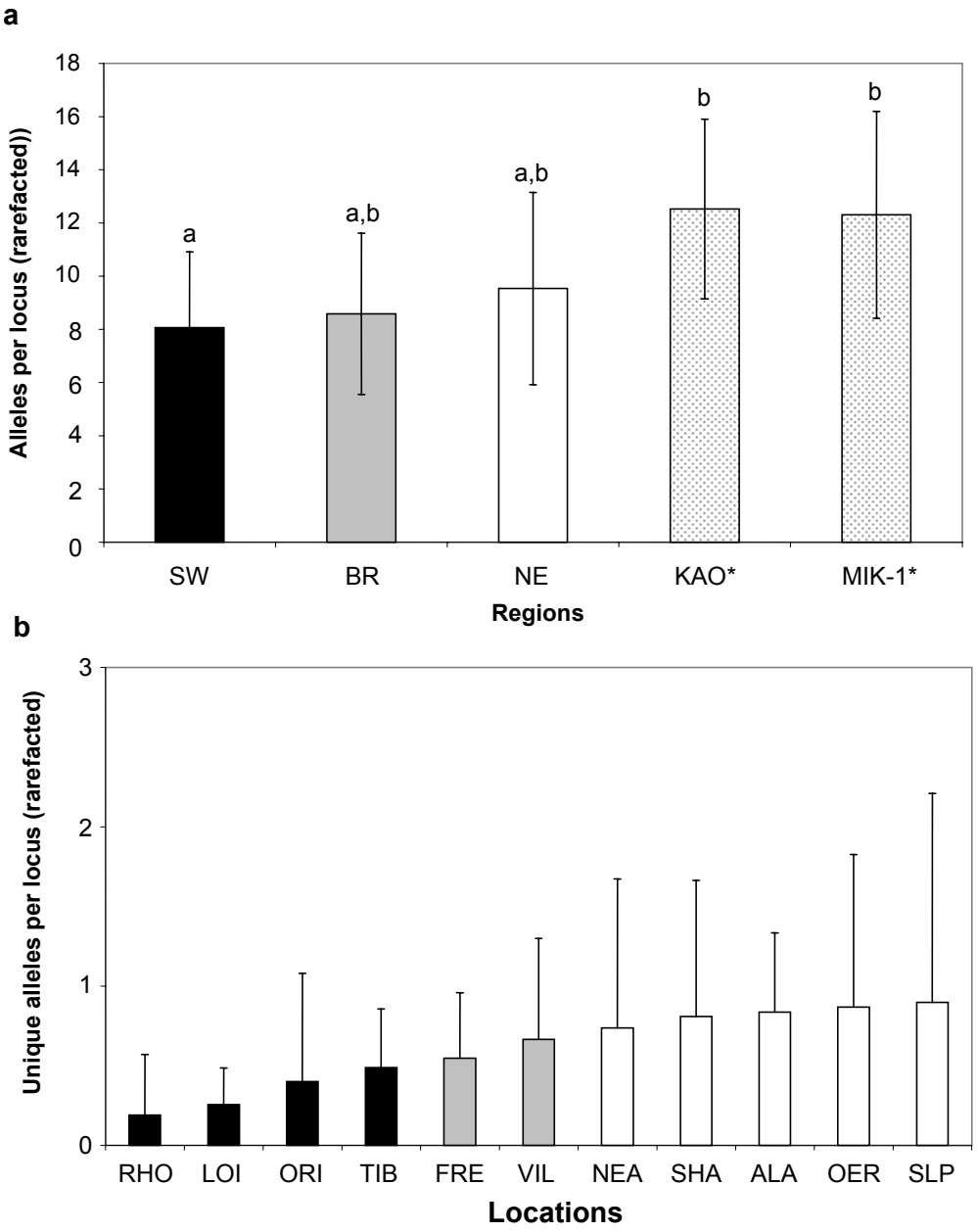

Figure 3.5 Box plot representation of **a**) mean allelic richness (rarefacted number of alleles per locus) between invasive European and native Asian (*) regions; SW (black), South-Western European locations; BR (grey), Brittany locations; NE (white), North-Eastern European locations; KAO, Kao-Ping, Taiwan (dotted); MIK-1, Mikawa Bay 1, Japan (dotted); and **b**) mean allelic uniqueness (rarefacted number of private alleles per locus) among European localities only; bar colors display geographic grouping as indicated in plot a). Error bars indicate the SD of arithmetic means. Means not significantly different from one another share the same small letters (a, b) above the error bars ($\alpha = 0.05$).

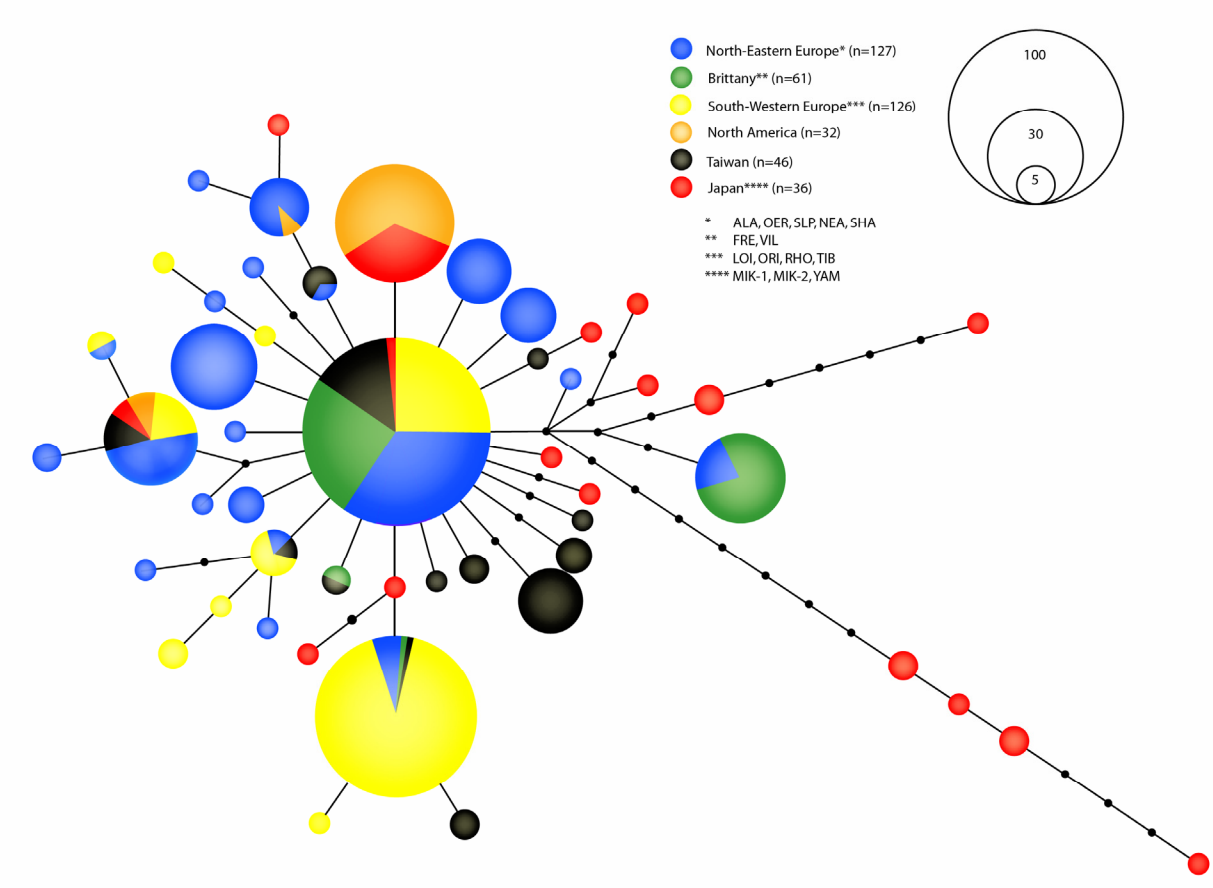

Figure 3.6 Minimum spanning haplotype network of mitochondrial COI-haplotypes. Circles represent one mutational change toward either connection; the areas of circles and circle sections are directly proportional to the number of individuals sharing the same haplotype sequence. Unsampled haplotypes are represented by small black dots. All haplotypes sequences have been submitted to GenBank (Accession nos. EU376536-EU376954).

Table 3.1 Sampling locations of *Anguillicola crassus* specimens including information on habitat characteristics, geographic position, sample sizes used for genetic analyses, year of catchment and two parasitological parameters (Bush *et al.* 2001), i.e., parasite prevalence (relative numbers of eels infected) and mean infection intensity (number of adults per infected eel swimbladder).

#	Ref	Site (Country)	Hab	Geographic Position		Sample Sizes		Date of Collection	Prevalence [%]	Mean infection intensity	Sample collector
				Latitude (dec)	Longitude (dec)	mtDNA	STR				
1	ALA	Åland Islands (SF)	R	60.12N	19.90E	16	15	Jul- Aug 2005	25.5	3.6	HPF
2	OER	Kullen, Øresund/ Kattegat (S)	M	56.18N	12.27E	30	24	Oct 2003	46.4	8.6	HW;PC
3	SLP	Slapton Ley (GB)	R	50.24N	3.68W	15	15	May- Jun 2005	53.9	1.8	PB
4	NEA	Lake Neagh (GB)	L	54.65N	6.22W	31	40	May 2005	100	7.8	DE
5	SHA	Lough Dergh, Shannon (IRE)	L	52.67N	8.63W	30	37	Sept 2005	n.d.	n.d.	KMcC
6	FRE	Bois Joli, Frémur (F)	L	48.56N	2.08W	31	39	Oct 2006	57.1	6.3	SW;JG;JMC
7	VIL	Brain-sur-Vilaine (F)	R	47.7N	1.90W	30	44	Jun 2005	64.9	3.9	CB
8	LOI	Angers, Loire (F)	R	47.5N	0.57W	32	50	Feb 2005	71.7	3.7	TW
9	ORI	Oria (E)	R	43.27N	2.03W	30	17	Jun- Sep 2005	25.0	3.7	ED
10	RHO	Camargue, Rhône (F)	R	43.50N	4.50E	30	42	Oct 2004- Mar 2005	n.d.	6.3	AC
11	TIB	Roma, Tiber (I)	R	41.80N	12.60E	30	40	1996	66.3	5.2	EC; FB
12	KAO	Tung-chiang, Kao-Ping (RCA)	R	22.51N	120.42E	46	44	Oct 2006	60.0	2.8	HT;YSH
13	MIK	Mikawa Bay (JP)	R	35.47N	137.07E	29	29	Aug 2005- Feb 2006	1.57	2.8	JA
14	YAM	Yamaguchi, Fushino (JP)	R	34.17N	131.48E	7	9	Oct 2006	n.d.	2.5	HT;HS
15	STJ	St.Jones River (USA)	R	39.07N	75.42W	32	45	Aug 2005	34.0	n.d.	CC;DF

#, consecutive number; Ref, reference name; Hab, habitat characteristic; R, riverine; L, lacustrine; M, marine; mtDNA, mitochondrial DNA; STR, microsatellites; HPF, Hans-Peter Fagerholm; PB, Polly Bown; HW, Hakan Wickström; PC, Patrik Clevestam; DE, Derek Evans; KMcC, Kieran McCarthy; CB, Cédric Briand; ED, Estibaliz Diaz; AC, Alain Crivelli (AC); SW, Sébastien Wielgoss; JG, Jérome Guillouët; JMC, Jean-Marie Caraguel; EC, Eleonora Ciccotti; FB, Federica Berrilli; CC, Colette Cairns; DF, Dewayne Fox; JA, Jun Aoyama; HT, Horst Taraschewski; HS, Hiroshi Sato; YSH, Yu-San Han.

Table 3.2 Pairwise θ_{ST} values for all sampling locations (above diagonal mitochondrial COI locus; below diagonal 7 microsatellite markers combined).

	Regions	SOUTH-WEST				BRITTANY		NORTH-EAST					TAIWAN	JAPAN			USA
Ref	(S;M)	RHO	TIB	ORI	LOI	VIL	FRE	SHA	NEA	SLP	OER	ALA	KAO	MIK-1	MIK-2	YAM	STJ
RHO	(42;32)		**0.097****	**0.094****	**0.074****	0.483*	0.607*	0.437*	0.517*	0.711*	0.478*	0.620*	0.496*	0.496*	0.648*	0.584*	0.741*
TIB	(40;30)	0.036*		-0.016	-0.019	0.256*	0.444*	0.206*	0.284*	0.453*	0.256*	**0.278***	0.227*	0.378*	0.437*	0.364*	0.536*
ORI	(17;30)	0.042*	**0.023***		0.008	0.253*	0.453*	0.225*	0.305*	0.461*	0.256*	0.299*	0.248*	0.377*	0.446*	**0.371***	0.536*
LOI	(50;32)	0.025*	0.016*	**0.016****		0.289*	0.454*	0.227*	0.304*	0.462*	0.289*	0.305*	0.256*	0.392*	0.435*	0.375*	0.537*
VIL	(44;30)	0.116*	0.041*	0.049*	0.053*		**0.144***	**0.104***	**0.065****	0.300*	**0.107***	**0.080****	0.093*	0.294*	0.324*	**0.173****	0.388*
FRE	(39;31)	0.169*	0.103*	0.087*	0.114*	0.042*		0.287*	**0.218***	0.465*	0.345*	**0.347***	0.364*	0.373*	0.460*	**0.218****	0.550*
SHA	(37;30)	0.080*	0.025*	0.043*	0.041*	0.042*	0.100*		0.000	0.318*	0.155*	**0.096***	0.098*	0.325*	0.338*	**0.237***	0.423*
NEA	(40;31)	0.092*	0.036*	0.061*	0.049*	0.040*	0.089*	**0.015***		0.292*	0.142*	**0.069****	0.093*	0.329*	0.327*	**0.217***	0.406*
SLP	(15;15)	0.135*	0.083*	0.087*	0.099*	0.084*	0.097*	0.067*	0.051*		0.235*	0.382*	0.320*	0.346*	0.523*	0.422*	0.644*
OER	(24;30)	0.092*	0.034*	0.034*	0.045*	0.038*	0.077*	0.038*	0.040*	0.066*		**0.055**	0.094*	0.303*	0.326*	0.264*	0.377*
ALA	(15;16)	0.097*	0.039*	0.037*	0.058*	0.038*	0.068*	0.040*	0.038*	0.056*	0.001		**-0.011**	**0.293***	0.384*	**0.294***	0.496*
KAO	(44;46)	0.083*	0.038*	0.042*	0.044*	0.035*	0.060*	0.035*	0.035*	0.056*	0.030*	0.020*		0.386*	0.358*	0.307*	0.414*
MIK-1	(12;12)	0.090*	0.037*	**0.038***	0.047*	0.042*	0.056*	**0.029***	**0.026***	**0.045***	**0.022****	**0.023***	**0.017***		**0.312***	0.125	0.486*
MIK-2	(17;17)	0.210*	0.161*	0.150*	0.163*	0.160*	0.173*	0.163*	0.176*	0.199*	0.146*	0.139*	0.120*	0.146*		0.367*	0.025
YAM	(09;07)	0.202*	0.148*	0.126*	0.147*	0.134*	0.121*	0.130*	0.132*	0.170*	0.112*	0.114*	0.084*	0.123*	0.156*		0.557*
STJ	(32;32)	0.165*	0.079*	0.110*	0.118*	0.090*	0.115*	0.090*	0.097*	0.105*	0.082*	0.069*	0.069*	0.088*	0.174*	0.129*	

***P* < 0.05; **P* < 0.01 significantly different (10,000 Permutations); bold values were not significant after Bonferroni-correction (P<0.0004);
Ref, Reference Name; S, STR markers; M, mitochondrial marker.

Table 3.3 Summary of global indices of differentiation and correlations between genetic differentiation were performed according to Mantel (1967) using straight geographic distance among European sampling localities.

Differentiation index	*Global value*	*r*	*P*
θ_{ST}	0.057*	-0.100	0.67
θ'_{ST}	0.320*	-0.052	0.80
$\theta_{ST\ (ENA)}$	0.056*	-0.083	0.70
$\theta'_{ST\ (ENA)}$	0.320*	-0.084	0.51
ρ_{ST}	0.059*	**0.39**	**0.028**
$\rho_{ST\ (ENA)}$	0.053*	**0.41**	**0.012**

Genetic differentation indeces are defined as follows: θ_{ST} and ρ_{ST}, raw data; θ'_{ST} , standardized data; $\theta_{ST\ (ENA)}$ and $\rho_{ST\ (ENA)}$, null-allele-corrected data; $\theta'_{ST\ (ENA)}$ both standardized and null-allele-corrected data. Asterisks indicate genetic differentiation significantly different from zero. Bold values represent significant Mantel tests with $P < 0.05$.

Chapter 4:

Parasite communities in eels of the Island of Reunion (Indian Ocean): a lesson in parasite introduction

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Published in

Parasitology Research **102**, 1343–1350

4.1 Abstract

Eel populations from the small rivers on the Island of Reunion (French Overseas Department in the Indian Ocean) were investigated with respect to the occurrence and abundance of helminths during the autumn of 2005. The native species *Anguilla marmorata* (n=80), *Anguilla bicolor* (n=23), and *Anguilla mossambica* (n=15) were studied. Six species of helminths were identified, four of them having a definitely nonnative status. Furthermore, unidentified intra-intestinal juvenile cestodes and extraintestinal encapsulated anisakid nematode larvae were present in a few eels. We found that the invasive swim bladder nematode *Anguillicoloides* (*Anguillicola*) *crassus* had been introduced into the island. Six specimens were collected, four from *A. marmorata*, one from *A. bicolor* and one from *A. mossambica*. The maximum intensity of infection was two worms. The other helminths also showed a low abundance. These species were the monogenean gill worms *Pseudodactylogyrus anguillae* and *Pseudodactylogyrus bini* and the intestinal parasites *Bothriocephalus claviceps* (Cestodes), *Paraquimperia africana* (Nematodes), and the acanthocephalan *Acanthocephalus reunionensis* Warner, Sasal, and Taraschewski, 2007. The latter species, found as intraintestinal immatures, is thought to utilize amphibians as required hosts; its status, introduced or native, could not be determined. *P. africana* was described from *A. mossambica* in South Africa and has not been recorded outside Africa. The other species are known from populations of European and American eels. However, *A. crassus* and the two *Pseudodactylogyrus* species originate from East Asia, where they are indigenous parasites of *Anguilla japonica*. Both an assignment test based on seven specific microsatellite loci and subsequent sequencing of mitochondrial haplotypes of a partial

fragment of cytochrome c oxidase 1 strongly suggest that the *A. crassus* may originated around the Baltic Sea. According to the results presented here, populations of the indigenous eel species from Reunion can be considered to harbor extremely isolationist alien parasite communities. Our findings support the hypothesis that during the present time of global biological change, invasion by a nonnative species into a target island is more likely to reflect the political affiliation of the colonized environment and the pathways of trade and tourism than geographic proximity between donor and recipient areas or other natural circumstances.

4.2 Introduction

Theoretical considerations on the structure of animal communities, including parasites, on islands and their continuous supplementation by new species arriving from adjacent continents have long been considered under the premise that the processes involved are governed by nature. According to the "colonization time hypothesis", the helminth species richness in a fish host on an island is related to the time since the respective host arrived (Rohde 1989; Guégan & Kennedy 1993). Natural invasions and colonization of islands have always taken place, but since humans have begun increasingly and dramatically to alter the earth, the vast opportunities for anthropochore transport have accelerated and reinforced this phenomenon, making island environments very vulnerable to exotic invaders (Taraschewski 2006). For the indigenous freshwater fishes of tropical oceanic islands, a characteristic pattern can be determined: species belonging to the families Gobiidae, Eleotridae, Kuhliidae, Anguillidae, and a few others dominate the species poor communities. These fishes have an amphidromous or catadromous mode of life (Tesch 2003; Font 2007; Froese & Pauly 2007). The native or endemic species share their habitats with varying numbers of introduced species, belonging to families such as the Poeciliidae (guppy, sword-tail, platy, etc.), Cichlidae (tilapia, Nile tilapia, etc.), and Cyprinidae (common carp, grass carp, etc.). The anguillid genus *Anguilla* Shaw, 1803 comprises 15 species with different distributional ranges (Watanabe 2000; Aoyama *et al.* 2001; Tesch 2003). Due to their catadromous biology combined with a pronounced migratory behavior, eels are typical elements of the native fish fauna of rivers and lakes of marine islands in many geographical regions (Froese & Pauly 2007). Along the Southwest Indian Ocean, four species have been identified: *Anguilla bicolor bicolor* McClelland, 1844, *Anguilla marmorata* Quoy and Gaimard, 1824, *A. mossambica* Peters, 1852 and *Anguilla nebulosa labiata* Peters, 1852 (Tesch 2003; Keith *et al.* 2006). For Reunion Island, a recent study revealed that *A. marmorata* is the most abundant eel species, *A. mossambica* and *A. bicolor* being less frequently caught. *A. nebulosa labiata* is considered to be very rare (Robinet *et al.* 2007). The Japanese eel (*Anguilla japonica*, Temminck and Schlegel), the European eel (*Anguilla Anguilla*, Linnaeus) and to a lesser extent the American eel (*A. rostrata*, Lesueur) have been intensively studied due to their great economic importance, but much less is known about the species occurring around the Indian Ocean (Tesch 2003). The same is apparent for the parasites of eels. Within the last 25 years, several helminths of the Japanese eel attained a huge interest after colonizing Europe, North Africa, and finally North America, where the two recipient host species *A. anguilla* and *A. rostrata* turned out to be highly susceptible and vulnerable, especially with respect to infections by the swim bladder nematode *Anguillicoloides* (*Anguillicola*) *crassus* (see Kirk 2003; Knopf 2006; Taraschewski 2006). The parasite was named *Anguillicola*

crassus until it was recently transferred to the genus *Anguillicoloides* by Moravec (2006). In contrast, for long, there were no reports on alien parasites having invaded populations of the eel species occurring around the Indian Ocean (Taraschewski *et al.* 2005), but, recently, the East Asian monogenean *Pseudodactylogyrus anguillae* (Yin & Sproston, 1948) has been recorded from juvenile *A. mossambica* in South Africa (Christison & Baker 2007). In the present survey, we investigate for the first time the macroparasite community of the three common eel species of Reunion Island. The parasites of *A. marmorata* and of *A. bicolor* have not as yet been surveyed. Moreover, it was the aim of this work to estimate the origin of the introduced swim bladder nematode and consider this introduction in the light of global change.

4.3 Materials & Methods

The volcanic island of Reunion is situated in the Western Indian Ocean about 1,600 km east of Africa, about 800 km east of Madagascar, and about 160 km southwest of the island of Mauritius. Reunion, politically belonging to France, has a length of about 70 km and a width of about 50 km (**Fig. 4.1**). Eels were collected by electrofishing in the main rivers of the island during September 2005. These small, swiftly flowing streams can be seen in **Fig. 4.1**. Altogether, 118 eels (80 *A. marmorata*, 23 *A. bicolor* and 15 *A. mossambica*) were collected (**Table 4.1**). The fishes were brought to the laboratory in oxygenated tanks and were killed by decapitation prior to dissection and parasitological examination. These were performed by the first two authors of this communication. Eel species identification was done following Ege (1939), considering the coloration of the back and the ratio between the anterior end of the anal (LA) and the dorsal (LD) fins and total body length (TL; ratio = [(LA − LD) / TL)×100]). This ratio is particularly important for small fishes or when coloration is not clear enough. It allows differentiation between short-fin species (ratio<2% for *A. bicolor bicolor*) and long-fin species (ratio>14% for *A. marmorata*, <14% for *A. nebulosa labiata* and around 14% for *A. mossambica*; Ege 1939; Tesch 2003). Specimens belonging to *A. marmorata* from about 15 cm in length have a well-marked marbled dark brown-greenish coloration. All eels were measured to the nearest millimeter (total length in centimeter) and weighed to the nearest gram (empty weight in grams). The gills and digestive tract were removed and examined under a binocular-dissecting microscope for parasites. The swim bladders were opened and inspected for adult helminths with overhead light. Worm larvae inside the swim bladder wall were sought in squash-prepared tissue. The parasites collected were fixed according to different procedures prior to their identification: nematodes in 70% alcohol, cestodes were relaxed overnight in chilled tap water followed by adaptive frequency allocation and 40% buffered formalin, acanthocephalans were also relaxed overnight in chilled tap water followed by 5% formalin, monogeneans were fixed under a microscopic slide with Malmberg fluid. The

two monogeneans *P. anguillae* and *P. bini* were differentiated up to species level for five specimens of *A. mossambica*. For the other eels infected by these gill worms, we did not distinguish between the two species.

To trace the most likely geographic origin of the specimens of the invasive nematode *A. crassus*, a population genetic approach was applied. Whole DNA was extracted (Bruford *et al.* 1992) and gel-quantified. First, seven dinucleotide microsatellite markers were amplified as described (Wielgoss *et al.* 2007). Genotypes were analyzed and size-called by ABI's Genescan and Genotyper softwares (vers. 4), respectively. A statistical evaluation was performed in GENECLASS2 (Piry *et al.* 2004) in order to assign individuals to previously sampled invasive and endemic populations (n=490) using two different Bayesian models (Rannala & Mountain 1997; Baudouin & Lebrun 2000). A Monte Carlo resampling algorithm of Paetkau *et al.* (2004) simulated 10,000 random individuals which were compared to real data using the default α-value of 0.01. The higher the relative likelihood of stemming from a given population, the higher the assigned match score in percent by GENECLASS2. Thus, a 99% score is considered very highly likely if statistically different from chance assignment. Because DNA extracted from one individual (REU101) was found to be severely degraded, typing for this sample was limited to only a few markers. Second, a part of 552 bp of the mitochondrial gene cytochrome c oxidase subunit I (CO1) was directly sequenced from the amplicon using the recommended polymerase chain reaction protocol for universal invertebrate primers HCO2198 and LCO1490 (Folmer *et al.* 1994). Sequence data for mitochondrial haplotypes were integrated into a large dataset consisting of 419 specimens from invasive and endemic *A. crassus* populations and were analyzed under maximum parsimony criteria to infer a haplyotype network in TCS version 1.20 (Clement *et al.* 2000).

4.4 Results

A total of eight helminth species were found in the eel species investigated, six of these could be identified, while two species, occurring as intra- or extra-intestinal juveniles, remained unidentified (**Table 4.2**). We encountered the gill monogeneans *P. anguillae* Yin and Sproston, 1948 and *P. bini* Kikuchi, 1929. These parasites commonly occurred in *A. mossambica*, rarely in *A. marmorata*, and were not found in *A. bicolor*. In *A. mossambica*, they reached intensities of up to 30 worms (**Table 4.2**). For five specimens of this eel species, specific identification was done. *P. anguillae* was far more abundant (90% of the worms) than *P. bini*. The swim bladder inhabiting nematode *Anguillicoloides crassus* (Kuwahara, Niimii, and Itagaki, 1974) was detected in all three native eel species, its prevalence ranging from 4% to 8%. Intensity did not exceed two worms (**Table 4.2**). No eggs with L2-larvae were found in the lumen of the swim bladders, which might reflect the fact that just one eel (*A. marmorata*) contained two worms belonging to the same sex. *A. crassus* is

recorded for the first time from these three *Anguilla* species (see Taraschewski 2006). Inside the gut, three identifiable helminths were found, the most prevalent being *Paraquimperia africana* Moravec *et al.* 2000 occurring in about 20% of the available *A. marmorata*. *A. mossambica* was less frequently infected, whereas *A. bicolor* did not harbor this parasite. Intensity was equally low in both hosts. *A. marmorata* is a new host record for this parasite (see Moravec *et al.* 2000). The acanthocephalan *Acanthocephalus reunionensis* Smales *et al.* 2007 occurred as a satellite or as a rare species (*A. marmorata*). In *A. mossambica*, it reached intensities of up to six individuals comprising female as well as male specimens. However, even when both sexes were present, no gravid females were encountered. This is the first record of this spiny-headed worm for all three eel species (see Smales *et al.* 2007). *Bothriocephalus claviceps* (Goetze, 1782) was a rare parasite of *A. marmorata* in this study, being demonstrated in this host for the first time (see Taraschewski 2006). The unidentified intra-intestinal immature cestodes and encapsulated extra-intestinal nematode larvae were only found in *A. marmorata* (**Table 4.2**). Double infections of two different species of helminths appeared in nine eels and triple infections in one. Two of the five eels infected with *A. crassus* also showed another helminth infection. *A. marmorata* occurred in all sampled waters; conversely, the other two eels were more abundant in two or three rivers at the east coast of the island and almost restricted to these places. For all host species, the average weight and the mean length were comparably low, very large eels being absent from the samples (**Table 4.3**; compare Tesch 2003). The available data are insufficient and too scattered (in terms of abundance and prevalence) for a statistical evaluation of overdispersion and size class aggregation of the parasites in their host populations. Three of the helminth species detected in the indigenous eels of Reunion are native parasites of the Japanese eel *A. japonica* (*P. anguillae*, *P. bini*, *A. crassus*) and one (*B. claviceps*) of the two Atlantic eels (*Anguilla anguilla* and *A. rostrata*; see Taraschewski 2006). In contrast, *P. africanus* seems to be native in the East African region were it has been recorded from *A. mossambica* (see Moravec *et al.* 2000). The status of *A. reunionensis* remains doubtful (Smales *et al.* 2007).

The six specimens of *A. crassus* were subjected to a molecular analysis in order to trace their geographic origin (**Table 4.4**). Overall, DNA quality was acceptable and only DNA extracted from one individual (REU101) was found to be severely degraded. Consequently, microsatellite typing for this sample was limited to only a few markers (three out of seven). The remainder of the specimens could be more accurately typed for at least five microsatellite markers. For all but one individual (REU115), a partial sequence of 552 bp of the mitochondrial locus CO1 could be unambiguously determined for both strands. Based on GENECLASS2, Rannala & Mountain's (1997) Bayesian method (RMB) retrieved assignments of consistently high confidence (up to 99.997%), with nine out of 12

assignments being significant (**Table 4.4**). On the contrary, Baudouin & Lebrun's (2000) Bayesian method could not match this accuracy, though the population assignments were almost identical. Considering invasive populations only using RMB, individuals were assigned to the Northeastern European population, which includes the Baltic Sea (OER, ALA) and Ireland (SHA). To provide more confidence, a haplotype network based on the mitochondrial CO1 locus was considered and as a result, 50% of the samples shared an extremely seldom private haplotype, which is found only in the Baltic population of Aland (ALA). The remainder did not allow for exclusion of any locations except that from Northern America (STJ).

4.5 Discussion

This work presents the first parasite survey of fishes from Reunion Island. The parasite communities of the four species of eels from eight neighboring biotopes reveal a remarkably high species richness (eight species), coinciding with a low degree of dominance of the single parasite species. No core species exist; instead, the helminths in all hosts either occur as satellite or as rare species. Only in *A. mossambica* do *Pseudodactylogyrus* spp. reach a prevalence of more than 50%; but, here, we combined the two species *P. anguillae* and *P. bini*. Moreover, except for this host–parasite association, the intensities of all parasites are less than about ten and, thus, rather low. This is in contrast to results from a comparable study on *A. mossambica* from small rivers of the Eastern Cape on the Southern African mainland where only four (native) helminths occurred. A dominant core species showing prevalences of 70–100% and intensities partly of more than 50 worms was present there: the stomach-dwelling nematode *Heliconema longissimum* (see Taraschewski *et al.* 2005). This species is also widely distributed in populations of various anguilliform hosts in Asia and Australia (Moravec *et al.* 2006, 2007) but was absent in eels from Reunion Island. Furthermore, the intestinal nematode *P. africana*, also recorded from Reunion in the present study, reached prevalences of between 50% and about 65% and mean intensities of up to 15 worms in the eels from the African mainland. Interestingly, in that study, no monogeneans were found on the gills of the eels, but *P. anguillae* as recently detected (Christison & Baker 2007). Most populations of the two Atlantic eel species (*A. anguilla* and *A. rostrata*) were free of gill-dwelling monogeneans prior to the arrival of the East Asian parasites, *P. anguillae* and *P. bini* (Kikuchi, 1929; see Taraschewski 2006). In these two eel species, the microhabitat of the swim bladder was also unoccupied until the early 1980s when the invasive nematode *A. crassus* colonized Europe and, during the 1990s, North America (Kirk 2003). According to Kennedy and Guégan (1996), eels are generally considered to harbour species poor component- and infrapopulations with many vacant niches. Nevertheless, in tropical

mainland Northern Australia, populations of *Anguilla reinhardtii* with conspicuously diverse parasite communities have been described (Kennedy 1994). According to Esch *et al.* (1988), two categories of fish parasitic helminths are recognized in terms of parasite colonization: autogenic species which mature in fish and allogenic species which mature in vertebrates other than fish and have a greater colonization potential and ability than the ones using freshwater fishes as final hosts. This hypothesis is supported by the findings of a survey on macroparasites of sticklebacks (Gasterosteidae) on Sable Island situated about 250 km east of the Canadian Atlantic coast in which five allogenic and two autogenic species (*Gyrodactylus canadensis* Hanek and Threlfall, 1969 and *Thersitina gasterostei* Pagenstecher, 1861) were recorded (Marcogliese 1992). On Hawaii, the few native or endemic freshwater fish species reflected an extreme ecological isolation with respect to their parasite communities. Prior to the introduction of exotic freshwater fishes and parasites, they only carried infections with allogenic parasites maturing in gulls and marine mammals (Font 1998). Meanwhile, autogenic as well as allogenic nonnative parasites show a higher diversity and are more widely distributed among the native and introduced fishes of Hawaiian streams than the native allogenic ones using the fish as intermediate hosts (Font 2007). Among the alien parasites of Hawaiian stream fishes are the cosmopolitan cestode *Bothriocephalus acheilognathi* of Asian origin as well as the hirudinean *Myzobdella lugubris* Leidy, 1851, which is an autochthonic species of the Southern USA. Thus far, however, none of the introduced parasites has been traced back to its geographic origin (Font 2007). Our findings from Reunion resemble the situation described for native endemic Hawaiian gobiids, although the species richness of exotic autogenic parasites recorded from the eels in our study is even higher. Both examples of oceanic islands reveal that in the present times of global change, colonization of islands by parasites does not depend on an island's distance to the respective mainland or its size, but reflects the territory's political affiliation, the prevailing routes of trade, and other anthropogenic features.

As to the occurrence of *A. crassus* in Reunion, the genetic information seems strong enough to rule out North American and Southwestern European origins. When including mitochondrial DNA, the most likely scenario is an introduction of eels from Northern European countries, with the Baltic Sea being the best candidate. Populations from the Baltic Sea display very high heterozygosities and are genetically more diverse as compared to Atlantic or Mediterranean populations (Wielgoss *et al.* 2007). This makes such a brackish, cool water body a huge reservoir for dispersal. The 50% frequency of the private haplotype shared only with specimens from Aland (ALA) makes a single secondary spread of European origin more likely than an independent and mixed import from Asian source populations from both Japan and Taiwan, as suggested by microsatellite data including these samples. An alternative explanation may be that a yet unsampled Northern European location gave rise to

the small population found in Reunion, thus, giving more weight to the Asian signals in the Bayesian analysis, which founded both the European and American invasive samples a mere 30 years ago.

Frenot *et al.* (2005) reviewed the literature on alien microbes, fungi, plants, and animals occurring on most of the sub-Antarctic islands which are under the administration of France or Britain, as well as parts of the Antarctic continent. They found that the large majority of aliens are European in origin. In the present case, the introduced species also came from Europe, although most are of East Asian origin. On the island of Reunion, we identified two fish farms as well as a supermarket where European eels were temporarily kept alive. It is, then, highly probable that some European eels may have escaped from a fish tank or pool, leading to the spread of its exotic parasites. The geographic origin of *A. crassus* in Reunion followed up in this study reveals a degree of globalization which has not yet reached its climax. If this was the case, European and Japanese eels would have been imported from China, where gigantic eel aquacultures have been built up out-competing the formerly successful eel farming first in Japan and then in Taiwan (see Taraschewski 2006). In its native host *A. japonica* and its natural distributional range, *A. crassus* reveals a moderate abundance and a low degree of pathogenicity. Conversely, in populations of European and the American eels, the prevalence and intensity are significantly higher which coincides with a substantially increased size of parasite individuals and conspicuous pathogenicity. This difference seems to result from a lack of adaptation between host and parasite in the novel Atlantic hosts, which do not develop a concomitant immunity like the well-adapted natural host (Würtz & Taraschewski 2000; Lefebvre *et al.* 2002, 2004; Knopf 2006; Münderle *et al.* 2006; Taraschewski 2006). One should assume that the invasion of *A. crassus* (and parts of the other nonnative eel parasites encountered on Reunion) will be followed or is already being followed by its spread and establishment in the eel populations of Mauritius, Madagascar, as well as South and East Africa. However, the eel species occurring in this region should not be as naïve as *A. anguilla* and *A. rostrata* with respect to their defense against the swim bladder nematode because they occur together with *Anguillicoloides papernai* Moravec and Taraschewski, 1988 which, however thus far, has only been recorded from *A. mossambica* in South Africa (Taraschewski *et al.* 2005). Thus, it is doubtful whether *A. crassus* will attain the same conspicuous speed of dispersal, high abundance, and pathogenicity as described from Europe in its novel African range. In Europe, it first appeared around 1982 in Germany, and then colonized most populations of the European eel throughout the continent in less than 10 years before it finally reached Ireland after 16 years (Kirk 2003). Its spread was largely facilitated by anthropogenic transfers. After the parasite had invaded England, its dispersal followed the routes taken by the lorries transporting eels for stocking purposes. The resting points of the lorry drivers,

where they exchanged the maintenance water of the eels, could be identified as the stepping stones in the dispersal of the exotic nematode (Kennedy and Fitch 1990). As for the two invasive monogeneans encountered in the native eels of Reunion, the host species should be completely naive because they obviously did not have previous contact with dactylogyrids and perhaps with other monogeneans as well. Both *Pseudodactylogyrus* species are very successful colonizers (Buchmann *et. al.* 1987; Hayward *et al.* 2001) and, thus, the very recent first record of *P. anguillae* from *A. mossambica* kept in a fish pond in South Africa (Christison & Baker 2007) is not surprising. The low abundance of all helminths species recorded from eels of Reunion, introduced as well as authochthonic (*P. africana*), appears to result from the ecological conditions prevailing within the swift, small rivers with their low diversity of invertebrates potentially serving as intermediate hosts. Even in Europe, where the prevalence of *A. crassus* in *A. anguilla* ranges around 60–90%, streams revealed a lower abundance of this copepod- and ostracod-transmitted parasite than lake biotopes (Münderle 2005).

4.6 Conclusion

Summarizing, there is currently an increasingly globalized world fisheries industry. Isolated oceanic islands may show a higher parasite species richness for the same host species from the adjacent continent if the island is affiliated with a country which is more developed and has a higher rate of trade than the respective mainland. For the eels of Reunion, we found an assemblage of native and introduced species with global players which have East Asia as their source area, being the dominating element. Thus, the hypotheses related to island colonization, which were elaborated 30 or more years ago (MacArthur & Wilson 1967) at a time when the large scale international displacement of species had not yet become a major ecological phenomenon, no longer fit the facts.

4.7 Acknowledgements

Financial support for the field work was provided by the Centre national de la recherche scientifique, Departement des Sciences de la Vie for P. Sasal and by Karlsruher Universitätsgesellschaft for H. Taraschewski. Participation of F. Moravec in this study was supported by a grant (524/06/0170) from the Grant Agency of the Czech Republic. T.N. Petney kindly checked the English. Thomas Bücher has typed the manuscript.

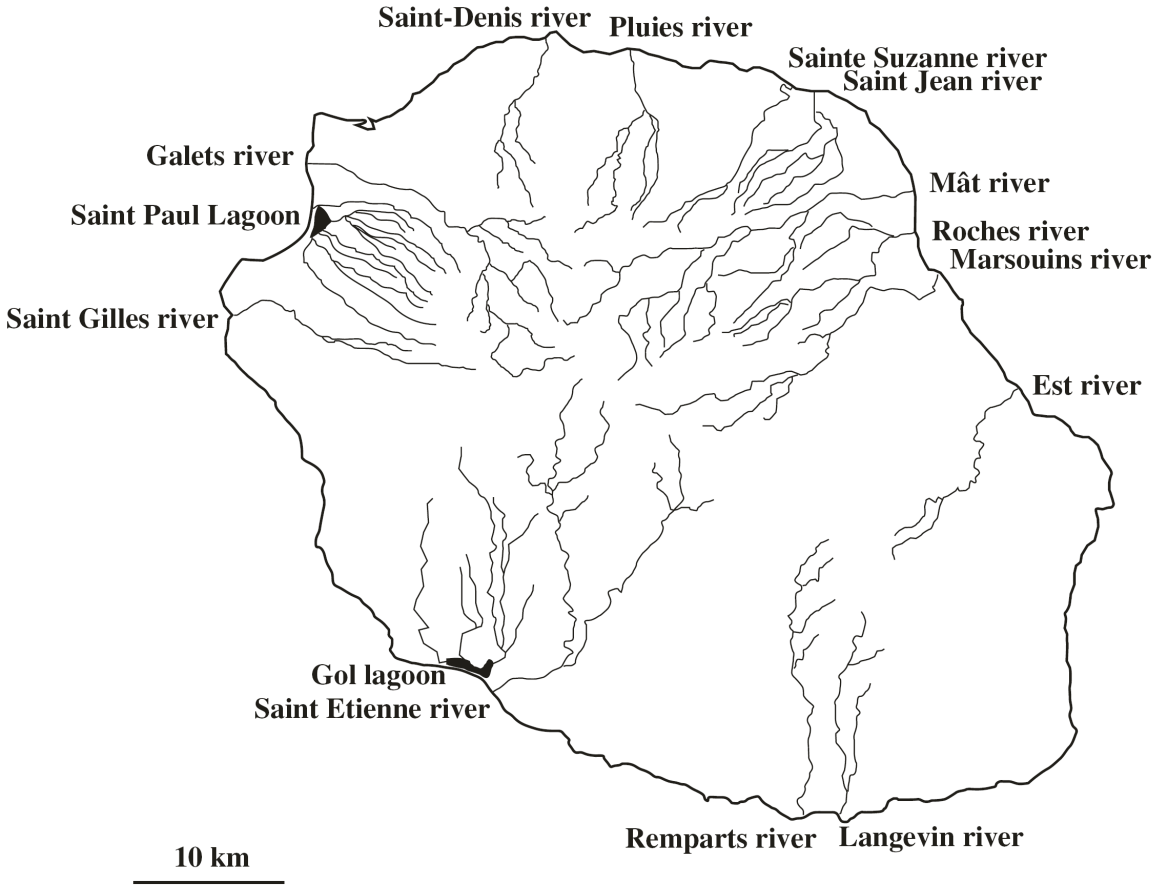

Figure 4.1 Map of the Reunion Island with the sampled rivers. 165×128 mm (600×600 DPI)

Table 4.1 Sample size of the eels from the rivers and lagoons of the Reunion Island which were dissected (compare **Fig. 4.1**).

River	*Anguilla bicolor*	*Anguilla marmorata*	*Anguilla mossambica*
Saint Jean River	14	12	9
Roches River	5	15	6
Saint Gilles River	4	14	0
Remparts River	0	21	0
Marsouins River	0	13	0
Gol Lagoon	0	3	0
Saint Paul Lagoon	0	1	0
Mât River	0	1	0
Total	23	80	15

Table 4.2 Prevalence and intensity (min–max) of the parasites species found in the collected eels from Reunion Island.

Species	*Anguilla marmorata*	*Anguilla bicolor*	*Anguilla mossambica*
n	80	23	15
Pseudodactylogyrus spp. (*P. anguillae* & *P. bini*)	2.5% (1)	0	60% (1-30)
Anguillicola crassus	3.8% (1-2)	7.7% (1)	6.7% (1)
Paraquimperia africana	21.3% (1-2)	0	13.3% (1-2)
Acanthocephalus reunionensis	1.3% (1)	13.0% (1-4)	20% (1-6)
Bothriocephalus claviceps	2.5% (1-2)	0	0
Unidentified intra-intestinal cestode larvae	7.5% (1-3)	0	0
Unidentified extra-intestinal nematode larvae	10% (1-12)	0	0

Table 4.3 Mean weights (empty weight in grams ± SE), lengths (total lengths in cm ± SE) and range of LA-LD ratio (min-max in %) of the dissected eels.

Species	*Anguilla bicolor*	*Anguilla marmorata*	*Anguilla mossambica*
Mean weight	41.8 ± 41.4	58.6 ± 61.9	34.6 ± 22.9
Mean total length	31.0 ± 9.0	29.4 ± 8.6	25.8 ± 6.4
LA-LD ratio	0 – 3.4	10.8 – 22.1	12.1 – 16.6

Table 4.4 Assignment of individual nematode specimens from Reunion to known European and American subpopulations according to log likelihood ranking.

Individuals	*n*	Microsatellites												mtDNA type	Origin
		Rannala & Mountain (1997)						Baudouin&Lebrun (2000)							
		Colonies			+Endemic			Colonies			+Endemic				
		Pop	Score (%)	*P*	Pop	Score (%)	*P*	Pop	SCORE (%)	*P*	Pop	SCORE (%)	*P*		
REU44a	6	SHA	88.861	**0.018**	KAO	99.997	**0.027**	ORI	60.271	0.003	KAO	28.416	0.001	ALA	Ireland
REU44b	5	OER	99.105	**0.085**	KAO	99.989	**0.265**	OER	55.970	0.008	KAO	80.234	**0.108**	ALA	Baltic
REU113	7	OER	99.982	0.008	YAM	99.346	0.003	OER	64.709	0.000	YAM	90.395	0.001	ALA	Baltic
REU115	7	ALA	96.692	**0.011**	MIK	99.998	**0.130**	ALA	69.785	0.009	MIK	64.201	**0.240**	-	Baltic
REU131	7	ALA	94.673	**0.030**	MIK	95.552	0.000	ALA	49.554	0.009	MIK	86.123	0.000	all	Baltic
REU101	3	OER	91.834	**0.221**	KAO	45.254	**0.116**	OER	64.672	**0.296**	OER	27.210	**0.121**	not US	Baltic

n, # loci; Bold probability values (*P*) indicate significant relationship between assigned and proposed population of origin (α>0.01) using 10,000 simulated samples according to Paetkau *et al.* (2004). REU, Reunion Island; SHA, Shannon River (Ireland); OER, Oeresund (Baltic Sea); ALA, Aland (Baltic Sea); KAO Kao-Ping (Taiwan), YAM, Yamaguchi (Japan); MIK, Mikawa Bay (Japan); ORI, Oria (Atlantic)

Chapter 5:

Assessing the use of an invasive eel parasite, *Anguillicola crassus*, as biotag for eel migratory behaviour and stock management using a population genetics approach

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Unpublished

5.1 Abstract

The utilization of parasites as tags for lowly structured marine and migratory fishes has a long tradition in stock management. However, the use of population genetics tools is not widely applied in this field of parasitology. Thus, combining the latter approach to study parasitized, but lowly structured fish hosts offers a great potential for both conservation and stock management. As recruitment levels of European glass eels (*Anguilla anguilla* L.) have collapsed by 90-99% points since the early 1980s, the European Commission has recently proposed a Council Regulation to establish management actions for protection and restoration of the eel spawner stocks. Here, relying on microsatellite markers and genetic data from a phylogeography study, we demonstrate the usefulness of the omnipresent invasive nematode, *Anguillicola crassus*, as indicator of eel stocking by contrasting samples of two European rivers differing in management of eel recruitment. We can show that while under natural eel recruitment nematode samples meet the expectations of Hardy and Weinberg for a single panmictic population, frequent stocking of farm eels is reflected by a low but significant F_{ST} value among within-host populations (infrapopulations) along with high inbreeding indeces F_{IS} consistent over all loci. Moreover, these signals are not sex-specific or biased by marker defects. Utilizing statistics tools, we demonstrate high levels of admixture and the presence of first generation migrants from mostly Northern European locations, and conclude that a Wahlund effect due to very recent geneflow gives rise to increased F_{IS} values. Finally, we find evidence for frequent introduction of pairs of related and unrelated individuals into the same infrapopulations in one river most likely due to larval cluster transmission via sufficiently large intermediate hosts.

5.2 Introduction

Recruitment levels of European glass eels (*Anguilla anguilla* L.) have collapsed by 90-99% points since the early 1980s (Moriarty 1986, 1996; Dekker 2000, 2003). Thus, in response to the dramatic economic consequences for fisheries (Stone 2003), the European Commission has recently proposed a Council Regulation to establish management actions for protection and restoration of the eel stocks (COM 2005, 472). Since freshwater eels are unevenly distributed over the European content and parts of North Africa (Dekker 2000), the corner stone of this initiative is the development of regional and local restoration programs, ultimately involving many independently acting organizations of its member states. Thus, a "Study Leading to Informed Management of Eels" (Acronym: SLIME) was initiated under the 6th framework programme of the European Union. The programme assessed different regional and global models aiding eel stock management (SLIME 2006). One main conclusion highlighted that measures for securing the productiveness and stability of European eel stocks, are undermined by the only poorly understood population dynamics of eels. Thus, in the short term impact of local stocking in freshwater on survival and spawner escapement needs to be understood in order to devise fruitful management action plans.

The eel's peculiar life-cycle comprises a long-distance migration loop (Tsukamoto *et al.* 2002) and a continental resident stage covering up to 6,000 km in the open ocean to reach its spawning grounds in the Sargasso Sea. Importantly, natural distribution of eel recruits is considerably distorted by anthropogenic intervention. For example stocking compromises migratory behavior of stocked eels in the Baltic Sea (Westin 1990; Westin 2003) and displacement of millions of glass eels from Western Europe (e.g., the British Isles, North-Western France, and the Netherlands) into Eastern and central parts considerably increases distances to the spawning grounds. More worryingly, a considerable amount of recruits is automatically lost to serve the Asian food market, where eels are an important part of the culinary tradition (Ringuet *et al.* 2002). Consequently, highest priority is assigned to the restoration of spawning stocks capable of escaping from the continental waters towards the open ocean (SLIME 2006).

However, the direct assessment of escapement success (local stock identity) or the control of stocking regulations is difficult to monitor. Usually, it is necessary to apply external tags (e.g., Carlin tags; Westin 1990) to follow eel migration, but this does not allow for routinely tracking eels on a global scale. Thus, the use of biological (genetical) tags appears most suitable. Given the eel's very low population genetic structure across the whole distribution range (Lintas *et al.* 1998; Wirth & Bernatchez 2001, 2003; Maes & Volckaert 2002; Dannewitz *et al.* 2005), we suggest to use the eel's parasite fauna as a surrogate for monitoring eel transfer and migration behaviour. The movement and connectivity of stocks of

lowly structured marine and migratory fishes by means of their parasite communities has a long and successful tradition (e.g., Herrington *et al.* 1939; Mosquera *et al.* 2000; MacKenzie 2002). Recently, Criscione *et al.* (2006) demonstrated that the assignment of salmonid steelhead trouts (Onkorrhynchus mykiss) back to their river of origin is more reliable if a freshwater-dependent trematode parasite is used instead of the host itself. However, while the application of genetics tools to trace movement animals by means of their related parasites and pathogens has been proposed repeatedly (Wirth *et al.* 2005; Nieberding & Olivieri 2007), this feature remains largely unexplored for aquatic organisms, and there is no example of parasite tags to observe catadromous fish movement in the literature.

One strong candidate for tracking European eel residents and migrating spawners is the omnipresent rhabditid nematode *Anguillicola crassus* (Superfamily: Dracunculoidea: Family: Anguillicolidae). Sampling and classification of nematodes is easy and it reaches average abundance over 50% and mean infection intensities around 5 nematodes /eel (Wielgoss *et al.* 2008a). It is native to South-East Asia, where it parasitizes the swimbladder of its obligate final host *Anguilla japonica*. However, following intercontinental trading routes of eel stocks, the nematode has occupied new freshwater eel host species around the world. Within the past 30 years, *A. crassus* has firmly established in Europe (Kirk 2003) and also spreads in the American eel, *A. rostrata* along the US East Coast (Barse *et al.* 2001). The only observed barriers to the aquatic parasite's dissemination are average temperatures below 4°C and high salinity (i.e., marine sea water; see Kirk 2003 for a general review), and thus, the distribution range of *A. crassus* almost matches that of its eel host, except for Iceland (Kristmundsson & Helgason 2007). The North Atlantic eel does not show specific immunological defense mechanisms (Knopf 2006), and most European wild eels harbour several adult nematodes in their swimbladder (Kirk 2003; Wielgoss *et al.* 2008a). The nematode is purely sexual and its life cycle comprises one obligate intermediate host, typically (epi-)benthic copepods, and the eel as final host. Since many small prey fish of the eel, such as ruffe, sticklebacks and gobids, are also infected, they appear to be the most important sources of Anguillicolosis for large priscivorous eels (Kirk 2003; **Fig. 5.1**).

Relying on microsatellite markers, Wielgoss *et al.* (2008a) demonstrated population genetic structure in *A. crassus* among eleven evenly distributed sampling locations, following a pattern of isolation-by-distance along the European coastline. Three populations are separated in reminiscence of Briggs's zoogeographic break (1974) at the British Channel. Using this data as a baseline, Sasal *et al.* (2008) inferred that the recent parasite colonization event on the remote Island of Reunion in the Indian Ocean reflects close historical and economic bonds with Europe, while the North American parasite invasion appears to have stemmed from an independent introduction route (Wielgoss *et al.* 2008a).

Here, utilizing a population genetics approach, we assess the usefulness of the nematode as biological tag for monitoring eel stock management and eel migration behavior. Relying on microsatellite markers and using the genotype data from previously described locations across Europe (Wielgoss *et al.* 2008a) infrapopulation samples of two European rivers differing in management of eel recruitment are contrasted. First, paying special attention to parasite structure into infrapopulations, as well as sex and marker defects, we assess Hardy Weinberg expectations and population differentiation. Second, the role of admixture and the presence of first generation migrants is weighed against increased levels of relatedness, which both can lead to deviations from HWE.

5.3 Material & Methods

5.3.1 Sample Material

All eel samples were collected with fyke nets in October 2006. The first eel sample (n = 62; *A. crassus*: Prevalence = 0.32; Mean infection intensity = 5.6) derived from a small side arm along the River Rhine (RHI) in Karlsruhe, Germany (Rußheimer Altrhein; Lat 49.212N; Lon 8.398E, while the second one (*n* = 70; *A. crassus*: Prevalence = 0.57; Mean infection intensity = 6.3; Lat 48.56N Lon 2.08W) was sampled upstream a dam system called Bois Joli in the River Frémur (FRE), France. While the Rußheimer Altrhein is strongly influenced by annual eel stocking with 1,000-2,000 glass eels of lengths 10-15 cm derived from aquafarms in Germany (Hartmann, Karlsruhe, personal communication), eels are recruited naturally each year in the Bois Joli, which is situated only 6km upstream from the sea, connected by the River Frémur. This river system is equipped with eel ladders and an eel lift surveyed by the company FISHPASS in Rennes (FR). Eels were gutted and viscera were removed and processed in the lab. All adult nematode parasites were assigned a label indicating its respective eel host infrapopulation and singly stored in screw-cap tubes in 70% of Ethanol until being further investigated. For both localities, the largest infrapopulation samples were investigated with *n* = 76 (RHI) and *n* = 108 (FRE), respectively.

5.3.2 Morphometrics and sexing

Each nematode was classified and sexed according to Moravec & Taraschewski (1988), photographed and wet weights were determined to the first decimal of the mg-scale on a calibrated fine balance. Total lengths were approximated from the photographs using the CAD-programme AB Viewer version 6.3 (Softgold Ltd). The same mm-grid placed below each nematode specimen facilitated the conversion of pixel measures to the metric mm-scale (up to the first decimal). Since measures along the nematodes' central lines were highly concordant with the measures of the respective nematodes' circumferences (*n* = 62; R^2 = 0.997), the former measure was used for all nematodes. Male nematodes below the

balance's scale (<0.1mg) were excluded from the morphometric analyses, as were some female specimen due to body rupture (RHI: 2 females, 6 males; FRE: 8 females, 1 male). Arithmetic means, standard deviations and confidence intervals were calculated for individual total weights (TW), total lengths (TL), as well as the ratio of weight and length, representing the corpulence or condition factor (CF). All standard statistical evaluation procedures were performed in Excel® version 2003 (Microsoft).

5.3.3 Molecular Analyses

Genomic DNA was extracted using the high salt precipitation technique for animal tissues devised by Bruford *et al.* (1992). Individuals were screened at four microsatellite markers, *AcrCT27*, *AcrCT53*, *AcrCT54*, and *AcrCA102*, respectively, following a multiplex PCR approach (Wielgoss *et al.* 2007). The PCR products were diluted 1:20 in fully deionized water, and 1.2 µL of the bulk dilution was added to a sequencing plate containing 10.8 µL of HiDi- Formamide and 0.2 µL of internal size standard. Due to an upgrade of sequencer hardware and chemistry during the project from ABI's 3100 to a 3300xl Genetic Analyzer a total of 32 previously scored individuals were re-run on the new system to account for consistent size calling in GeneMapper.

5.3.4 Population-based microsatellite analyses

Microsatellite loci were tested for Linkage Disequilibrium and Hardy-Weinberg equilibrium using Fisher's exact test in GENEPOP ON THE WEB (Raymond & Rousset 1995). The markers were specifically tested for the presence of marker defects using the programme MICROCHECKER (van Oosterhout *et al.* 2004). The observed and expected heterozygosities, and sex-specific F-statistics were assessed using Génétix version 4.05 (Dawson & Belkhir 2001), and allelic richness after correcting for unequal sample sizes (rarefaction) were inferred using HP-RARE (Kalinowski 2005). Pairwise relatedness r_{xy} (Queller & Goodnight 1989) was calculated considering infrapopulations using IDENTIX version 1.1 (Belkhir *et al.* 2002). An AMOVA was used to compare the distribution of the overall genetic variance among locations, infrapopulations and individuals (Excoffier *et al.* 1992) in ARLEQUIN version 3.1 (Excoffier *et al.* 2005). All standard statistical evaluation procedures were performed in Excel® version 2003 (Microsoft).

5.3.5 Individual-based microsatellite analyses

Subsequently, two different Bayesian cluster techniques of individuals were utilized to estimate population genetic structure and degree of admixture without using a priori information on individual sampling locations. First, a factorial component analysis (FCA) implemented in GENETIX version 4.05 (Dawson & Belkhir 2001) extracted a set of orthogonal

axes of variation ranked by informativeness. The two-dimensional scatter plot based on the output matrix of eigenvalues was recalculated in MATLAB® version 7.1 (Release 14, SP3; The MathWorks Inc). Second, genetic admixture in either population was assessed in STRUCTURE version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). Here to cover all European populations, the dataset was complemented by previously genotyped samples from South-Western European (n = 41; Camargue, FR) and North-Eastern European (n = 29; Neagh, IR) populations. The expected number of European populations in the dataset, K = 3 (Wielgoss *et al.* 2008a), was highly supported from sampling 200,000 MCMC repeats after discarding the first 50,000 steps (burn-ins). Hence these settings were used to infer average individual population membership coefficients (*Q*) and confidence intervals ($P > 0.90$).

To identify first generation migrants in our dataset, we used Rannala & Mountain's (1997) Bayesian methods in Geneclass version 2.0h (Piry *et al.* 2004). In brief, we derived the likelihood statistics L_{home} and $\Lambda = (L_{home} - L_{max})$ (Paetkau *et al.* 2004) for individuals of either sampling site, RHI and FRE, respectively, to be first generation immigrants from a known baseline dataset comprising 362 individuals derived from 11 broadly distributed European localities (Wielgoss *et al.* 2008a). Subsequently, the probabilities for being a resident were derived for 10,000 simulated individuals, accepting P-values above 0.05.

5.4 Results

5.4.1 Morphometric differentiation: regions, sexes

Sex ratios (SR) are slightly skewed toward females in both sampling locations, with $SR_{RHI} = 1.23$, and $SR_{FRE} = 1.38$, respectively. There is a marked sexual dimorphism in *A. crassus* with females growing larger and much heavier than males in both RHI and FRE, respectively (**Table 5.1**; and **Fig. S5.1**; Appendix 1). Moreover, by separating sexes among locations, the female RHI specimens differentiate significantly based on a t-test comparing their corpulence factors ($P = 0.029$), whereas other measures show no significant difference (**Table 5.1**; and **Fig. S5.1**; Appendix 1).

5.4.2 Test for Linkage Disequilibrium and Hardy-Weinberg Equilibrium

According to an exact test for Linkage Disequilibrium among markers, no comparison indicated significant deviations at either sampling locality (P>0.05, each). While the Frémur (FRE) sample is in agreement with Hardy-Weinberg expectations (HWE) at all four loci, neither marker matched HWE in the Rhine (RHI), according to Fisher's exact test ($P < 0.0001$, each). The F_{IS} values were consistent among marker loci (**Table 5.2**), while they are not sex-specific (**Table 5.3**). Heterozygote deficits in the RHI are evenly distributed over all size classes at each locus, according to the MICROCHECKER programme (van Oosterhout *et al.* 2004; data not shown). Moreover, when applying a jack-knifing procedure, removing one

locus at a time, all loci contributed similarly to the given F_{IS} (**Table 5.4**). Thus, we conclude that the deviation from HWE are independent of null alleles and other marker defects, but are connected to a population-level effect.

5.4.3 Relatedness

Using infrapopulations as input structure, observed mean values of pairwise relatedness are not significantly different from zero compared to the computation of the expected value retrieved from 1,000 simulations (**Figs. 5.2 a-d**). However, the observed value of the variance was statistically higher than the mean of the test distribution (P = 0.0008) in the FRE sample, which indicates the joint clustering of related and unrelated individuals in the same swimbladder and might rely on a peculiar mode of transmission of infective larvae in this habitat. No such trend was visible in the RHI sample. In conclusion, since the FRE sample is in HWE, relatedness has no influence on panmixia and does not cause the high F_{IS} values in the RHI sample.

5.4.4 Hierarchical F statistics

An AMOVA indicated that the component adding to the overall genetic variance least was the variance among infrapopulations within regions (0.93%), whereas 4.85% of the variance is confined among regions, FRE and RHI. The highest values of genetic variation were found at the individual level, where genetic variance was confined within the individuals (85.1%), and within individuals among infrapopulations (9.13%); Φ_{IS} = 0.09691; Φ_{SC} = 0.00975; Φ_{CT} = 0.0485; Φ_{IT} = 014905. A further analysis of among infrapopulations within localities revealed a low but significant F_{ST} = 0.014; $P < 0.05$ in the RHI. On the contrary, the FRE sample was not differentiated among infrapopulations (F_{ST} = 0.0007; $P > 0.05$).

5.4.5 Signals of admixture & presence of first generation migrants in the stocked system

According to a factorial component analysis (FCA) the only split of the dataset occurs along the first axis, separating FRE from RHI samples (**Fig. 5.3**). While most samples cluster within close range of their respective group members, parts of the RHI samples scatter widely in variance space. Consequently, assuming population structure, a high proportion of admixed individuals in the RHI sample is apparent (**Fig. 5.4a**). Using Q estimates in STRUCTURE only one third of individuals appear to have a pure (here Northern) genetic background (**Fig. 5.4b**). And several single individuals appear to have been introduced as first generation migrants into the RHI. This hypothesis could be verified using GENECLASS. While there is no single instance of immigration in the FRE, the presence of three first generation migrants is supported for the RHI (**Table 5.5**). As a side note, both statistics, L_{home} and Λ, respectively, identified the same suspect individuals for the given computation method

used. Because the latter formula is most appropriate if all relevant source populations have been sampled (Paetkau *et al.* 2004), it follows that the European invasion is reasonably well represented by the sampling effort of Wielgoss *et al.* (2008a).

5.5 Discussion

5.5.1 Wahlund effect and detection of first generation migrants

The present study demonstrates the usefulness of *A. crassus* as a biological tag for eel host movement and migration. While random mating of nematodes is apparent in naturally recruiting eel hosts (FRE), the RHI sample shows strong deviation from Hardy-Weinberg expectations consistent over all loci and among sexes. While there is no sign for increased levels of relatedness to explain this observation, several independent measures give strong evidence for the detection of a Wahlund effect (Hartl & Clark 1997), as a direct consequence of annual stocking of infected farm eels. First, the highly polymorphic microsatellite markers show consistent patterns of high heterozygote deficiency (average F_{IS} = 0.25), and markers contributing most to the overall significant F_{ST} are also the ones showing the highest F_{IS} values (r = 0.953). This pattern is absent in the FRE (r = -0.855). Second, genetic differentiation among infrapopulations in the RHI is markedly higher than between samples on opposite sides of the Baltic Sea (Wielgoss *et al.* 2008a), while allelic richness corrected for sample size is double the number of the FRE found in the FRE sample. Third, the presence of first generation migrants in the RHI is highly supported using both individual clustering and assignment approaches based on Bayesian statistics.

Importantly, the origin of a Wahlund effect, thus the lumping of separate population (signals) into one, can be quickly simulated using Easypop version 1.7 (Balloux 2001). Given a single invasion event with only low genetic drift in the founder population, and assuming three regional populations after possibly 50-100 generations (Wielgoss *et al.* 2008a) with low migration rates (m < 0.01; 1D-stepping stone or island model), lumping of populations compared to separate treatment leads to results already observed in our real dataset, i.e., a) rapidly increasing F_{IS} values over generations and consistent over loci, b) positive correlation of marker-specific F_{ST} and F_{IS} values; c) inflated allelic richness.

A similar pattern of HWE deviation has previously been detected in a parasite host system comprising a marine Anguilliform species, *Conger conger*, and its trematode parasite fluke, *Lecithochirium fusiforme*. Based on 6 polymorphic allozyme makers, Vilas *et al.* (2003) inferred the influence of a Wahlund effect due to temporal mixing of divergent parasite populations in the unstructured marine habitats of conger eels, because of highly correlated F_{ST}- and F_{IS}-values. The authors attributed this effect to the high mobility of known transport fish hosts, and the possibility of low effective population sizes in parasite populations due to low survival in a coarse-grained parasite environment (Price 1977).

5.5.2 Evidence for aggregate transmission in *A. crassus*

There is evidence of mixing of related and unrelated nematodes in the same infrapopulations, as a significantly larger variance of pairwise relatedness-estimates r_{xy} (Queller & Goodnight 1989) is apparent in the FRE sample (P = 0.0008) compared to the null model of "all unrelated" individuals within infrapopulations according to IDENTIX. However, as Prugnolle *et al.* (2005) could show using a modelling approach, even the presence of several clones cannot explain increased F_{IS} levels, when there is random mating in the final host. This is exactly what is found for the natural recruitment system FRE, and thus, there is strong evidence against inbreeding, or non-sexual propagation in *A. crassus*. On the other hand, this finding is evidence for frequently clumped transmission of individual nematodes into single intermediate eel hosts in the Frémur. Such aggregate transmissions are found in some parasite-host-systems comprising sheep and frog final hosts (Boag *et al.* 1989; Zelmer *et al.* 1999). An explanation might derive from the behaviour of larval L2 stages of *A. crassus*, which are known to rapidly stick to substratum in the aquatic environment and start undulating vividly to attract potential intermediate hosts (Kirk 2003). Freshly hatched L2 already show the same behaviour within the swimbladder lumen, where they stick to loose tissue particles, constantly undulating (own observation). Given that only a limited number of females are present in one swimbladder, larvae can already be released as clumps of relateds into the environment. The difference among river systems then might simply reflect the *in situ* differences in intermediate host composition (Thielen *et al.* 2007) and the individual copepod's carrying capacity of nematode larvae (Kirk *et al.* 2000b; Thomas 1993).

5.6 Conclusion

In summary, here using standard and modern population genetics methods it is shown that the nematode invader *Anguillicola crassus* is not structured among different host vectors within freshwater systems. Thus, the mating strategy in this nematode is clearly panmictic, but depicts marked deviations from Hardy-Weinberg equilibrium when subjected to stocking with infected eels, which signal is highly consistent over all markers applied. Occasional clumped transmission of related individuals into the same eel host does not influence panmixia over generations due to the random process of eel infections in each generation. Thus, the nematode can be very useful to assist detection of stocking activities in eels (and other infected fish in the same system). Moreover, spawners in the open ocean could be traced back to both their approximate origin given a known population baseline of major river systems, and to the management system practiced there, i.e., stocked versus natural recruits.

5.7 Ackownledgements

This study was supported by a studentship of the Landesgraduiertenförderung Baden-Württemberg to SW. We owe thanks to Prof. Horst Taraschewski and Urszula Weclawski (TH Karlsruhe) for contributing samples from the RHI. Further we thank Jérôme Guillouët, Jean-Marie Caraguel, and Antoine Legault from FISHPASS in Rennes-Champie for offering logistic help with on-site sampling in the FRE, as well as Prof. Pascal Lafaille and Dr. Aurore Baisez for kindly providing us with laboratory space and equipment at the University of Rennes. We thank Kathryn Elmer for helpful comments on an earlier version of the manuscript.

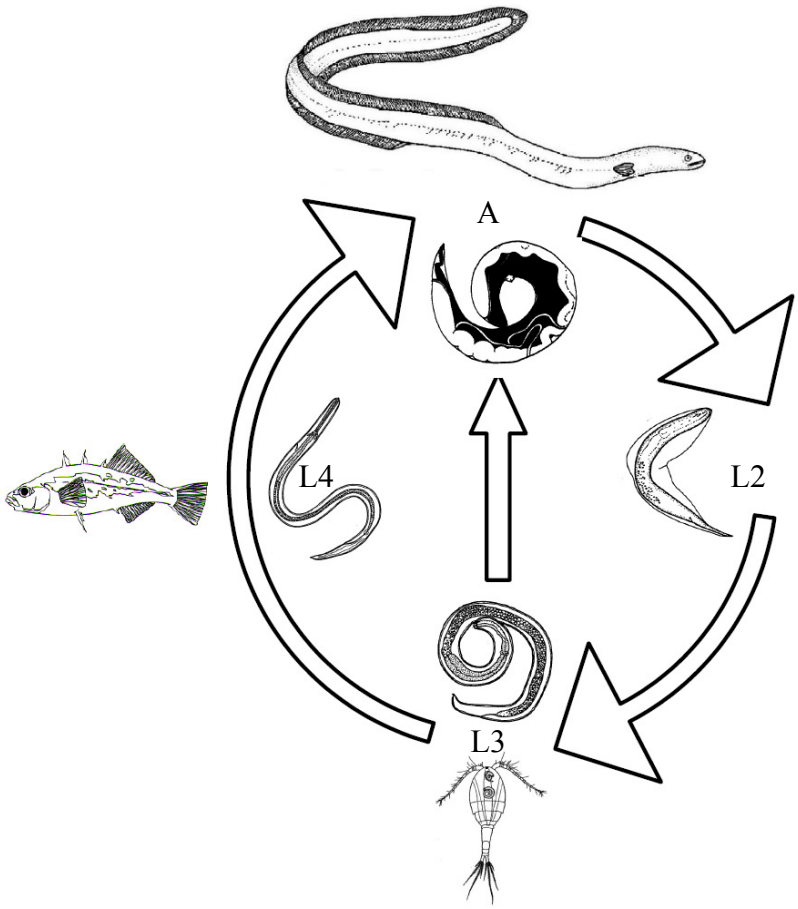

Figure 5.1 Schematic life cycle of the invasive nematode parasite *Anguillicola crassus.* The nematode reproduced sexually in the swimbladder lumen of its novel fish host, *Anguilla anguilla* in Europe. All nematodes are moulting four times (larval stages L1-L4), before getting adult (stage A). The first moulting stage is already performed within the egg sheath in the eel host's swimbladder lumen. L2 larvae are extruded to the aqueous environment over the *ductus pneumaticus*, which connects the swimbladder with the eel's intestinal tract. In the open water, L2 larvae attach to substratum and attract zooplanktonic predators by undulating. Upon ingestion, they subsequently infest in obligate copepod intermediate hosts (not species specific), and moult to the L3 larva. These larvae are infective for many freshwater fishes, in which they can moult to an L4 larva. However, life-cycle is only completed in the final host's swimbladder. Thus, other fish hosts are paratenic hosts, and this part of the life cycle is facultative. Eels can get infected by ingesting both infected crustaceans and prey fish. When inside the eel gut, larvae penetrate the mucosa and enter the swimbladder wall's connective tissue, in which they moult until they are pre-adult. Finally, individuals enter the organ's lumen after several weeks. Sexual dimorphism is apparent among males and females, and after mating females constantly release eggs and internally hatched L2 larvae into the swimbladder lumen. Adults do not exit their hosts and decay inside the swimbladder lumen, thereby causing fibrosis or inflammation of the organ.

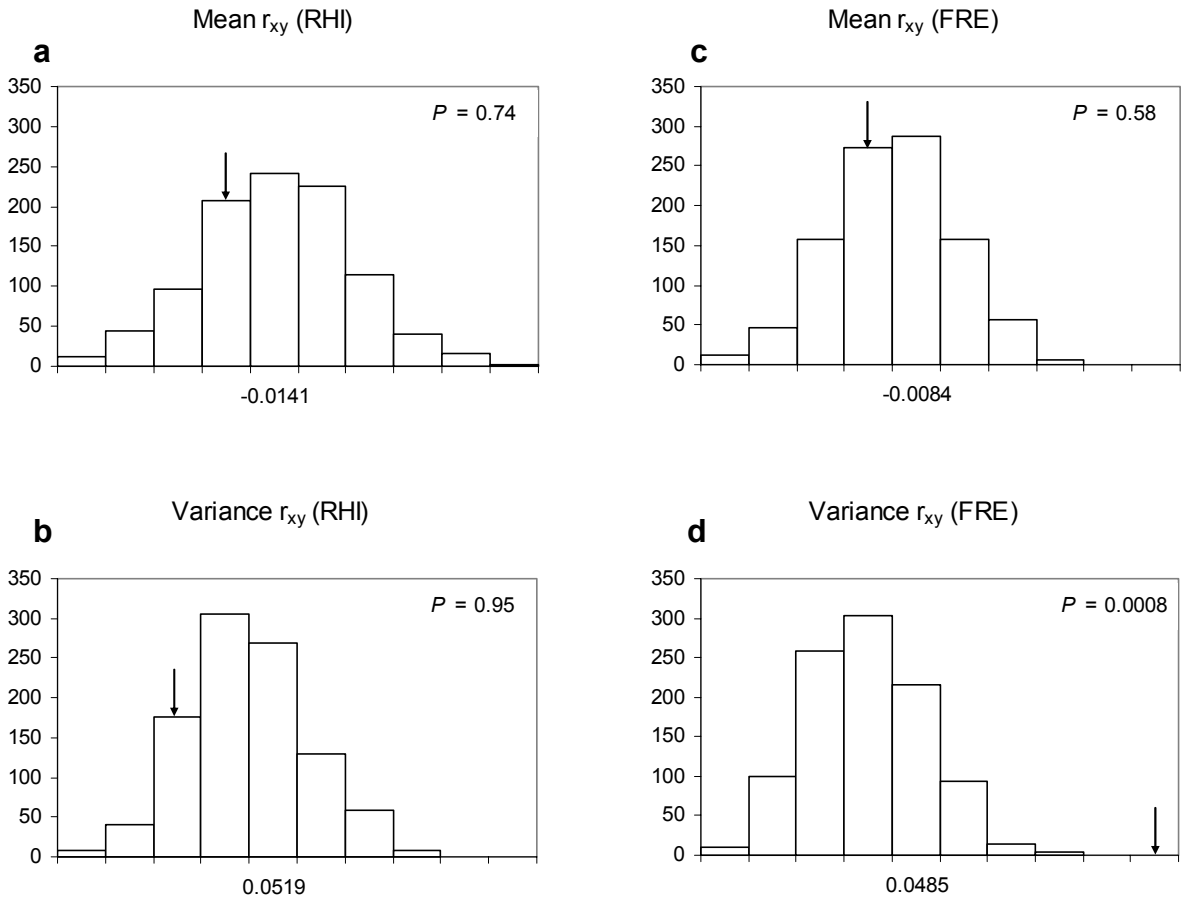

Figure 5.2 Assessment of simulated and observed measures of pairwise relatedness r_{xy} (Queller & Goodnight 1989) using IDENTIX version 1.1 (Belkhir *et al.* 2002). While **a)** & **c)** represent means of relatedness for RHI and FRE, respectively; **b)** & **d)** show variances of relatedness. Black arrows indicate the relative position of the observed value in the frequency plot, and *P*-values for the one-sided test are given to indicate significantly higher means or variances than expected from the simulation approach.

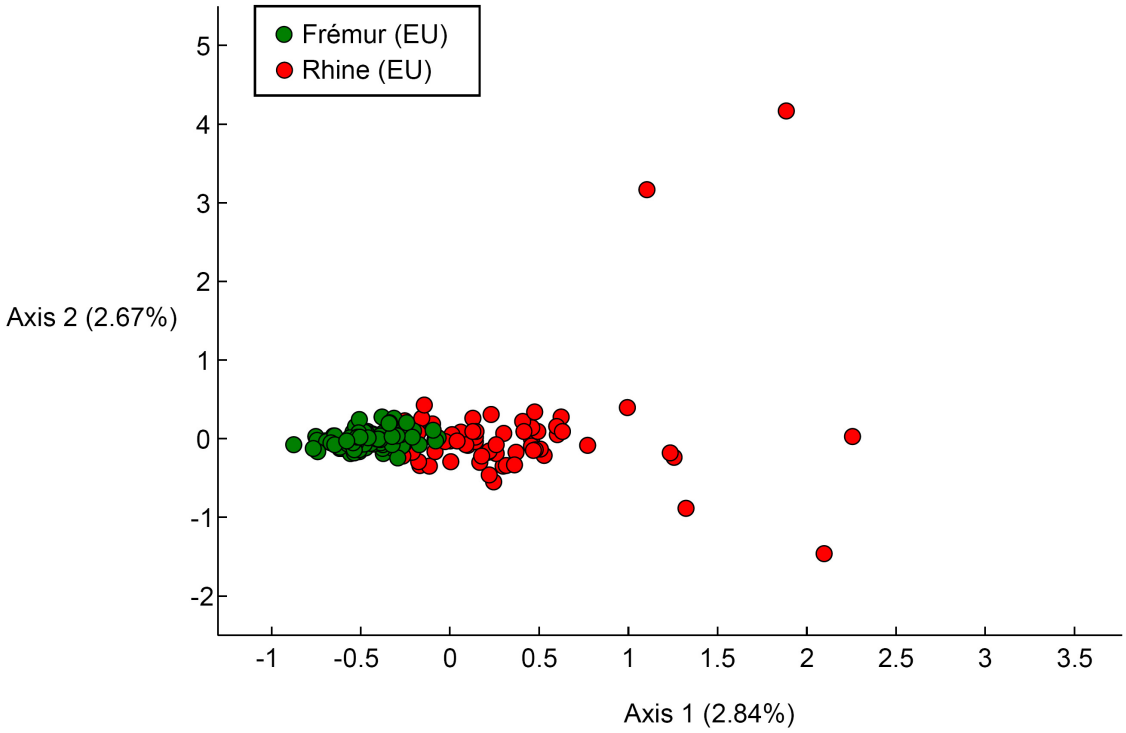

Figure 5.3 Factorial component analysis highlighting individual clustering of specimens of *Anguillicola crassus* for the first two dimensions of variance. The only split of the data is apparent among localities. While most individuals cluster in close vicinity, several outliers indicate differentiation within the RHI sample.

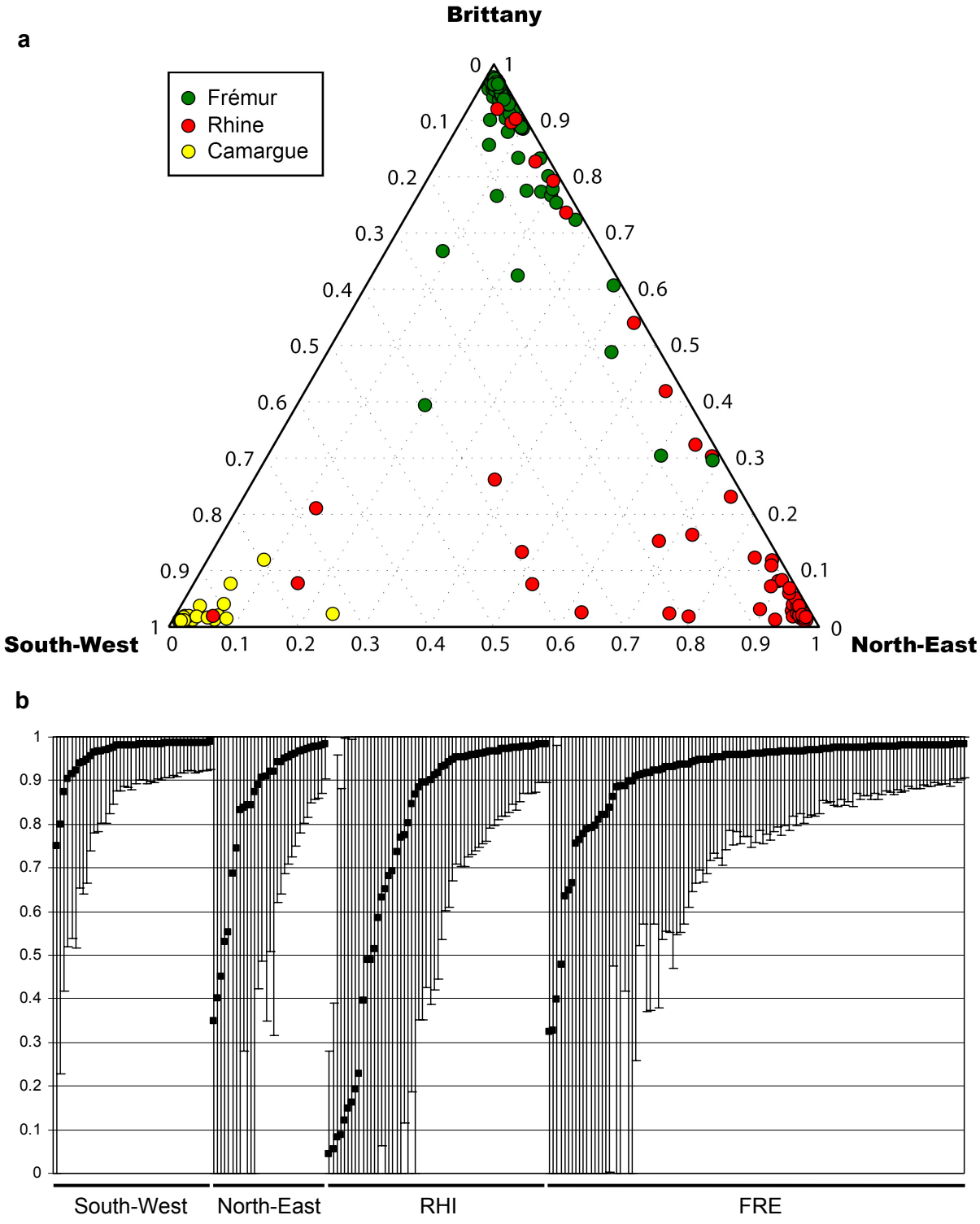

Figure 5.4 Levels of admixture of specimens of *Anguillicola crassus* within and among European sampling localities. **a)** Ternary plot of ancestry proportions (*Q*) highlights identiy of individuals according to previously detected population clusters in Europe (Wielgoss *et al.* 2008a). Pure ancestry is indicated for corner positions, whereas admixed states are present at intermediate ranges; **b)** The presence of expatriates is emphasized when using average and variance of ancestry proportions *Q* for individuals. The maximum (Q_{max} = 1.0) on the y-axis represents pure origin for each population sample, i.e., South-West for Camargue; North-East for Neagh and the Rhine, respectively, and Breton for the Frémur, while the minimum Q_{min} = 0 highlights different origins.

Table 5.1 Sample sizes, total weights, total lengths and corpulence factors of adult parasitic nematodes, *Anguillicola crassus*, listed separately for sampling locality, infrapopulation and sex.

Infrapopulation	Females				Males			
	n	TW [mg]	TL [mm]	CF [mg/mm]	n	TW [mg]	TL [mm]	CF [mg/mm]
River Rhine								
RHI04	2	82.4	25.7	2.98	3	8.17	16.3	0.48
RHI06	4	38.1	24.3	1.45	2	3.75	13.3	0.30
RHI11	5	72.7	27.3	2.38	3	25.1	32.7	0.84
RHI12	3	44.0	24.1	1.81	3	18.3	20.0	0.73
RHI21	5	40.0	25.5	1.53	6	14.2	21.1	0.65
RHI25	10	156	34.7	4.16	5	36.1	26.1	1.35
RHI40	5	247	40.5	4.64	5	31.9	24.2	1.14
RHI42	4	55.2	29.9	1.98	2	7.50	29.5	0.25
Average	38	100	30.1	2.83	29	20.8	23.1	0.813
River Frémur								
FRE04	5	105.2	27.6	3.80	3	11.0	14.0	0.81
FRE17	8	111	28.0	3.76	2	17.8	20.1	0.81
FRE18	7	86.5	24.4	3.31	4	9.69	15.8	0.59
FRE26	5	132	29.2	4.07	5	19.3	20.5	0.95
FRE45	15	136	25.5	4.95	13	30.7	21.8	1.20
FRE55	9	69.2	27.6	2.36	8	16.3	20.5	0.71
FRE56	3	88.8	21.9	3.73	4	8.00	16.2	0.42
Average	52	108	26.5	3.83	39	19.6	19.5	0.88
P_{ttest}		0.686	0.070	0.029		0.776	0.062	0.664

n = sample size; TW, total weight; TL, total length; CF, Corpulence Factor; ttest, P_{ttest}, two-sided, unequal sample sizes, H_0: RHI = FRE

Table 5.2 Measures of genetic diversity for *Anguillicola crassus* listed separated for sampling locality and microsatellite marker.

Location	$H_{E\ (n.b.)}$	H_O	F_{IS}	*A*	AR	SD	$CI_{5\%}$
River Rhine							
AcrCT27	0.9453	0.7308*	0.2269	30	25.0		
AcrCT53	0.9610	0.7273*	0.2432	39	36.2		
AcrCT54	0.7608	0.5636*	0.2592	11	9.83		
AcrCA102	0.6272	0.4483*	0.2852	9	8.69		
Total	**0.8249**	**0.6175***	**0.2514**	**22.3**	**19.9**	**13.1**	**12.9**
River Frémur							
AcrCT27	0.8912	0.8839	0.0082	14	12.9		
AcrCT53	0.8979	0.8929	0.0056	19	16.6		
AcrCT54	0.7241	0.6786	0.0628	7	7.00		
AcrCA102	0.7717	0.7411	0.0397	9	7.62		
Total	**0.8221**	**0.7991**	**0.0280**	**12.3**	**11.0**	**4.54**	**4.45**

$H_{E\ (n.b.)}$= Nei's unbiased estimate of the expected heterozygosity F_{IS}, inbreeding coefficient calculated as ($H_{E\ (n.b.)}$ - H_O) / $H_{E\ (n.b.)}$; *, deviation from Hardy-Weinberg expectations for P = 0.05; A, number of alleles; AR, allelic richness after rarefaction (104 genes).

Table 5.3 Single and averaged F_{IS} values of *Anguillicola crassus* listed separately for sampling locality, marker and sex.

		Single locus F_{IS}						
Location	**Sex**	***AcrCT27***	***AcrCT53***	***AcrCT54***	***AcrCA102***	**Mean**	**SD**	**$CI_{5\%}$**
River Rhine	F	0.316	0.283	0.203	0.301	0.276	0.050	0.049
	M	0.131	0.309	0.260	0.462	0.291	0.137	0.134
River Frémur	F	0.025	0.004	-0.026	0.003	0.011*	0.012	0.014
	M	0.048	0.040	0.080	0.085	0.063	0.023	0.022

F_{IS}, inbreeding coefficient calculated as ($H_{E\ (n.b.)}$ - H_O) / $H_{E\ (n.b.)}$; F, Female; M, Male.
*, asterisk denotes excluded negative F_{IS}-value from calculating the mean.

Table 5.4 Jackknifing procedure to depict inbreeding measures upon excluding one marker at a time highlighting the influence of removing related mating pairs from the dataset.

Jackknive (JK)	F_{IS}	F_{IT}	F_{ST}
River Rhine			
w/o AcrCT53	0.268	0.279	0.0156
w/o AcrCT54	0.271	0.286	0.0198
w/o AcrCT27	0.283	0.297	0.0194
w/o AcrCA102	0.255	0.257	0.00270
Mean	**0.267**	**0.278**	**0.0133** $^{s, 5\%}$
SD	0.0175	0.02547	0.0120
River Frémur			
w/o AcrCT53	0.0348	0.0368	0.00209
w/o AcrCT54	0.0149	0.0187	0.00391
w/o AcrCT27	0.0387	0.0349	-0.00394
w/o AcrCA102	0.0197	0.0237	0.00411
Mean	**0.0259**	**0.0276**	**0.00183** $^{ns, 5\%}$
SD	0.0173	0.0131	0.00565

Table 5.5 Detection of F_0 migrants from a baseline dataset comprising 362 individuals of 11 European sampling localities (Wielgoss *et al.* 2008a) using Rannala & Mountain's (1997) Bayesian computation method, and two different likelihood statistics –log (L_{home}) and Λ (Paetkau *et al.* 2004) as given in GENECLASS version 2.0h (Piry *et al.* 2004). Probabilities of being a resident were performed using Paetkau *et al.*'s (2004) sampling method, comparing observed genotypes with 10,000 simulated genotypes.

Individual	Λ	*P*	Assigned to	-log(L_{home})	*P*	Assigned to
Rhine04c	0.239	**0.015**	NORTH Baltic (ALA)	12.176	**0.029**	NORTH Baltic (ALA)
Rhine04d	1.780	**0.002**	NORTH Irish (SHA)	12.333	**0.002**	NORTH Irish (SHA)
Rhine06e	0.809	0.584	NORTH Irish (NEA)	7.531	0.456	NORTH Irish (NEA)
Rhine12h	0.332	0.071	WEST Breton (VIL)	10.521	0.068	WEST Breton (VIL)
Rhine21f	1.569	0.372	NORTH Irish (NEA)	8.129	0.212	NORTH Irish (NEA)
Rhine25n	1.604	**0.024**	NORTH Irish (SHA)	10.342	**0.016**	NORTH Irish (SHA)
Rhine40h	0.535	0.181	SOUTH Mediterranean (TIB)	9.218	0.174	SOUTH Mediterranean (TIB)

Λ = -log(L_{home})/ -log (L_{max}); P = probability; ALA, Aland Island (FI); SHA, Shannon (IR); NEA, Neagh (IR); VIL, Vilaine (FR); TIB, Tiber (IT); bold values denote significant assignment below the 5% level into the given locality

Part C: Natural and anthropogenically driven hybridization in freshwater eels and its consequences on eel stock management.

Chapter 6:

Introgressive hybridization and latitudinal admixture clines generate isolation by distance patterns in the European eel.

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unpublished

6.1 Abstract

The North Atlantic freshwater eel species, *Anguilla anguilla* (European eel) and *A. rostrata* (American eel) display a remarkable catadromous life cycle. Despite the importance of their marine life stages, dispersal and migration strategies remain largely unknown. Moreover, the presence of hybrids in Iceland indicates overlapping spawning areas in the vicinity of the Sargasso Sea. No study to date has explicitly focused on the influence of hybridization on genetic differentiation in North Atlantic eels. Here, using both microsatellite and mitochondrial markers, we investigated the occurrence of genotypic clines over the whole North Atlantic distribution area for these species. While mitochondrial lineages remain 100% distinct on both sides of the Atlantic, the hybridization signal expands further to continental stocks in the nuclear lineage, with a latitudinal admixture cline that peaks in the northern areas and decreases linearly approaching the Southern range limits on both continents. No pure American expatriate was apparent in Iceland, while the average ancestry proportion of Icelandic eels carrying American haplotypes was exactly intermediate between continental eel stocks. When simulating increasing proportions of F1 individuals from the Southern to the Northern-most locations we were able to generate a highly significant isolation by distance (IBD) pattern, reminiscent of previously published data (Wirth & Bernatchez 2001). Therefore introgressive hybridization alone is sufficient to explain the correlation of geographic and genetic distances reported in the European freshwater eel. The contrasting information gathered from mtDNA and nuclear markers provides evidence for a recent onset of gene flow between the two Atlantic eel species after a secondary overlap. Several mechanisms and models of hybridization are presented and discussed in detail, and consequences of our results are highlighted for the management of declining North Atlantic eel stocks.

6.2 Introduction

Hybridization, the interbreeding of diagnosable populations, is a major focus in evolutionary studies (Barton & Hewitt 1985, 1989; Harrison 1990) as it is a key concept to understand demographic and/or evolutionary cohesiveness of natural populations (Wirth *et al.* 1998; Waples .& Gaggiotti 2006). Hybridization has great potential to rapidly introduce variability into a recipient population, if barriers to recombination can be overcome. In contrast, the time needed to accumulate beneficial mutations and fitter genotypes is significantly longer. For example, it took 33,000 generations in a non-recombining laboratory strain of *E. coli* to make use of previously unutilizable citrate (Blount *et al.* 2008). In this system which basically relied on historical contingency, a couple of preceding mutations were necessary to finally enable mutants to compete with 'wildtype' cells in the same demographic "population". On the other hand, Cooper (2007) put forth strong experimental evidence that sexual recombination speeds up the adaptation process in an artificial environment compared to purely clonal strains of *E. coli*, thus supporting the theoretical predictions from the Fisher-Muller model (Fisher 1930, Muller 1932). Based on the evidence derived from empircal data, including invasive species (Cox 2004) and signatures of massive horizontal gene transfer in a paradigmatic long-term asexual species (Gladyshev *et al.* 2008), it might be generalized that in order to quickly adapt, any mechanism of lateral gene transfer or recombination is highly favoured in novel or unstable environments. Importantly, the same might apply for secondarily overlapping populations after a pahse of allopatry, such as is assumed for the North Atlantic eels (Avise *et al.* 1990).

Traditionally, zoologists are reluctant to consider hybridization as an important evolutionary process that generates new species, since the pre- and post-mating barriers to establishing F1 hybrids are often considerable (Arnold 1997). Thus, many evolutionary biologists have held that natural hybrid populations among different nominal species are unstable or ephemeral at best (Darwin 1872, Mayr 1942; Dobzhansky 1937; Wilson 1965). However, this view is in stark contrast to the plethora of known examples of reticulate evolution in nature (Arnold 1997), and above all in plants and fungi (e.g., Arnold *et al.* 1990, 1991; Rieseberg *et al.* 1990, 1995; Xie *et al.* 2008). Arnold (1997) highlighted that the extent to which hybridization might become evolutionary relevant must necessarily depend on the frequency of mating opportunities to finally produce successful recombinants which are better adapted to certain environments than are their homotypic parents. In general, hybrid zone dynamics are characterized using one of three different models that differ in their assumptions concerning the relative hybrid fitness and the genotype-environment interactions. The most commonly referenced scenario in animals, termed the 'Tension Zone Model' (Barton & Hewitt 1985), holds that hybrid zones are smooth transects across population ranges that stabilize due to a shifting balance of dispersal and selection against

viable hybrids, regardless of environment (e.g., fire-bellied toad hybrid zone among *Bombina bombina* and *B. variegata* near Krakow in Southern Poland; Szymura 1993). A second concept, the 'Mosaic Model', agrees with the 'Tension Zone Model' in that hybrid fitness is assumed to be uniformly lowered, however, it assumes a patchy hybrid zone distribution characterized by abrupt trait changes (e.g., *Gryllus* crickets in Harrison 1986, 1990; Rand & Harrison 1989). In stark contrast with the former concepts, in the 'Bounded Hybrid Superiority Model' (Endler 1977, Moore 1977) hybrids are supposed to be better adapted to peculiar environmental conditions (called ecotones) relative to their parents, and cannot stably occur beyond certain boundaries irrespective of their dispersal capabilities. Thus, assessing hybrid fitness and categorization into first and later generation hybrids is a prerequisite in order to be able to make a clear statement about actual fitness differences (Arnold & Hodges 1995). However, direct estimates of fitness often remain difficult in natural populations.

A very well known feature of hybrid zones are joint changes at several independent characters, resulting in parallel frequency gradients (clines), and such a linkage is measurably exemplified in North Atlantic eel species, *A. anguilla* and *A. rostrata*. Here, a cytonuclear disequilibrium has been identified in the narrow zone of species overlap in Iceland (Avise *et al.* 1990; Asmussen & Arnold 1991; Arnold 1992), which scales well with a transition zone based on vertebral counts, a nearly diagnostic charcter trait among the two species of eels (Williams *et al.* 1984; Avise *et al.* 1990).

The North Atlantic eel species, *Anguilla anguilla* (Europe) and *A. rostrata* (America) display a remarkable catadromous life-cycle that comprises two long-range migrations ("loops") in the open ocean, a continental growing phase, and a spawning stage in the Sargasso Sea (Tesch 2003). Despite the key importance of the marine phase (Knights 2003), Maes *et al.* 2006a concluded that most of the scientific investigations focused on the continental phase, and hence disregarded differential selection regimes that influence the eel's life-history characteristics. To this day, migration routes and exact spawning places remain hypothetical (Schmidt 1925; Tesch *et al.* 1979; Schoth & Tesch 1982; Kleckner & McCleave 1988). Fifty years ago, to the great surprise of the scientific community, Tucker (1959) proposed to consider the European eel as an evolutionary dead-end and attributed vertebral count increase in European freshwater eels to the elongation of the larval stage compared to the American eel. However, with the advent of new molecular markers the genealogical cohesiveness of the two species was largely clarified (**Table 6.1**). Several studies provided strong evidence for the two-species-status (deLigny & Pantelouris 1973; Avise *et al.* 1986; Minegishi *et al.* 2005), originally proposed by Schmidt (1925).

Recently, three independent studies reported isolation by distance (IBD) in *A. anguilla* which is clear evidence against the long-held paradigm of panmixia (Wirth & Bernatchez 2001; Maes & Volckaert 2002; Dannewitz *et al.* 2005). This signal is congruent with the

frequent measurement of very low, but highly significant genetic structure within European eels (**Table 6.1**). Interestingly, no IBD pattern was detected in the American eel (Wirth & Bernatchez 2003). Moreover, Maes *et al.* (2006a) showed that the inter-generational signal of isolation by time (IBT) in *A. anguilla* is even more pronounced than IBD for certain years. Thus, several scenarios of larval homing were suggested to explain these different models of genetic structure in *A. anguilla*.

Wirth & Bernatchez (2001) suggested that IBD patterns are due to a stable temporal delay of spawning migration in eels from Northern habitats, as distances are markedly extended compared to Western and Southern European habitats. Alternatively, more than one reproductive area is used and different currents carry the leptocephali back to their parent's original freshwater habitat. Thirdly, but less likely, assortative mating among regional groups might be responsible. Recently, Maes *et al.* (2006a) suggested that, given the random factors affecting spawning success in the open ocean, a sweepstake strategy (Hedgecock 1994) might explain genetically patched recruits in sampling locations across Europe (Pujolar *et al.* 2006), and thus cause a strong IBT signal. Finally, IBD might be produced by inter-species hybridization clines and these might explain the emergence of an IBT signal if the number of hybridization events is fluctuating over time.

The literature on hybrid eels is rather scarce (**Table 6.2**). Williams *et al.* 1984 found matches between vertebral count and nuclear genetic intermediacy in Icelandic eels. Later, the same authors (Avise *et al.* 1990) estimated the hybrid fraction in Iceland to be 2-4%. Recently, Albert *et al.* (2006) evaluated the extent of hybridization and tested for the occurrence of hybrids beyond the first generations using 376 AFLP markers. A total hybrid fraction of 15.5% was identified, of which 30% were assigned to the later generation hybrids group, a result that implies hybrid survival. Moreover, the frequency increase of hybrid individuals in the resident yellow eel stages relative to the recruiting glass eels suggests hybrid vigour and increased hybrid fitness. Interestingly, recent data on otolith microstructure and microchemistry suggests, that Iceland represents an intermediate habitat among North Atlantic eels (Kuroki *et al.* 2008). Moreover, Icelandic eels lack the usually sharp changes in in Sr:Ca-ratios upon mainland arrival, illustrating therefore the peculiar environmental conditions that glass eels have to cope with in Iceland.

No study to date has explicitly tested the influence of hybridization among North Atlantic eels on the extent of genetic differentiation. Here, using microsatellite and mitochondrial markers and by combining and extending available data (Wirth & Bernatchez 2001, 2003), we investigate the occurrence of genotypic clines over the whole North Atlantic distribution area of the eel . We then use a simulation approach to explicitly test if the original IBD in European eels (Wirth & Bernatchez 2001) could be explained by admixture clines.

Finally, we quantify the amount of gene flow that would be necessary to generate such a correlation.

6.3 Material & Methods

6.3.1 Samples

A total of 1,263 North Atlantic eels were collected in 1999. The study includes twelve European (n = 561), one Icelandic (n = 300) and eight North American (n = 402) samples (see Wirth & Bernatchez 2001, 2003). In order to test the power and reliability of the microsatellite markers, three Pacific eel species were also included in the dataset, i.e., the Australian shortfin eel (*A. australis*, n = 110), the New Zealand longfin eel (*A. dieffenbachii*, n = 96) and the Japanese eel (*A. japonica*, n = 94) (**Table S6.1**; Appendix 2).

6.3.2 Mitochondrial DNA diagnosis and sequencing

All North Atlantic eel samples were screened by PCR-RFLP analysis of a 362 bp segment of the cytochrome b (cytb) (Tagliavini *et al.* 1995). This test is based on a diagnostic HinfI restriction site, specific to American eels. In order to confirm this quick screening approach, all American haplotypes detected in Iceland were directly sequenced for the cytb fragment on an ABI 377 automated sequencer. Incorporating known sequence data from Genbank, a haplotype network based on maximum parsimony was constructed in TCS version 1.20 (Clement *et al.* 2000) relying on an alignment of 278bp. This network was compared to a maximum likelihood tree to correct for ambiguous, multiple connections. In brief, all redundant sequences were removed from the data set, and the best-fit model of sequence evolution (HKY + G) was chosen based on the agreement of all Information Criteria (cAIC, AIC2, BIC) used in MODELGENERATOR version 0.85 (Keane *et al.* 2006). Based on the estimated shape parameter of the γ-distribution, $\alpha = 0.02$, the proportion of invariable sites, Pinvar = 0.8849, and an expected transition-transversion ratio of 6.28, a phylogenetic maximum-likelihood tree was inferred in PHYML Online version 3.0 (Guindon & Gascuel 2003) and compared to the haplotype network derived by the parsimony approach in TCS.

6.3.3 Microsatellite genotyping

Considering a subset of 125 Icelandic eels, a total of 1088 North Atlantic eel specimens were genotyped using nine microsatellite markers. Original genotypes for seven microsatellite loci (Wirth & Bernatchez 2001, 2003) were supplemented by two additional loci, *Ang075* and *Aro146* (Genbank AF237903 and AF237904). The same procedure was applied for the Pacific eels. The fragment sizes were determined by reference to a size standard run using the software GENESCAN v2.1 and GENOTYPER v2.0, respectively.

6.3.4 Data analysis

Allelic diversity, genetic variation and deviation from HWE were calculated with GENEPOP on the web (Raymond & Rousset 1995) and GENETIX version 4.05 (Dawson & Belkhir 2001). Genetic differentiations were calculated with ARLEQUIN version 3.1 (Excoffier *et al.* 2005). Individual ancestry proportions in North Altantic eel species were estimated using STRUCTURE version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007) performing 100,000 burnin steps followed by 1,000,000 MCMC repeats and three iterations to reach chain convergence. Resulting ancestry proportions for the most likely number of populations (K = 2) were compared in two different models. First, we ran an admixture model without informed prior to infer the individual ancestry proportions and to detect putative clines of admixture over the whole sampling area. Second, an admixture model including prior information on sampling localities was used, except for Icelandic eels, to estimate the admixture proportions more precisely. A threshold level for the posterior probability of $Q = 0.9$ was used, in order to reach maximum assignment efficiency. Due to the low degree of genetic differentiation in North Atlantic eels ($F_{ST} = 0.015$; $P < 0.001$), the efficiency and performance of hybrid identification may be hampered given the limited amount of markers available (Vähä & Primmer 2006). Thus, to test if Icelandic eels with American mitochondrial haplotypes (“suspects”; n = 16) are actually intermediate rather than pure expatriates, another test was performed analogous to an urn model. Using POPTOOLS version 2.5.5 (Hood 2005), groups of 16 individuals were drawn a thousand times at random from either *Anguilla* gene pool, excluding Iceland. Frequencies of average ancestry proportions were plotted, and the 16 suspects' average value was compared to either gene pool, and to the average of the remaining Icelandic samples, respectively.

As tendencies for geographical groupings were apparent from distance-based phenograms (Wirth & Bernatchez 2001, 2003; Mank & Avise 2003), the eel samples were clustered according to the 7-year-average of sea surface temperatures (SST) in the North Atlantic Ocean (NASA 2008). Hence, European samples were categorized into Northern (Baltic Sea, Elbe, Imsa), Western (Grand-Lieu, Couesnon, Severn) and Southern (Mediterranean Sea, Minho, Adour) groups. The American eels were split into Southern (St. Johns River, South Edisto); intermediate (Wye River, Hudson River, Boston Harbour,) and Northern (Prince Edwards Island, Trinité, Medomak River) samples. To test our hypothesis, that the IBD signal detected in Wirth & Bernatchez (2001) could have been generated by inter-species admixture, 12 virtual hybrid populations were generated in reminiscence of the original samples with increasing F1 proportions (1 - 4% increments per population) following a latitudinal cline. In brief, assuming random mating, virtual F1 offspring were generated for each hybrid population using the shuffle and recombination options in POPTOOLS 2.5.5 (Hood 2005) to cross individuals from the purest American population (River South Edisto, Florida)

with individuals from the purest European population (River Minho, Portugal). Genetic D_{CE} distances (Cavalli-Sforza & Edwards 1967) were calculated for each of the four gene flow scenarios using PHYLIP version 3.68 (Felsenstein 2008). Given the apparent admixture clines, we assumed that hybridization increases linearly from Southern-most to Northern-most populations in the real data, and thus, we matched the genetic distance data from our 12 virtual populations to geographic distance data to account for the original dataset (Wirth & Bernatchez 2001). Moreover, to estimate the gene flow necessary to establish the degree of correlation (and significance) in the original dataset (Wirth & Bernatchez 2001), both correlation coefficients (r) and the slope of the trend line (b) were matched against the simulated gene flow levels.

After evaluating the weak genetic differentiation among North Atlantic eels using basic summary statistics, Mank & Avise (2003) concluded that the large overlap in their allelic frequencies is generated by extensive homoplasy associated with a mutation-driven saturation effect. This strong argument casts doubt on the usefulness of rapidly evolving microsatellite loci for short term evolutionary and hybridization studies. However, some other aspects were neglected in this study, including any reference to the low sampling effort of continental eels, or the use of modern statistical approaches to evaluate microsatellite data Thus, many obvious questions remain unanswered: how can a saturated marker generate statistically significant patterns of IBD (Wirth & Bernatchez 2001)? Why do Icelandic samples always appear in an intermediate position, between both North-Atlantic species (Wirth & Bernatchez 2003; Mank & Avise 2003)? And ultimately, can low levels of hybridization be enough to generate the observed microsatellite patterns in the nearly panmictic North-Atlantic eels ? In order to solve these questions, we investigated the genetic structure of the Atlantic eels closest relatives. According to mitochondrial DNA *A. australis* and *A. dieffenbachii* are the sister group of the Atlantic eels, therefore we genotyped 110 and 96 individuals from each species, respectively, with the same 9 microsatellite loci. We also included a Japanese eel sample (n = 96) as a neutral reference. If Mank & Avise's (2003) "saturation" hypothesis is correct, then it should hold for the other and rather older eel lineages. F_{ST} values must be weak and delineating hybrids should be nearly impossible. Assuming no prior in the program NEWHYBRIDS v1.1beta3 (Anderson & Thompson 2002), another Bayesian method that implements a more specific inheritance model than STRUCTURE (Pritchard *et al.* 2000), we calculated the hybrid frequencies for the Australian and New Zealand eels. Thus, we created virtual hybrid classes (F1, F2 and two classes of backcrosses, Bx) using the program HYBRIDLAB (Nielsen *et al.* 2006) from which the first or later hybrid generations were reassessed under the Bayesian procedure (burn-in period of 30,000 steps, followed by another 50,000 MCMC steps). We set the assignment efficiency (A_e) threshold at $Q = 0.9$.

6.4 Results

6.4.1 Testing the power of the microsatellite markers

While eight out of nine markers depicted a high degree of primer site conservation across eel species, one marker, *Aro146*, failed to amplify in Pacific eels. Thus, subsequent comparisons among all species relied on eight markers only, whereas all loci were used to infer intra-specific relationships among North-Atlantic eels. Overall, levels of observed polymorphism were high in North Atlantic eels, ranging from H_o= 0.38 at locus *Aro121* to H_o= 0.90 at locus *Ang101*, with a mean of H_o= 0.78. The same was true for the average numbers of alleles when correcting for sample sizes, ranging from A_r= 10.9 in *Aro054*, to A_r= 17.9 at locus *Ang114*. The average H_o and number of alleles in Pacific eels resembled those found in the North Atlantic eels (**Table S6.1**; Appendix 2), clearly depicting suitability of the marker system.

All inter-specific comparisons (F_{ST}) were highly significant (**Table S6.2**; Appendix 2). The genetic differentiation of the two Pacific eel species was approximately ten times higher (F_{ST} = 0.157; P < 0.001) than the one observed for the North Atlantic eel species (F_{ST} = 0.0146; P < 0.001) (**Table S6.2**; Appendix 2) and the assignment of pure individuals into the nominal species was A_{aus} = A_{dieff} = 1.0, even when an equal proportion of virtual F1 hybrids is present in the dataset, while assignment success in the F1 hybrid class reaches $A_{admixed}$ = 0.94 (**Fig. 6.1a**). When 20 individuals from each of the four different classes of hybrids are added, the efficiency slightly decreases to A_{aus} = 0.81, A_{dieff} = 0.86, and $A_{admixed}$ = 0.83 respectively (**Fig. 6.1b**). Finally, when the hybrids outnumber the pure nominal species, the assignment success dramatically drops to A_{aus} = 0.50; A_{dieff} = 0.61, but remains high for hybrids, $A_{admixed}$ = 0.90 (**Fig. 6.1c**). In summary, the number and sizes of different hybrid classes in the dataset largely influenced the power of assignment. However, these simulations illustrate how later generation hybrids will lower the power of delineation, a situation that might be encountered in North Atlantic eels (**Fig. S6.1 a-c**; Appendix 1).

6.4.2 Haplotype network and Bayesian assignments

Based on diagnostic restriction digests of *cytb* fragments, 16 out of 300 Icelandic eels (i.e., a fraction of 0.0533) carried American haplotypes (labelled “suspects”), whereas none of the continental North Atlantic eels showed restriction patterns corresponding to the other species (**Fig. 6.2**). Thus, as already described, there is a clear-cut sorting of mitochondrial lineages (Avise *et al.* 1986, 1990). The divergence was less pronounced based on ancestry proportions at nuclear markers, which consistently separated American and European gene pools for the most likely number of populations, K = 2. As expected, the average ancestry proportion of “suspects” in Iceland (Q = 0.40) based on nine microsatellite markers was intermediate (P < 0.0010) compared to those generated from 1,000 blind draws of 16 random

individuals from either species (**Fig. 6.3**), and thus most likely represent true hybrids. In addition, the mean ancestry proportion of non-suspect Icelandic eels is not significantly different from the European mean, albeit slightly shifted toward American eels (Q = 0.69; P = 0.32). When adding prior geographic information on continental stocks, no American expatriate (Q < 0.90 + American mtDNA) was detected in Iceland (**Fig. 6.4**), but two eels carrying a European haplotype were assigned as pure Americans given their nuclear data. When roughly grouping European samples into three cohorts based on average sea surface temperature, a clear cline of ancestry proportions is apparent in the North Atlantic, though less pronounced in North America. Means of ancestry proportions decline from South to North, whereas the opposite is true for the standard deviation (**Fig. 6.5a**). As expected, samples from the northern distribution have the lowest numbers of private alleles on either continent (**Fig. 6.5b**). Moreover, Iceland has the highest average level in observed heterozygosities (**Table S6.1**; Appendix 2).

Finally, the simulation of an IBD pattern in the European eel revealed increasing values of both IBD correlation coefficients and significance levels of the Mantel tests (**Fig. S6.2**; Appendix 1), when increasing levels of gene flow were applied. Assuming that the real IBD pattern (Wirth & Bernatchez 2001) is explained solely by the hybridization cline, an average F1 proportion of ca. 15% among real populations and culminating at 30% in Iceland can be held responsible for the IBD signal detected. Whereas a linear regression fits the data best for correlation coefficients r (**Fig. 6.6a**), exponential curve fitting performed slightly better for the slopes of the trend lines b (**Fig. 6.6b**).

6.5 Discussion

6.5.1 Microsatellite markers provide more information than noise

In our attempt to test the reliability of the hypervariable microsatellite markers to address the main issues raised in the present study, we came to the following conclusions. The much higher F_{ST} values observed for the Pacific eels, the clear assignment of the 16 Icelandic suspects sharing an American mtDNA to first or later hybrid generations (P < 0.010) and the presence of admixture clines provide clear evidence for the presence of information in the dataset. We do not exclude the presence of homoplasy but the generated noise does not erase the overall information gathered from the fish genotypes.

6.5.2 Hybridization pattern

Here we show that introgressive hybridization among European and American eels is sufficient to explain IBD patterns observed in the European eel using neutral microsatellite markers (Wirth & Bernatchez 2001; Dannewitz *et al.* 2005). Hybrid latitudinal clines are most likely due to a very recent onset of gene flow after a secondary overlap (Futuyma 2005) and

reflect superior hybrid fitness in the northern parts of the Atlantic. Several lines of argument support our inference.

First, we confirm that Icelandic samples display intermediate admixture proportions when compared to the continental samples. Hybrid frequencies in Iceland are high; the proportion of American haplotypes reached 0.053. These findings are in good congruence with reported cyto-nuclear disequilibria (Avise *et al.* 1990, Asmussen & Arnold 1991, Arnold 1993), which is a hallmark of hybrid zones (Hewitt & Barton 1985, 1989; Arnold 1992). Second, while mitochondrial lineages in eels remain 100% distinct on both sides of the Atlantic, the hybridization signal expands further to continental stocks in the nuclear genes, with decreasing latitudinal allelic richness and admixture portions. Thus, this diffusion most likely depicts recent on-going gene flow introducing new alleles into each continental nuclear gene pool by back-crossing in the absence of maternal lineage mixing. Third, when simulating declining proportions of F1 hybrids from North to South, a stepwise F1 decrease of approximately 5%/ 1,000km of coastline would explain the IBD signal reported in Wirth & Bernatchez (2001), whereas no or low gene flow will fail to do so.

6.5.3 Range limits

Even though Iceland is at an intersect of the North Atlantic eel distributions, Icelandic eels show more affinity towards the European eel gene pool. Iceland is obviously situated outside pure American leptocephali distribution ranges, where larvae are known to develop much faster than their European congeners (Tesch 2003). Utilizing a Bayesian clustering technique and an urn model, we were able to show that the suspect individuals carrying American haplotypes in Iceland could not be American expatriates ($Q < 0.9$). Instead these "suspects" had intermediate ancestry proportions compared to continental eels. This observation is in good accordance with results from Albert *et al.* (2006), who could not identify pure American eels in Iceland either. Moreover, from the sparse literature on off-continental eel samples (**Tables 6.1** and **6.2**), one can assume that Iceland reflects the only known stable habitat for early generation hybrids. The mechanisms responsible for this finding are not immediately obvious and require additional information on environmental characteristics and hybrid fitness.

6.5.4 Hybrid fitness & ecological peculiarities in Iceland

Albert *et al.* (2006) quantified the hybrid proportions in both recruiting and resident eel stages for several years. A total of 70% of putative hybrids fell into the first generation category, whereas 30% belonged to later generation hybrids. The authors observed an approximately two-fold increase in hybrid proportions from the recruiting glass eel to the resident yellow eel stages. These results suggest a higher hybrid survival upon residency.

The presence of second and later generation hybrids is the proof that hybrids transmitted their genes to the next generation and this would explain why the admixture extends further South on both continents. The increased hybrid fitness model would then already discount the possibility of both a tension zone and mosaic model of hybridization.

Nordic habitats, especially Iceland and Greenland (Boëtius 1985) might represent ecotones compared to the much warmer continents. Freshwater habitats in these areas were definitely uninhabitable during extensive glaciation events in the Pleistocene and must have been colonized afterwards (not earlier than 10,000 years ago). It is suggested that environmental peculiarities characterize eel habitats in Iceland, as freshwater temperatures are typically much lower there as compared to most potential continental habitats (Albert *et al.* 2006; Kuroki *et al.* 2008). This anomlay is directly refelcted in microstructure and mictochemistry of otoliths in glass eel from Iceland, which lacked both the usual sharp decrease in Sr:Ca ratios and elevated increment accumulations (Kuroki *et al.* 2008). Thus, eels appear to have retarded upstream migration into rivers and gather in cold seawater offshore until the upcoming short summer period allows them to enter the rivers. In addition, it is worth noting that the diffuse otolith increment zone after metamorphosis has never been observed in any other eel species outside Iceland. In summary, both intermediate travel distance and environmental opportunity might favour F1 hybrids in Iceland and other Nordic habitats.

6.5.5 Explanation for IBD and IBT signals

Iceland is a mere habitat of co-existence just after completion of long-range larval dispersal from their spawning grounds as far as 5,000 km away in the mid-Sargasso Sea. Thus, as eel larvae rely on long-range dispersal aided by oceanic currents, any model ignoring dispersal, such as the bound hybrid superiority model (Endler 1977, Moore 1977) can safely be discounted to fully explain the hybridization of North Atlantic eels. Even though the catchment areas for the earliest larval stages (group 0) are largely overlapping among eels (e.g., Tsukamoto 2006, but see also McCleave 2008), distinct spawning areas might be largely “allopatric”, due to low or fluctuating degrees of temporal overlap during the spawning seasons (Tesch 2003). Given that hybrid zones emerged secondarily after a longer phase of complete separation (i.e, some 1-2mya; Avise *et al.* 1986), the detection of only a small percentage of intermediate eels in Iceland indicates the presence of a narrow hybrid zone in the Sargasso Sea (Williams *et al.* 1984). Albert *et al.* (2006) showed that the proportion of hybrids reaching Icelandic waters seemed to decrease from 2000 to 2003. This trend is especially important in the light of both our present simulation data and previously inferred IBD patterns in independent studies dating back to samples from 1994 to 2002 (Wirth & Bernatchez 2001; Dannewitz *et al.* 2005). If a drifter simulation holds true and only a very

small percentage (0.8%) of hatched eel larvae successfully reach suitable habitats within two years of dispersal (Kettle & Haines 2006), fluctuations might be largely determined by random factors. Thus, sudden bursts of hybridization and hybrid arrivals might explain temporally unstable patterns of IBD and therefore give rise to IBT (Maes *et al.* 2006a). It can be assumed that the fluctuations in hybrid recruit portions reflect changes in the degree and timing of overlap of spawning grounds in an unstable oceanic environment (Arnold 1997). Therefore, the lower the overlap, the lower and less significant the correlation coefficients of the IBD signal on either continent.

6.5.6 Dispersal time and intrinsic factors to selection

Beside the environmental peculiarities, it is mandatory to describe and understand the North Atlantic eel hybrid zone in the light of eel biology and life history (van Ginneken & Maes 2005). The most obvious difference among the two North Atlantic eel species is their divergent larval dispersal strategy, coupled with differences in the on-set of metamorphosis (Palumbi 1994; Arai *et al.* 2000). These two strategies seem to be quite invariable in either species, as no single expatriate was found in the continental stocks. The timing of metamorphosis and recruitment rely on daily increments within the otoliths microstructure (Lecomte-Finiger 1994). However, these calculations provided distinct results between research groups, and, even more worrying, “back-calculated” larval migration times do not match the field observations (McCleave 2008). These inconsistencies suggest that daily increments are not suitable for calculating the whole time of dispersal, but rather that they provide a descent proxy for the timing of metamorphosis of leptocepahli into glass eels, which takes palce on the verge of open ocean and continental shelves (Tesch 2003). Kettle & Haines (2006) predicted a minimum of two years for larvae to successfully cross the Atlantic solely by passive drift, which was concordant with the early estimates based on larval growth rates by Schmidt (1923). This would suggest a three- to six-fold longer migration time for the European eel compared to its American congener (*A. rostrata)*, which arrives within some 7-12 months (Schmidt 1923).

The current view is that European and American leptocephali accomplish their migration back to the continental rivers within a year. This statement is based on both re-consideration of Schmidt’s work (Boëtius & Harding 1985) and recent analyses of otolith microstructure and microchemistry (Lecomte-Finger 1994; Wang & Tzeng 1998, 2000; Arai *et al.* 2000; Kuroki *et al.* 2008). However, in all studies the timing of metamorphosis is always longer for the European eel (200-350 days for *A*. *anguilla* and 150-200 days for *A. rostrata*) and is intermediate in Icelandic leptocephali with both American and European haplotypes (Arai *et al.* 2000; Kuroki *et al.* 2008).

6.5.7 Developmental retardation

Under a multi-year dispersal scenario, specific retardation of metamorphosis in European eels would be a major prerequisite to successfully complete dispersal towards freshwater or oceanic continental shelf habitats. Such developmental control over the timing of expression of a certain life-history stage is evident in several animals, as exemplified in the marine mollusc *Phestilla sibogae* (Miller & Hadfield 1990). Thus, developmental retardation could be an important factor regulating aging and senescence in animals with complex life cycles, and might be typical in several long-distance dispersers in marine environments (Palumbi 1994). Consequently, pure European larvae and early generation hybrids would not start to develop when close to the American continent, shortly after hatching and initial drifting. Analogously, lack of retardation in American eels could then explain why no single larval or adult expatriate is detected in the vicinity of the European continent, except for those with first or later generation hybrid origin. Just as for the marine mollusc, retardation needed to be broken based on environmental cues. Breaking of dormancy or developmental arrest have been described in animals including sea urchins (Footitt & Cohn 2001). Given that the European eel larval transport might span over 2 to 3 vegetative seasons, cold temperature periods and photoperiod changes accompany larval dispersal. Finally, the metamorphosis could be initiated near the continents upon olfactory freshwater cues.

6.5.8 Active locomotion and navigational control

Passive drift alone cannot explain the one-year dispersal scenario for the European eel (Kettle & Haines 2006), therefore highly efficient active locomotion and precise orientation must be considered in *A. anguilla*. Controlled navigation plays a crucial role in a plethora of diadromous and marine fishes (Leggett 1977). However, to explain the occurrence of obvious hybrids in Iceland only, Avise *et al.* (1990) suggested that this might only hold if directed navigation brings larvae specifically there, omitting earlier contact with European continental habitats. In fact, the intermediate age and peculiar otolith microstructure and microchemistry of Icelandic recruits would support this scenario (Kuroki *et al.* 2008). Though specific information on the open ocean stages is obsolete, intermediate homing might parallel the migratory behaviour of European warblers, which is under complete genetic control (Berthold & Querner 1981; Berthold 1988; Pulido *et al.* 1996). This migratory trait is inherited and hybrids of two different populations hold an exactly intermediate compass course when compared to their parents. Given the intermediate angular position of Nordic habitats, the presence of F1 hybrids would then suggest additive genetic control, possibly driven by only a few genes.

6.5.9 Mitochondrial constraints

The clear-cut differentiation of mitochondrial lineages on either side of the Atlantic is different from the admixture signal apparent in the nuclear genes. Given recent insight into the influence of mitochondrial DNA on senescence and proper zygote and embryological development (Rand 2008) one might speculate on the possible role of maternal or mitochondrial effects on the peculiar dichotomous distribution. In either long- or short-term dispersal scenarios (see above), special demands in terms of energy consumption arise. In a multi-year scenario, long-term developmental retardation might cause reduction in metabolism and thus down-regulation of components comprising the respiratory chain in the mitochondrial cell compartments, which are, in part, regulated or encoded by the mitogenome. On the contrary, in a single-year-scenario, increased swimming performance combined with navigational control during active migration requires a highly efficient supply of energy to omit early exhaustion, and might be coupled with the availablitiy of suitable nutrition (e.g., algal and zooplantic blooms) along the dispersal paths. Rapid changes in the mitochondrial mutation rates, which might be equivalent to the introduction of a new but very closely related mitochondrial lineage by hybridization, can directly influence the organism's senescence (Stewart *et al.* 2008). Therefore, highly divergent senescence regulation could be a prerequisite for successful dispersal into habitats of either range extremes, while first-generation hybrids might only be well equipped to reachintermediate habitats, such as Iceland.

6.5.10 Genetic models of hybridization

We thus reason that the bounded superiority model (Endler 1977; Moore 1977) best fits the derived from adult eel data, as environmental factors appear to increase hybrid survival in peculiar environments (Albert *et al.* 2006). However, with regard to the peculiar life cycle of catadromous eels, there is special need to refine the model in terms of differential survival depending on eel's multiple life stages. Even though the actual hybridization events and its annual rates remain to be determined, it might be speculated that different selection regimes apply for dispersal, residency and back-migration. Furthermore, special attention on the presence of mitochondrial selection signatures should be taken into account for future studies, to search for possible signs of cyto-nuclear interactions and more efficient methods must be developed to segregate hybrids from "escapers" and misplaced or artificaially introduced individuals. In the long run, the occurrence of hybrids might reinforce assortative mating (Dobzhansky 1937; Howard 1993). Such a scenario might be accompanied by novel selection pressures imposed on the eel immune system that were previously not acting, such as the recent introduction of new species of parasites (Wielgoss *et al.* 2008a).

The fascinating life history of Atlantic eels, their economic impact and the concomitant international trade induced numerous studies in the past 30 years. Therefore it is surprising that despite the high efforts taken, still so many questions remain unanswered. Ironically, the panmixia paradigm that was recently wavering due to major population genetics advances seems to hold and the most parsimonious scenario today consists of two random mating populations with fluctuating introgression rates. This result alone will affect the large-scale management of these endangered species.

6.6 Acknowledgements

SW was funded by grants from the Landesstiftung Baden-Württemberg. We thank Julia Jones for reading a draft of the manuscript and add valuable comments on style and rigour.

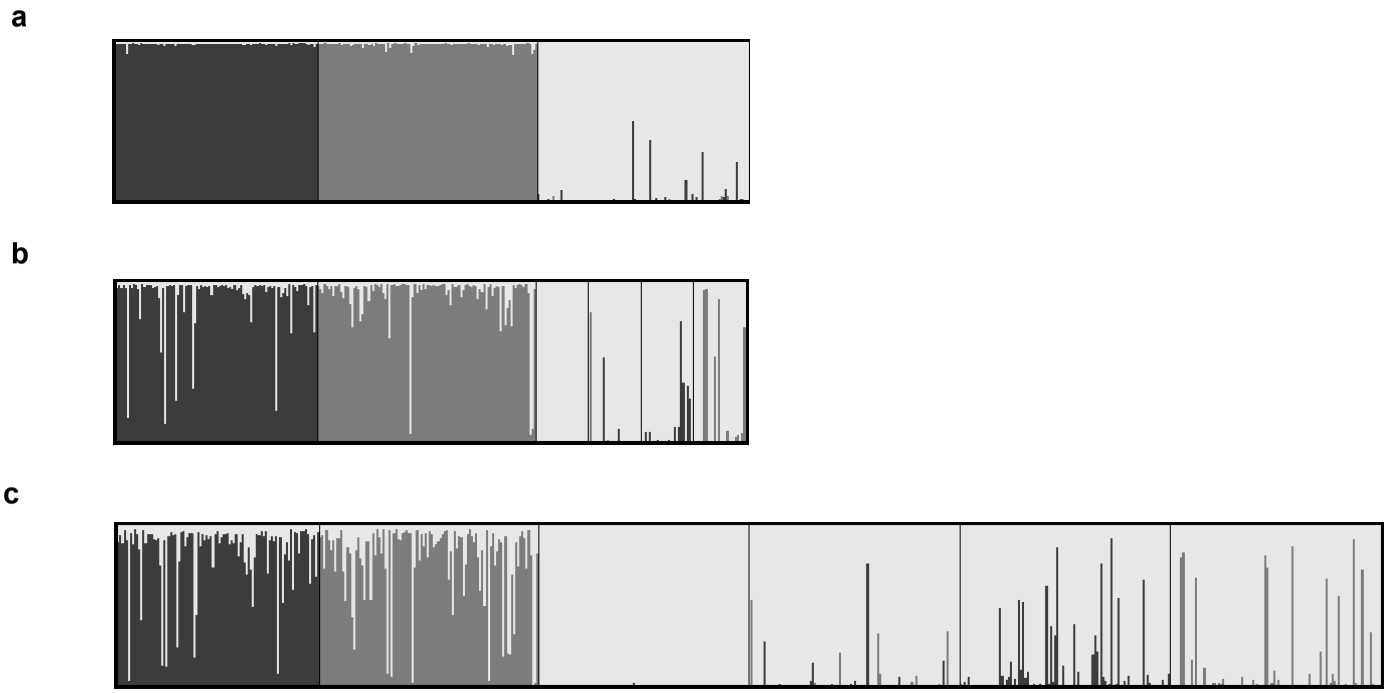


Figure 6.1 Individual Bayesian cluster plots depicting influence of both quantitatively and qualitatively changing hybrid fractions on clustering efficiency. Parental data comprising of *A. dieffenbachi* (black, *n* = 96) and *A. australis* (dark grey, *n* = 104) eel species was supplemented by varying numbers of artificial first and later generation hybrids (light grey) using HYBRIDLAB 1.0 (Nielsen *et al.* 2006), i.e., **a)** artificial F1 hybrids (n = 100); **b)** artificial F1, F2, two-way backcross hybrids (n = 20 each); **c)** same as b) including (n = 100, each). Assignment of pure, first and later generation hybrids in *A. dieffenbachi* and *A. australis* was performed for eight microsatellite loci using NEWHYBRIDS v1.1.3beta (Anderson & Thompson 2002). The figures were generated using DISTRUCT (Rosenberg 2004).

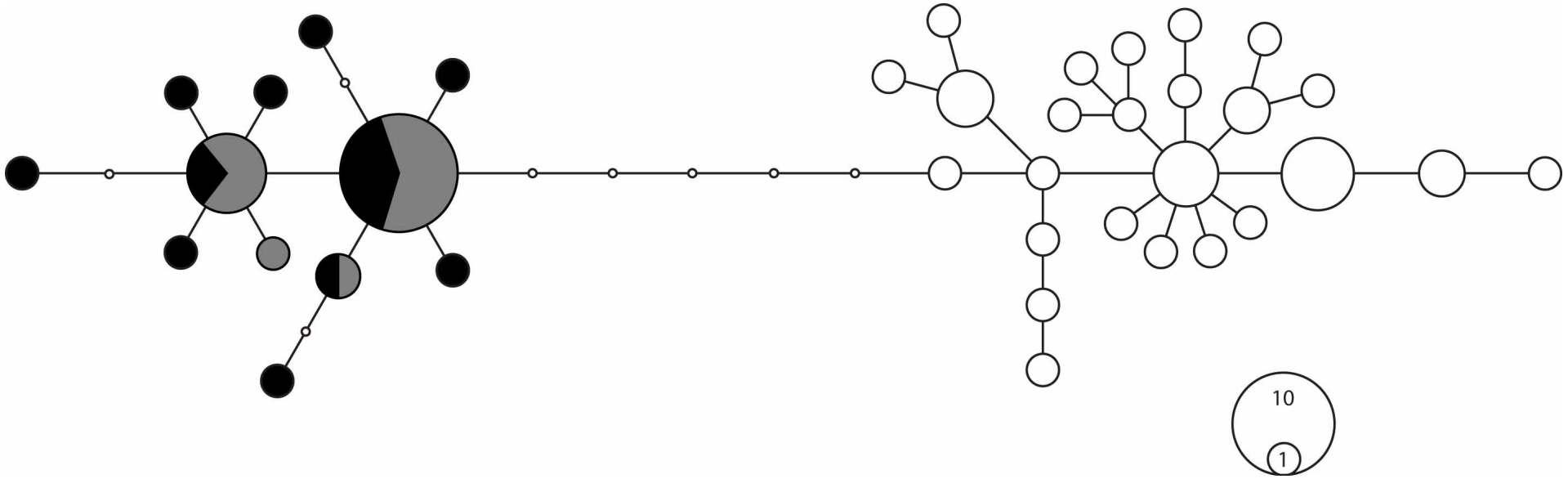


Figure 6.2 A minimum-spanning haplotype network based on a partial sequence of *cyt b* (276bp) shows the non-overlapping haplotype distribution among continental samples of American (black, *n* = 16) and European eels (white, *n* = 36). A fraction of eels (n = 16) sampled in Iceland carry a haplotype typical of American eels (grey), and are thus suspected to be of hybrid origin. A legend on the bottom right-hand side, indicates the number of individuals sharing a given haplotype.

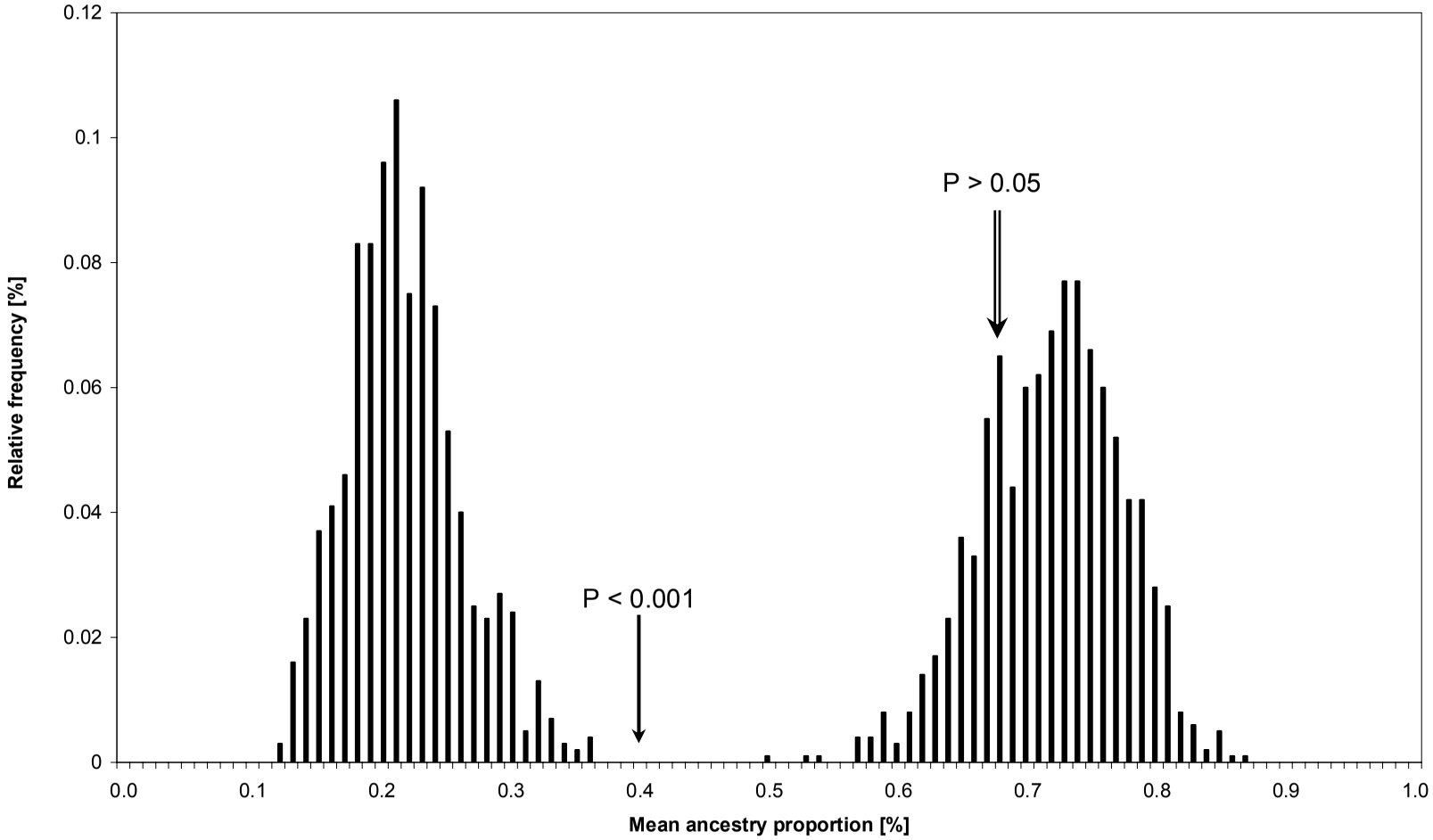


Figure 6.3 Frequency distribution deriving from a permutation test, which illustrates genetic intermediacy of suspect Icelandic eels compared to either continental sample. Analogous to the urn model and in reference to the group of 16 suspect Icelandic eels, groups of 16 eels were drawn 1,000 times from either continental eel population using POPTOOLS v2.5.5 (Hood 2005). Subsequently, the mean ancestry proportion was calculated for each draw. The permutation test illustrates bimodality, as expected under a two-species concept, and the mean ancestry proportion of the suspect eel group is exactly intermediate (black simple pointer; $Q_{suspects}$ = 0.40; P < 0.0010), whereas the mean for Icelandic eels with European haplotypes is not significantly different from the European eel population (black double pointer; Q_{mean} = 0.65; P > 0.050).

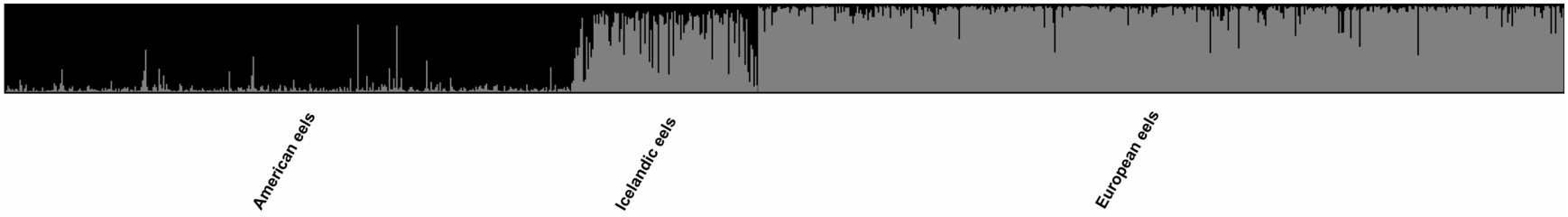


Figure 6.4 Bayesian cluster plot highlighting on-going gene flow in Iceland between European and American eels, in the absence of pure American expatriates. Prior geographic information was added for all continental eels (European ancestry, dark grey; American ancestry, light grey) to infer admixture levels in Icelandic eels. Nine microsatellite markers were used and the genotypes were analysed using STRUCTURE v2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). Pure species status was accepted for Icelandic individuals when the ancestry proportions *Q* were greater than 0.9.

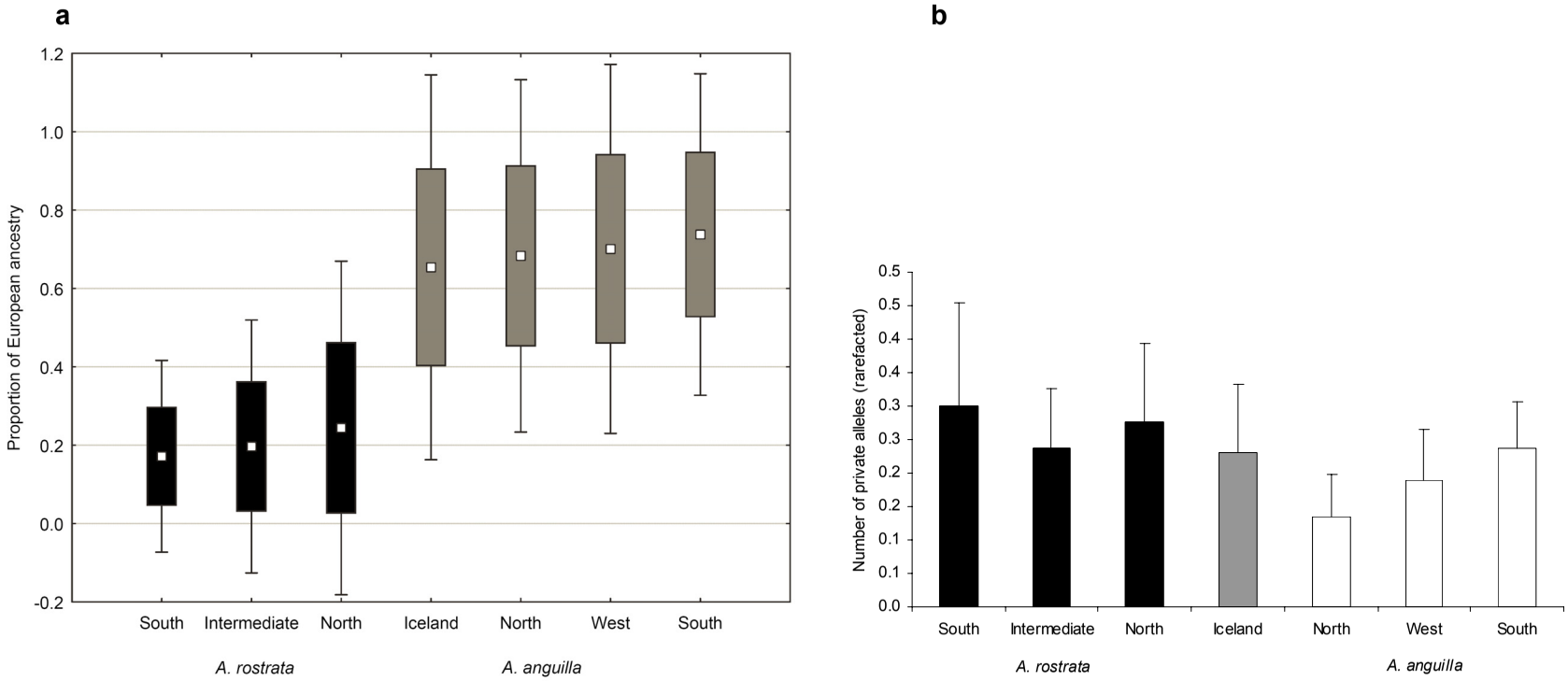
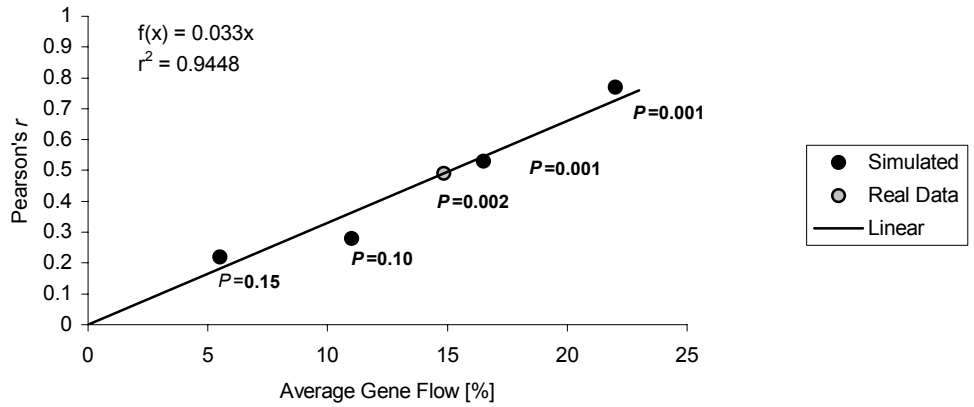


Figure 6.5 a) Box plot representation of clinal geographic change in admixture levels for sampling locations including European and American eels. Ancestry proportions were inferred from 9 microsatellite loci using STRUCTURE v2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). Values are relative to the European eel samples. Individual eels were partitioned according to distinct geographic entities within continents based on mean surface water temperature categories. **b)** Plot of average numbers of private alleles after rarefaction for the same geographic partitions. Error bars correspond to the confidence intervals of 95%.

a



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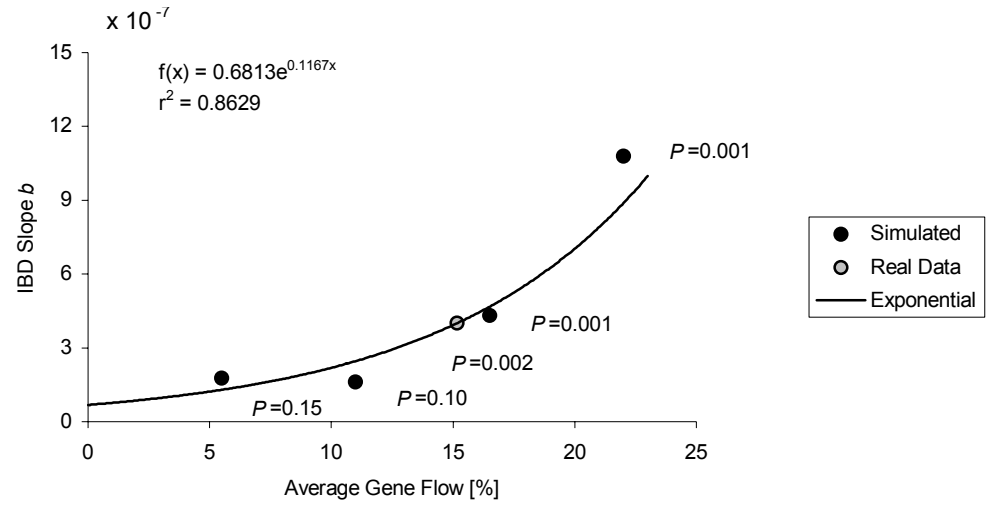

Figure 6.6 Inference of gene flow necessary to generate signficant IBD signal found by Wirth & Bernatchez (2001) using best-fit regression. **a)** Pearson's correlation coefficients *r*; and **b)** slopes of the trendlines *b*. are plotted over linearly increasing hybridization intensity. Gene flow in real data was estimated according to the curve fitting functions.

Table 6.1 Summary of the molecular and population genetics literature in European and American eels.

Allozymes								
Author(s)	**Year**	**Marker(s)**	**Sample Sizes (*n*)**					**Results**
			Continental		**Oceanic**			
			Europe	**America**	**Iceland**	**Sargasso**	**Azores**	
Fine *et al.*	1964	Transferrins*	44	0	0	0	0	Candidate markers for eel species differentiation: transferrins
Fine *et al.*	1967	Transferrins*	142	104	0	0	0	Heterogeneity among North Atlantic eels (not significant**)
Sick *et al.*	1967	Haemoglobin	848	666	0	0	0	Polymorphism in American eels only, monomorphy in European eels
Pantelouris *et al.*	1970	Transferrins*	40	0	37	0	0	Differentiation among European continental & Icelandic eels (significant**)
Pantelouris *et al.*	1971	Transferrins*	0	63	96	0	0	Differentiation among American continental & Icelandic eels (significant**)
de Ligny & Pantelouris	1973	MDH	300	70	0	0	25	First available diagnostic marker: MDH; Differentiation among American & European continental eels (significant**); No differentiation among eels from Azores & Europe.
Williams *et al.*	1973	ADH, PHI, SDH, MDH, EST	0	735	0	0	0	Latitudinal clines at three allozyme markers (MDH invariable) ADH & SDH clines establish at larval American eel stages PHI cline establishes during freshwater residency of American eels
Koehn & Williams	1978	ADH, PHI, SDH	0	n.d.	0	0	0	Latitudinal clines at SDH & PHI loci temporally stable ADH cline unstable and allele frequencies vary among years

Table 6.1 (continued) Summary of the molecular and population genetics literature in European and American eels

Allozymes *(continued)*								
Author(s)	**Year**	**Marker(s)**	**Sample Sizes (*n*)**					**Results**
			Continental		**Oceanic**			
			Europe	**America**	**Iceland**	**Sargasso**	**Azores**	
Comparini & Rodinò	1980	MDH-2	1079	696	0	126	0	Evidence for two eel species at spawning grounds in the Sargasso Sea
Williams *et al.*	1984	MDH-2	n.d.	n.d.	241	0	0	First indication of genetic hybrids in Iceland
Avise *et al.*	1990	MDH-2	0	0	197	0	0	Evidence for an eel hybrid zone: cyto-nucelar disequilibrium in Iceland
Maes & Volckaert	2002	12 loci	304	0	0	0	0	Evidence against panmixia in European eels: IBD (r =0.78; P = 0.030)
Maes *et al.*	2006	12 loci	840	0	172	0	0	No interannual differentiation in European eels: no IBT (r = 0.0050, P > 0.05)
Mitochondrial DNA								
Avise *et al.*	1986	RFLP (78 sites)	29	109	0	0	0	Strong evidence for two eel species in the North Atlantic
Avise *et al.*	1990	RFLP	17	27	197	0	0	Evidence for an eel hybrid zone: cyto-nucelar disequilibrium in Iceland
Lintas *et al.*	1998	D-loop (493bp)	55	0	0	0	0	Extensive variability in European eels
Daemen *et al.*	2001	Cytb (392bp)	253	0	0	0	0	Latitudinal haplotype diversity cline in European eels
AFLP								
Albert *et al.*	2006	373 fragments	186	193	748	0	0	Quantification of total fraction of hybrid eels in Iceland (15.5%); Latitudinal gradient of hybrid portions in Iceland; Indication of higher survival rates of hybrid eels in Iceland.

Table 6.1 (continued) Summary of the molecular and population genetics literature in European and American eels.

Microsatellites								
Author(s)	**Year**	**Marker(s)**	**Sample Sizes (*n*)**					**Results**
			Continental		**Oceanic**			
			Europe	**America**	**Iceland**	**Sargasso**	**Azores**	
Daemen *et al.*	2001	5 loci	107	0	0	0	0	Low, significant genetic differentiation in European eels (F_{ST}= 0.040; P < 0.050)
Wirth & Bernatchez	2001	7 loci	561	0	50	0	0	Evidence against panmixia in European eels: IBD (r = 0.46; P < 0.0070); Low, significant genetic differentiation in European eels (F_{ST} = 0.0017; P = 0.0014); Genetic intermediacy of Icelandic eels among North Atlantic locations.
Wirth & Bernatchez	2003	7 loci	561	402	50	0	0	Evidence for long-term population decline in North Atlantic eels; Differentiation among North Atlantic eels (F_{ST} = 0.018; P < 0.0010); No evidence against panmixia in American eels: no IBD (r = 0.0030; P > 0.40).
Mank & Avise	2003	6 loci	44	68	203	0	0	Mild genetic differentiation among North Atlantic eels (G_{ST} = 0.055; SE = 0.0049); Genetic intermediacy of Icelandic eels among North Atlantic locations.
Dannewitz *et al.*	2005	6 loci	2566	0	60	0	0	Temporal instability of IBD pattern in European eels Low genetic differentiation in European eels (F_{ST} = 0.0014; P < 0.010); Temporal genetic variation exceeds geographic variation.
Maes *et al.*	2006	6 loci	840	0	172	0	0	Evidence for interannual differentiation in Europe: IBT (r = 0.18; P = 0.043); Differs from allozyme pattern: no IBT, but IBD.

* reconsidered by Koehn (1972): listed markers agree with Mendelian inheritence and have objectively interpretable banding patterns; ** statistically re-evaluated by applying χ^2-statistics with correct degrees of freedom to test for Hardy-Weinberg equilibrium

Table 6.2 Frequencies of American haplotypes in Iceland.

Reference	***H***	***N***
Avise *et al.* (1990)	0.036	438
Kuroki *et al.* (2008)	0.060	311
Our study	0.053	300
H, mitochondrial frequency of A. rostrata haplotpyes in Iceland; N, sampling size		

Chapter 7:

Signatures of genetic pollution in commercial eel species as a consequence of aquaculture and intercontinental trade

THIERRY WIRTH, SÉBASTIEN WIELGOSS, LOUIS BERNATCHEZ

Unpublished

7.1 Abstract

Economically important eel species (*Anguilla japonica*, *A. anguilla* and *A. rostrata*) have declined dramatically over the last thirty years. The scarcity of this resource and the growing demand for eel in the Japanese food market promoted east-Asiatic eel farming and international living-eel trade. This anthropic driven potential for gene flow, the generation of artificial hybrids between *A. anguilla* and *A. japonica*, as well as panmixia in eels raise concerns about the possibility of interbreeding. Using microsatellite markers, we documented the genetic composition of the three main commercial species and two additional Austral species (*A. australis* and *A. dieffenbachii*) in order to assess whether commercial eel trade may have promoted introgressive hybridization. Admixture was detected both at the population and individual level. Atlantic and Japanese eels were shown to be genetically closely related, a result that contradicts the information gathered from the maternally inherited mtDNA. Moreover, using Bayesian statistics, signals of introgression from Japanese into European eels were depicted for at least one individual. This shows for the first time that commercial trade may impair the genetic integrity of eel species. Consequently farming habits must be changed and foreign imports drastically reduced to stop the ongoing genetic pollution. However, marker and sampling numbers must be considerably increased to routinely monitor eels for hybridization in future studies.

7.2 Introduction

The steep increase of man-driven translocation of nonindigenous species (NIS) depicts a great challenge for conservation biologists (Allendorf *et al.* 2001; Taraschewski 2006). Of significant importance are the surging imports of domesticated fish species, as natural hybridization among fishes is much higher than in other vertebrates (Campton 1987; Smith 1992). Salmonids are very well studied in this regard due to their high economic significance. Studies frequently report on fertile hybrids detected among domestic and wild brown trouts, but suggest that the impact are rather low, which is evidence for poor performance and low fitness of domesticated trout in the wild (Poteaux *et al.* 1998; Ruzzante *et al.* 2001; Hansen 2002). On the contrary, Roberge *et al.* (2008) find support that hybridization among fugitive farmed salmon and native wild consepcifics results in substantially modified genetic control of transcription, leading to unpredictable and potentially detrimental effects on the survival of admixed wild salmon. Moreover, introgression of transplaced individuals into massively declining fish populations, such as the Japanese freshwater eel, *Anguilla japonica*, should be of special concern to wildlife managers, as extinction risk is especially high for rare and disturbed species (Rhymer & Simberloff 1996).

The freshwater eel is an important dish on European and Japanese menus. Eel larvae are eaten as appetizers in Spain, while smoked eel is favoured elsewhere in Europe and North America. The Japanese culinary tradition offers a large spectrum of eel dishes, including different forms of grilled eel, *kabayaki*, *shirayaki*, *umaki* and the classical *sushi*. Consumed at both adult and glass eel stages, the market for unprocessed eel in Japan generates billions of Euros and ensures the livelihoods of 25 000 fishermen in Europe alone (Stone 2003). Commercial eel stocks have constantly declined over the last 50 years (Castonguay *et al.* 1994a,b; Haro *et al.* 2000; Dekker 2003). The causes of this dramatic decrease are still a matter of debate and several explanations have been proposed, which are not mutually exclusive. Climate induced changes in the Gulf Stream circulation might play a role in the North Atlantic eels' decline (*A. anguilla* and *A. rostrata*) decline, as well as the recent introduction and spread of *Anguillicola crassus*, an exotic swimbladder nematode (Kirk 2003; Wielgoss *et al.* 2008a). Moreover, anthropic factors such as river habitat destruction, dams, pollution and overfishing might be involved as well (see Feunteun 2002 for a review). Consequently eel demands and prices keep surging (Ringuet *et al.* 2002).

In the last 30 years, farming fry became more and more prevalent and Asian producers imported European and American glass eels on a massive scale, to satisfy the local consumer demand (Usui 1991; Ringuet *et al.* 2002). The eels are mostly caught at early stages in Western Europe and then exported to Chinese, South Korean and Japanese eel farms. The same is true the other way round whereby anguilliculture has driven European

and North American countries to import East-Asian eels and New Zealand eels (primarily *A. japonica and A. australis*) for a short period of time, which was enough to introduce and establish the alien parasite species, *Anguillicola crassus* (Taraschewski 2006; Wielgoss *et al.* 2008a). This global trade of living eels is raising many questions concerning the stock management, the domino effect induced by the growing eel demand in the Japanese food market, the introduction of exotic parasitism (Taraschewski 2006) and the preservation of the genetic integrity of eels in the face of potential introgressive hybridization. There is clear evidence for naturally occurring hybridization in Iceland, between the European and American eel (Albert *et al.* 2006; Avise *et al.* 1990). Therefore the presence of sexually mature European and American silver eels that might have escaped from culture ponds along the coasts and estuaries of Japan and Taiwan (Han *et al.* 2002), raises a legitimate concern about potential genetic pollution of the locally native species. This concern is reinforced by the successful production of artificial hybrids between *A. anguilla* and *A. japonica* (Okamura *et al.* 2004), and *A. australis* and *A. dieffenbachii* (Lokman & Young 2000), respectively.

Recently, two studies have demonstrated a high conservation level of microsatellite binding sites in Anguillid eels (Maes *et al.* 2006b; Wielgoss *et al.* 2008b), and assignment success using four species of eels reached levels > 90%, if prior information on geographic sampling was used (Maes *et al.* 2006b). However, though the latter authors found evidence for admixture among geographically isolated eel species, they did not specifically screen for hybrid individuals in their dataset. Moreover, Maes et al (2006b) chose loci according to maximal differentiation to the overall F_{ST} values, thereby possibly skewing the analysis toward high efficiencies in the pure category.

Here, including a total of 704 eel specimens from five different species of commercially traded eels, we assessed whether species allocations may have promoted introgressive hybridization,using eight microsatellite markers. Moreover, utilizing Bayesian clustering techniques, the influence of simulated species hybridization on assignment efficiency and accuracy are evaluated for real and simulated datasets.

7.3 Material and Methods

7.3.1 Sampling material.

Fin clips were sampled from three different species of eels, subject to intercontinental trade: *A. dieffenbachii* (North Island, NZ, *n* = 54; South Island, NZ, *n* =43), *A. australis* (Queensland, AU; *n* =111), *A. japonica* (Tokyo Bay, JP, *n* = 48; Taipeh Estuary, TW, *n* = 48) were collected during spring and autumn 1999, except for half of the Japanese sample which was collected in spring 2003 and stored in 70% of Ethanol. These samples were supplemented by published data derived from both North Atlantic eel species (Wirth &

Bernatchez 2001, 2003), i.e., *A. anguilla* (River Moulouya, *n* = 50; River Minho, *n* = 50; Lac Grand-Lieu, *n* = 50; River Elbe, *n* = 50) and *A. rostrata* (River St. Johns, *n* = 50; River Wye, *n* = 50; Boston Harbor, *n* = 50; River Medomak, *n* = 50).

7.3.2 Microsatellite genotyping.

Genomic DNA was isolated from adults' fin clips according to standard methods . The microsatellite flanking sequences and primers are available on Genbank under the accession numbers AF237896-AF237902. Polymerase chain reactions (PCRs) were performed in duplex or triplex with *Taq* DNA polymerase (Promega) and rhodamine-marked primers (Perkin Elmer) as detailed in Wirth & Bernatchez (2001). Briefly, reactions were pooled and alleles belonging to eight different loci were segregated on an ABI377 automated sequencer. The sizes of the fragments were determined in reference to a size standard running in each lane using the software GENESCAN version 2.1 and GENOTYPER version 2.0.

7.3.3 Genetic variability and population genetics parameters.

Allelic diversity, genetic variation (observed heterozygosity under HWE), deviation from HWE and genetic differentiation were calculated using GENEPOP version 4.0 (Raymond & Rousset 1995). Variation of allelic frequencies among samples was assessed by first testing the null hypothesis of homogeneity in allelic distribution using Fisher's exact test with Markov chain method and then by quantifying the standardized variance in allelic frequencies (θ_{ST}) as an estimator of F_{ST}, using a described method as implemented in GENETIX 4.0. Individual differences in genetic composition was visualised by performing a correspondence analysis (FCA), which graphically projects the individuals on the factor space defined by the similarity of their allelic states.

7.3.4 Population clustering and admixture using Bayesian statistics.

The program STRUCTURE was used for inference of population structure. This model-based Bayesian clustering software, aims at introducing population substructure by devising individuals into joint clusters using a Markov Chain Monte Carlo technique, thereby minimizing Hardy-Weinberg and gametic phase disequilibria between loci within groups (Pritchard *et al.* 2000). The number of clusters represented in our sample was estimated by calculating the probability of the data, for each of the models of $K \in \{1, 10\}$ populations, running 1,000,000 MCMC sweeps following a burn-in period of 100,000 steps. Subsequently, Evanno *et al.*'s (2005) ad-hoc statistics ΔK was used to evaluate the most likely number of clusters, by running 10 additional short runs of 10,000 Burn-In and 20,000 MCMC steps, respectively.

7.3.5 Detection of immigrants and hybrids.

The dataset was subjected to a second STRUCTURE analysis, aiming at the detection of first and later generation migrants, assuming a maximal migration of 0.05, which is equivalent to the average number of non-indigeneous eel species caught in Japan over more than a decade; i.e. 0.064 (Okamura *et al.* 2008), and a lower migration rate at 0.01. For this purpose, prior information on sampling locality was introduced (POPFLAG = 1), and eels with a less than 90% membership proportion considered. The results were compared to two more analyses. First, the number of first generation migrants apparent in the dataset was explicitly tested by running Geneclass 2.0 (Piry *et al.* 2004) using the given method suggested by Paetkau *et al.* (2004). Each individual's likelihood was computed by a resampling method drawing at random 10,000 virtual genotypes and assigning them to real dataset. Secondly, genotyped individuals of each possible species pair (North Atlantic eels were pooled as mentioned above) were assigned to each of the possible categories, either pure, first and second generation hybrids or backcrosses in either direction using NEWHYBRIDS v1.3c, which uses a Markov Chain Monte Carlo approach to categorize individuals of each of two species and their possible hybrids into their correct group (Anderson & Thompson 2002). In a second approach, species of eels were artificially crossed using the resampling and shuffling syntaxes available in Poptools for MS Excel (Hood 2005), and datasets were scored for efficiency and accuracy, following runs of 20,000 burn-in followed by 50,000 MCMC repeats.

7.4 Results and Discussion

All loci were highly polymorphic, showing a mean number of alleles per locus ranging from 13.0 (± 3.1) in *A. dieffenbachii* to 25.1 (± 4.3) in *A. japonica*. Observed and expected mean heterozygosities per species ranged from 0.68 (± 0.25) and 0.85 (± 0.08) to 0.75 (± 0.22) and 0.91 (± 0.03), respectively. Many alleles were species-specific (**Fig. S7.1**; Appendix 1), such that tests of genetic differentiation based on allelic frequency distribution, as well as the fixation index values (F_{ST} = 0.1059) over all samples were highly significant (P < 0.0001; 10,000 iterations). Moreover, all interspecific pairwise comparisons using F_{ST} were significant as well (**Table 7.1**). The three major commercial species, *A. anguilla*, *A. rostrata*, and *A. japonica*, were closely related with a maximal F_{ST} value of 0.045, only about half that among Australian and either Japanese or North Atlantic eels. This trend was confirmed by the factorial correspondence analysis (**Fig. 7.1**), where Atlantic and Japanese eels created a continuum rather than clear discrete entities, suggesting an admixed allelic composition of several individuals. This result is particularly surprising since it contradicts the phylogenetic relationships inferred from the maternally inherited mtDNA where Atlantic and Austral eels form a sister group that is clearly distinct from the Japanese eel clade (Lin *et al.* 2001;

Minegishi *et al.* 2005. The discrepancy between nuclear and mitochondrial genetic markers was confirmed by plotting pairwise F_{ST} values (nuclear microsatellite genes) against K2P distances (mitochondrial cytochrome b gene; **Table 7.1**). The Pearson's correlation (*r*) between the two genetic markers was not significant and close to zero (r = - 0.078, P = 0.554; Mantel 1967). In contrast to North Atlantic eels, admixture among the two Austral species was not evident, despite their sympatry (**Fig. 7.1**).

These results strongly suggest that introgression among Japanese and North Atlantic species of eels has been taking place, which is congruent with increased stocking activities among those species for the past 40 years (Ringuet *et al.* 2002). To further investigate this possibility the likelihood of the pooled data to cluster into more than one population was estimated using STRUCTURE, without prior geographic information. The Ln probability of the data was at a minimum for K = 1 (Ln = -31,502), increasing rapidly until K = 5 (Ln = -28,059), and finally stabilizing thereafter (**Fig. S7.2a**; Appendix 1). Evanno's ad-hoc statistic ΔK (Evanno *et al.* 2005), confirms the a priori assumption of K = 5 (**Fig. S7.2b**; Appendix 1). As differentiation among North Atlantic eels is low and to simplify the assignment and clustering methods among geographically separated units, we considered the Atlantic eels a single cluster for further analyses. The proportion of membership (q) of each species into four clusters representing the "cryptic" genetic populations was calculated (**Table 7.2**). Cluster I grouped the New Zealand eel *A. dieffenbachii* (q_1 > 0.97), cluster II grouped the Australian short-fin eel *A. australis* (q_2 > 0.97); cluster III grouped the Japanese eels *A. japonica* (q_3 > 0.95), and cluster IV grouped Atlantic eels (average q_4 = 0.90). Importantly, the analysis of individuals suggested possible hybridization among *A. japonica* and the Atlantic eels in the Atlantic Ocean (**Figs. 7.2a** & **b**; Appendix 1), where eight out of 378 eels split equally between both entities ($q_{3,4}$ = 0.45 … 0.55), which is indicative of their admixed ancestry (**Fig. 7.2b**). Pacific eels have low Atlantic ancestry proportions, and only two eels appear split among two clusters.

In a second approach, the same samples were subsequently assigned to one of the four predefined clusters in STRUCTURE (POPINFO = 1), this time making use of geographic information. According to this analysis (**Table S7.1**; Appendix 2), fifteen Atlantic eels, and nine Oceanic eels show ancestry proportions of 90% or lower. Two eels had a significantly different genotype from its expected ancestry, both found in Europe. The first one, Glieu.25, is identified as Japanese immigrant with high probability, P < 0.001. This was also highly supported when inferring its likelihood of being a first generation migrant using GeneClass (P < 0.0001). The second significant suspect, Glieu.6 is an F1 hybrid with a Japanese parent (P < 0.05), as there is no support for this individual to being a 1st generation migrant, neither in STRUCTURE nor in GeneClass (P > 0.05). Among the nine eels sampled in New Zealand and Australia, Aus.12 had the lowest proportion, with a 33.6% chance of having a Japanese

eel grandparent. Finally, Japo.04 has the highest chance of being admixed among the Japanese eels, with a 13.2% chance of having a grandfather from the Atlantic.

Using NewHybrids (Anderson & Thompson 2002), which specifically allows to test for several hybrid categories for a given species pair, pure fractions frequently reached assignment levels above 0.94 (**Table 7.3**), and no hybrids could be detected, neither at the 0.9 nor the 0.5 levels of probability. Moreover, Japanese and Atlantic eels only retrieved assignments of 0.69 and 0.68, respectively, which is most likely due to their low interspecific F_{ST} value. Based on this finding, we tested if the number of samples might have been too low to resolve hybrids specifically among Japanese and Atlantic eels using the NewHybrids program (Vähä & Primmer 2006). Thus, populations were generated to match Hardy-Weinberg expectations ("simulated"; F_{ST} = 0.040) for both species from the real dataset (F_{ST} = 0.043). Efficiencies were higher at 0.80 and 0.83, respectively, using comparable sampling sizes (n = 100 each, no F1 hybrids addded). When increasing simulated sampling size to n = 500, efficiencies were nearly perfect, above 0.98. From this it is apparent that small sampling sizes caused the lower than average assignment efficiency. As there is a big enough sampling size in Atlantic eels, at least Japanese eel sampling should be increased to efficiently detect pure individuals better in NewHybrids (whereas this was no problem using STRUCTURE).

However, undetected hybridization could still explain the lower observed assignment efficiency among Atlantic and Japanese eels than expected from simulation (**Table 7.3**). Thus, we assessed the effect on both assignment efficiency and accuracy in the presence of known (simulated) F1 hybrids, adjusted for equal sampling sizes in all comparisons. As a result, average assignment efficiencies decreased significantly by 9%, when adding an equal proportion of F1 individuals (**Table 7.3**; P = 0.013; ttest). This is however mainly due to the fact, that the JAP x NAT species pair shows the markedly lowest assignment accuracy reaching only 0.27. In turns, this suggests that the actual number of hybrid individuals in the real dataset is assumingly low and comparable to the numbers detected using STRUCTURE. Consequently, when assigning the real data back to the simulated Hardy-Weinberg populations, efficiencies reach nearly 100%, leaving out only those individuals already identified using STRUCTURE (Glieu.6; Glieu.25; Japo4). Vähä & Primmer (2006) recently assessed the detection limits of hybrids given different levels population differentiation, number of markers and number of hybrids expected. In analogy to our current case with an F_{ST} of around 0.05, but rather low number of hybrids in the dataset, up to 50 markers are needed to reach a detection efficiency of hybrids above 90%. Thus, it appears necessary to increase both, the number of samples, as well as the overall number of markers up to four-fold in order to highly statically infer a certain hybrid categry from its parental clusters (n = 500 and 50, respectively). Given that microsatellite flanking regions are quite well conserved

across the genus of freshwater eels (Maes *et al.* 2006b, Wielgoss *et al.* 2008b), one might reach this limit by pooling all makers currently available in Anguillid eels for future studies (Wirth & Bernatchez 2001; Ishikawa *et al.* 2001b; Tseng *et al.* 2001; Daemen *et al.* 2001; Wielgoss *et al.* 2008b).

A couple of recently published studies also tried to differentiate pure from hybrid individuals among different species of eels. Mank & Avise (2003) could not resolve potential hybrids and pure individuals among the North Atlantic species in Iceland using distance methods and basic summary statistics. The latter methods seem to be inadequate to retrieve hybrids, given the low inter-species differentiation F_{ST} = 0.015. The same issue was highlighted in Maes *et al.* (2006b), which instead utilized a Bayesian individual assignment method for the microsatellite genotype data. Assuming a threshold of q = 0.8, the authors could assign eels with high efficiency >90% to their expected species rank, when using informed prior for known samples. Importantly, a couple of admixed individuals were thus inferred. Efficiency data are similar to our findings reaching levels > 90%. However, Maes et al (2006b) find no evidence for interspecies admixture among Japanese and North Atlantic eels. This discrepancy likely derives from the different approaches to differentiate the dataset. While Maes et al (2006b) selected the most differentiating markers from the initial set, we instead used all markers and a higher sampling size thereby increasing power a priori, thus assignment proportions remain > 87% per species in STRUCTURE, even without assuming prior geographic information (**Fig. 7.2b**).

7.5 Conclusions

The following conclusions concerning natural and human driven hybridization in eels emerge from these analyses. First, natural hybridization does not only occur between the two Atlantic species but also occurs between the closely related Austral species (see AusN.45 ancestries in **Table S7.1**; Appendix 2). Second, natural hybridization occurred between geographically isolated species, with the clearest evidence for introgression of Japanese eels into the Atlantic species (Glieu.6), then Japanese eel into Austral eels (Aus.12) and possibly North-Atlantic eel into Japanese eel (Japo.04). These results are congruent with the introduction of Japanese and Australian eels from Taiwan and New Zealand into Northern-Europe in the late 1970s (Hartmann & Koops 1989). They are also consistent with the massive import of European and American eels to Japan and China since in the 1980s. Moreover, recent artificial crosses also support the potential for hybridization between European and Japanese eels (Okamura *et al.* 2004), as well as Among Australian and New Zeland eels (Lokman & Young 2000).

The introgression signals detected here might be only the tip of the iceberg, as increasing number of markers would increase the significance level of assignment into hybrid

classes (Vähä & Primmer 2006). As a result of the eel's panmixia and inefficient stock managements, long-term genetic pollution is likely. Indeed, the fact that evidence for introgressive hybridization was found after only two or three generations raise concerns for long-term consequences, including the loss of species integrity (Arnold & Hodges 1995), reduction in fitness (Dowling & Moore 1985; Rhymer & Simberloff 1996), or species replacements (Hale *et al.* 2004). The economical impact is important as well; anthropic driven hybridization will affect the Japanese market, where *unagi* are preferred over their Atlantic counterparts. Therefore farming security might improve, foreign imports might decrease and prizes might exponentially increase.

7.6 Acknowledgements

We thank J. Crumpton, E. Ciccotti, Simon Hoyle, Mike Dredge, Peter Smith, W-N Tzeng and the CEMAGREF for providing the samples used in this study. Michael Arnold and Katharine Webb for comments on the manuscript. The research program of L.B. on the evolution and conservation of northern fishes is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) research grants and by the Canadian Research Chair in Conservation Genetics of Aquatic Resources. This work is a contribution to the programme of Québec-Océan.

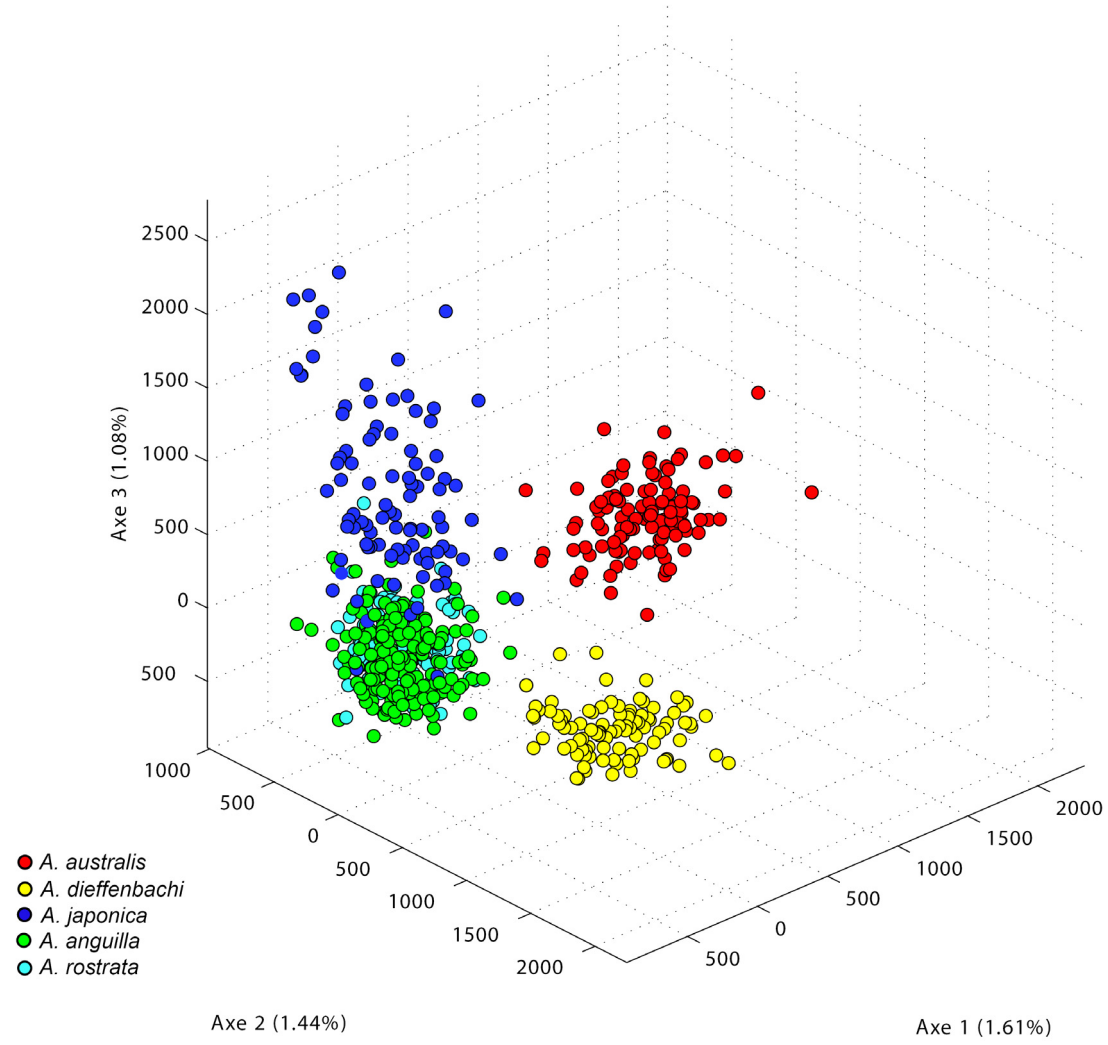

Figure 7.1. Three-dimensional correspondence analysis (FCA) of microsatellite genotypes from five eel species. Red, *A. australis*; yellow, *A. dieffenbachii*; green, *A. anguilla*; light blue, *A. rostrata* and dark blue, *A. japonica*.

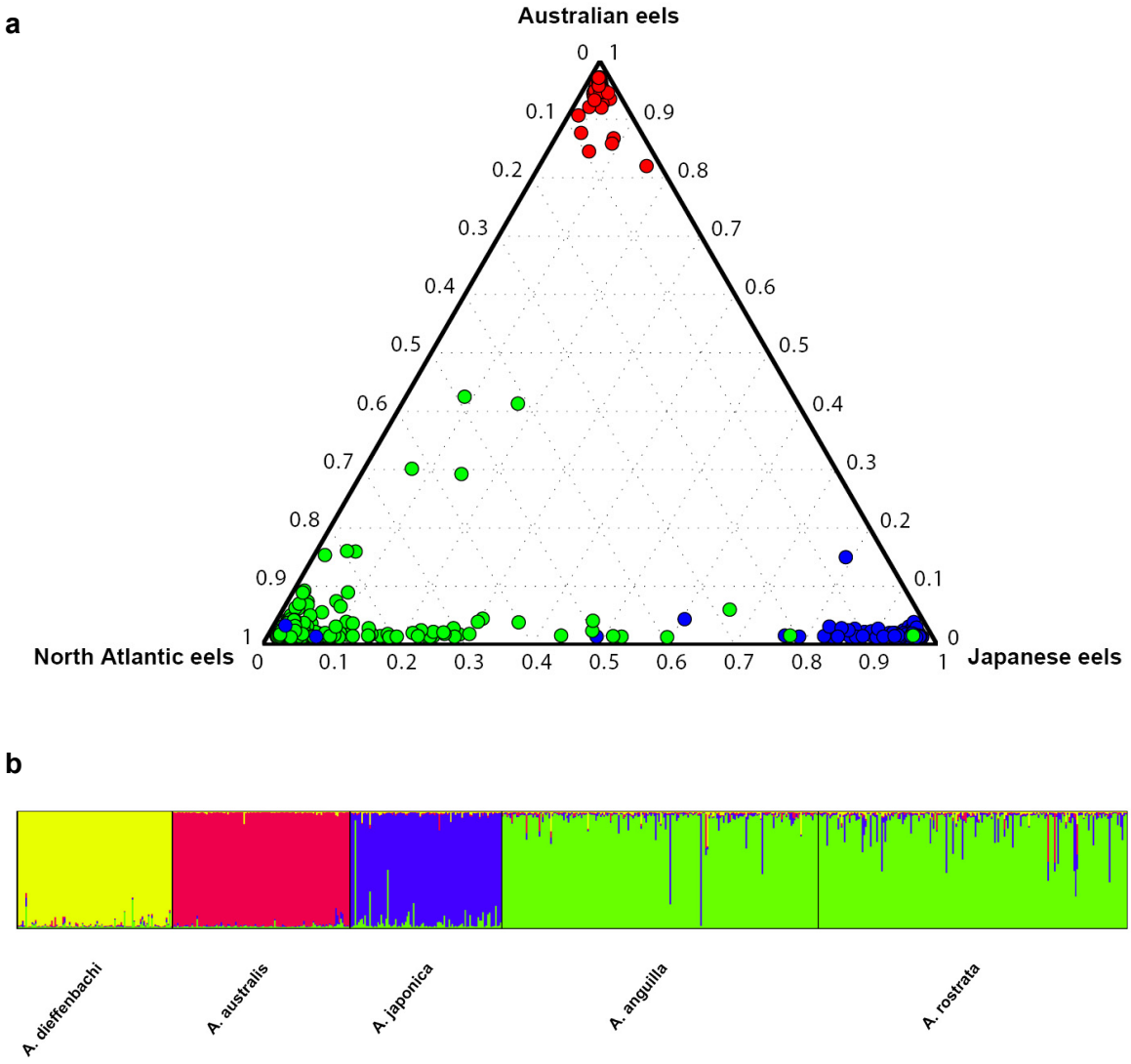

Figure 7.2. Estimated population structure. **a)** Ternary plots of proportion of ancestry from three inferred clusters of individuals estimated by STRUCTURE. Each data point corresponds to a single isolate whose proportion of ancestry from each of the three sources is represented by its proximity to the corresponding corner of the triangle. Note that the proportion of ancestry ranges between 0 and 1, regardless of the true genetic distances between the ancestral sources. **b)** Clustering assignments of 704 eels. Each individual eel is represented on the graph by a vertical line divided into coloured segments corresponding to different genetic clusters. The length of each coloured segment is equal to the estimated proportion of the individual's membership in the cluster of corresponding colour (designated on the y axis as a percentage).

Table 7.1 Pairwise genetic distance matrix of different eel species. The upper diagonal corresponds to the Kimura-two parameter model distance based on the mitochondrial cytochrome b gene. The lower diagonal corresponds to F_{ST} values between species inferred from microsatellite markers (all comparisons were highly significant, $P < 0.001$).

Fst/K2P	*A. anguilla*	*A. rostrata*	*A. australis*	*A. dieffenbachii*	*A. japonica*
A. anguilla		0.046 ± 0.006	0.089 ± 0.008	0.073 ± 0.008	0.103 ± 0.009
A. rostrata	0.015		0.087 ± 0.009	0.074 ± 0.008	0.098 ± 0.009
A. australis	0.098	0.094		0.061 ± 0.007	0.094 ± 0.009
A. dieffenbachii	0.122	0.115	0.155		0.080 ± 0.008
A. japonica	0.045	0.040	0.115	0.130	

Table 7.2 Bayesian clustering analyses for the pooled eel samples (704 individuals; 8 loci) performed using STRUCTURE.

Population	I	II	III	IV
A. dieffenbachii	0.970	0.008	0.009	0.013
A. australis	0.008	0.974	0.011	0.008
A. japonica	0.012	0.009	0.948	0.031
A. anguilla	0.015	0.014	0.041	0.930
A. rostrata	0.015	0.018	0.105	0.862

Table 7.3 Assignment of real and simulated genotype data including or excluding virtual F1 hybrids using NewHybrids version 1.3.

SPECIES PAIR		Efficiency ($P > 0.9$) Raw Data		Efficiency ($P > 0.9$) + Simulated F1			
SP1 (n)	SP2 (n)	SP1	SP2	SP1	SP2	+F1	Accuracy
Real Data							
AUS (96)	DIE (96)	1.00	1.00	0.98	1.00	0.99	0.99
AUS (110)	NAT (110)	1.00	0.94	0.97	0.87	0.78	0.88
AUS (94)	JAP (94)	1.00	0.98	0.99	0.91	0.81	0.90
DIE (94)	JAP (94)	1.00	0.97	0.98	0.87	0.85	0.90
DIE (96)	NAT (96)	1.00	0.99	0.97	0.83	0.72	0.84
JAP (94)	NAT (94)	0.69	0.68	0.19	0.63	0.00	0.27
Simulated							
JAP (100)	NAT (100)	0.80	0.83	0.63	0.30	0.03	0.32
JAP (500)	NAT (500)	0.98	0.99	0.76	0.72	0.43	0.69

SP; Species; AUS, *Anguilla australis*; DIE, *A. dieffenbachii*; NAT, North Atlantic eels; JAP, *A. japonica*; *n*, Sampling Size

ABSTRACT

In the presented PhD thesis, new light is shed on the impact of global eel transfer on the spread of exotic parasitism and the influence of genetic admixture in previously geographically isolated species of eels. The chapters cover basic research in parasite population genetics, the isolation of molecular markers, and more applied investigations in conservation biology. Here, one of the few available studies on parasite population genetic structure using microsatellite markers is presented, and the invasion history and extant phylogeography of the eel swimbladder parasite, *Anguillicola crassus*, is inferred for its novel habitats in the Atlantic and Indian Oceans. However, the omnipresent parasite invader does not only represent a potential threat to its naïve eel hosts, but is shown to assist fisheries managers in detecting local eel stocking. Furthermore, evidence from computer simulations indicate that natural introgressive hybridization among Atlantic eels is enough to explain genetic differentiation patterns previously published for European eels. This might consolidate recently derived explanations for these patterns. Finally, relying on molecular markers and using Bayesian clustering approaches statistical evidence is presented, that massive trade of live eels might have lead to hybridization among previously isolated species of eels within only a couple of decades. The presented population genetic approach should lead to a better understanding about the direct genetic impact of intentional stocking on both native and introduced nonindigenous species.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit gehe ich auf die durch weltweiten Handel hervorgerufene Verbreitung fremder Aalparasiten und die mögliche genetische Vermischung vorher isolierter Aalarten ein. Die aufgeführten Kapitel decken sowohl die Grundlagenforschung der Parasiten-Populationsgenetik, als auch die Isolierung molekularer Marker und angewandter Methoden des biologischen Artenschutzes ab. Es wird unter anderem eine der wenigen Arbeiten zur Populationsstruktur von Parasiten mittels neuer Mikrosatelliten-Marker vorgestellt. Dabei wurde die Invasionsgeschichte und bestehende Phylogeographie des Schwimmblasennematoden, *Anguillicola crassus*, in seinen neuen Habitaten im Atlantik und dem Indischen Ozean abgeleitet. Der mittlerweile weitverbreitete Parasit stellt jedoch nicht nur eine potentielle Gefahr für den Aal-Bestand dar, denn es zeigte sich, dass er sich auch gut dazu verwenden lässt, lokale Besatzmaßnahmen nachzuweisen. Dadurch könnte der Nematode für das ökologische Management von Aalen Bedeutung gewinnen. In einem weiteren Ansatz wird mit Hilfe von Simulationsstudien gezeigt, dass introgressive Hybridisierung zwischen nordatlantischen Aalen ausreichen würde, um die kürzlich publizierten genetischen Differenzierungsmuster im europäischen Flussaal zu erklären. Dadurch könnten die unterschiedlichen Erklärungsansätze für die Entstehung dieser Differenzierung zu einer Hypothese zusammengefasst werden. Aus den statistischen Daten moderner Bayes'scher Clustering-Methoden auf Basis genetischer Marker wird schließlich dargelegt, dass der massive globale Lebendhandel mit Aalen schon innerhalb weniger Jahrzehnte zu Hybridisierung zwischen verschiedenen Aalarten geführt haben könnte. Insgesamt soll die vorliegende Arbeit dem besseren Verständnis der genetischen Auswirkungen absichtlicher Einschleppung auf heimische und eingeführte Arten dienen.

GENERAL DISCUSSION

Despite their huge diversity, parasites are under-represented in the evolutionary biology literature (Criscione *et al.* 2005). Research on parasitic invaders remains scarce, although numerous parasites have rapidly expanded their ranges recently, and can seriously impact resident biodiversity and human society (Taraschewski 2006). For animal parasites, it has been suggested that host movement is one of the most decisive forces shaping population structure. Animal host migration facilitates gene flow over large distances (Blouin *et al.* 1995; McCoy *et al.* 2003; Criscione & Blouin 2004), and likely causes the breakdown of parasite population structure (Nadler *et al.* 1995). In this thesis, relying on newly derived microsatellite loci (**Chapter 1**), and standard mitochondrial markers, the population structure and phylogeography of an exotic nematode, *Anguillicola crassus,* is studied, which recently invaded the European freshwater eel, *Anguilla anguilla* from Asia. Here I provide evidence for extensive gene flow among parasite sampling localities over large parts of Europe (**Chapter 3**). This confirms the impact of known eel host movement on parasite populations. However, dispersal is punctuated in a North-to-South direction by an existing zoogeographic barrier to invertebrate species, in which direction genetic diversity decreases continuously. In combination with the topology of the distance tree among *A. crassus* nematode populations, my data suggest that Europe was invaded only once from Taiwan, and that subsequently, genetic diversity was lost due to random drift by successive spread of the parasite from North to South. The finding of reduced genetic diversity in invasive nematodes compared to native Asian populations is congruent with the patterns found in many other invasive species (Dlugosch & Parker 2008).

The clarification of the nematode's invasion history and distribution patterns also served as baseline to understand other recent geographic colonizations. Here evidence is presented, that previously introduced North American samples share nuclear and mitochondrial signatures with Japanese specimens, which indicates a separate source population (**Chapter 3**). In contrast, the spread of the parasite to the remote Island of Reunion situated in the Indian Ocean appears to be tightly linked with the European invasion (**Chapter 4**), which was based on the circumstantial findings of shared unique mitochondrial haplotypes and the presence of nonindigenous European eel hosts on this Island. Taken together, both chapters highlight that the spread of the eel parasite correlates with existing trading routes. Thus, invasions might not be sustainable by shipping contaminated fish tank water alone as suggested by Kennedy & Fitch (1990).

However, several aspects remain to be clarified in this parasite-host system. Among them is the need for temporal repetition of sampling (Strayer *et al.* 2006), as the invasion is still very young, and many local populations of the nematode have not yet reached migration-

drift equilibrium (**Chapter 3**). Moreover, the clinical consequences of the swimbladder infestations are likely to compromise the eel's spawning migration (Palstra *et al.* 2007; Lefebvre *et al.* 2007). Thus, because of a potentially increased selection pressure, adaptations on the host's side will presumably evolve rapidly. However, the utilization of neutral markers cannot give insights to adaptive responses at the molecular level, unless they are tightly linked to potential loci under selection, such as *MHCIIb*, involved in the molecular recognition of external parasites (Janeway *et al.* 2004). Therefore, future research in immunogenetics might unravel a strong MHC allele turnover after the arrival of the parasite in Europe. An alternative approach may be to identify variation in the transcriptome among native and nonindigenous populations of the parasite for example by using the advanced high-throughput technology of second generation sequencing. This strategy could detect possible targets for rapid adaptive changes in transcription regulation.

Beside the potentially negative influences of the nematode, its widespread occurrence in Europe can also offer useful potential. Relying on the identified population structure in Europe (**Chapter 3**), we have a genetic baseline to test the utility of parasites as biological tags, which has a long tradition in fish stock identification (MacKenzie 2002). However, the use of molecular markers to infer parasite movement as proxy for wild hosts that lack population genetic structure, has only recently been embraced more broadly (Wirth *et al.* 2005; Nieberding & Oliveri 2007). In **Chapter 5**, I demonstrate the usefulness of the nematode as biological tag of eel stocking, by contrasting two differently managed eel stocks. Stocks disrupted by recent stocking (River Rhine) display significant deviations from Hardy-Weinberg Equilibrium, compared to a system with natural glass eel recruitment (River Frémur). This signal is consistent over all four genetic markers used, and is due to the presence of first generation migrants from all around Europe. Therefore, this method can assist to discern anthropogenically driven stocking from naturally occurring eel recruitment. Despite evidence for occasional cluster transmission of related parasite organisms into the same eel host, local nematode populations are unstructured and random mating is apparent.

No publication to date has explicitly focused on the influence of hybridization on genetic differentiation signatures in North Atlantic eels. Therefore, in **Chapter 6**, available nuclear microsatellite and mitochondrial sequence data of 1,263 eel samples from across the Atlantic and from Iceland were statistically evaluated. When simulating continuously increasing proportions of F1 hybrid individuals from the southern to the northern-most locations in Europe, highly significant isolation-by-distance patterns arose, that are reminiscent of previously published data (Wirth & Bernatchez 2001). Therefore introgressive hybridization alone is sufficient to explain the correlation of geographic and genetic distances reported for the European freshwater eel. Moreover, contrasting signals among nuclear and mitochondrial lineages suggest a recent onset of gene flow, most likely after glacial retreat

following the last Ice Age (vicariant scenario; Avise *et al.* 1990). Importantly, our findings are in agreement with previous results on genetic isolation patterns in European eels, either based on geography (IBD; Wirth & Bernatchez 2001; Maes & Volckaert 2002) or interannual genetic composition (IBT; Maes *et al.* 2006a). If we can assume that the known overlap of the two species' spawning grounds (Tsukamoto 2006) is annually changing, the IBD signal should decline in some years (with low overlap), and increase in others. This will then automatically lead to an even higher IBT signal among annual recruitment waves within species. This hypothesis cannot yet explain the clear separation of mitochondrial lineages and therefore remains to be tested. We suggest that the lack of pure American expatriates in Iceland (see also Albert *et al.* 2006), is due to the American eel's much faster ontogenetic development and metamorphosis which might prevent its settlement in this northern region (comparable to an early exploding "time bomb"). All in all, evidence for higher hybrid survival rates in Iceland (Albert *et al.* 2006) favours the introgression hypothesis followed by subsequent backcrossing. This might not only hold for Iceland but for other Nordic regions as well, and might generate a North-to-South-hybrid gradient in both Atlantic eel species.

Finally in **Chapter 7**, due to the high conservation of the microsatellite flanking regions (Maes *et al.* 2006b; **Chapter 2**), the survey of eel stocks for hybrids was expanded to five commercial species using Bayesian clustering approaches. In line with the surprisingly low nuclear genetic differentiation among Japanese and European eels, we found evidence for the presence of an F1 hybrid among Japanese and European eels in France. These results are congruent with the introduction of Japanese eels from Taiwan into northern Europe in the late 1970s (Hartmann & Koops 1989). Moreover, natural hybridization might occur not only between the two Atlantic eel species (**Chapter 6**), but also between the closely related Australian shortfin and New Zealand longfin eels. The introgression signals detected here might be only the tip of the iceberg, as increasing number of markers would increase the significance level of assignment into hybrid classes (Vähä & Primmer 2006). As a result of the eel's panmixia and inefficient stock management, long-term genetic pollution is likely. Indeed, the fact that evidence for introgressive hybridization was found after only two or three generations raise concerns for long-term consequences, including the loss of species integrity (Arnold & Hodges 1995), reduction in fitness (Dowling & Moore 1985; Rhymer & Simberloff 1996), or species replacements (Hale *et al.* 2004).

ALLGEMEINE DISKUSSION

Trotz ihrer großen Diversität sind parasitische Organismen in der evolutionsbiologischen Literatur unterrepräsentiert (Criscione *et al.* 2005). Zudem gibt es nur wenige Studien über invasive (eingeschleppte) Parasiten, obschon sich diese in letzter Zeit stark ausgebreitet haben. Dies kann sich nachhaltig sowohl auf die heimische Biodiversität als auch auf unsere menschliche Gesellschaft auswirken (Taraschewski 2006). Für Zoo-Parasiten gelten Wirtsbewegungen als wichtigste Einflussfaktoren auf die Populationsstruktur; denn über die Tierwirte wird auch der Genfluss der Parasiten über weite Entfernungen aufrechterhalten (Blouin *et al.* 1995; McCoy *et al.* 2003; Criscione & Blouin 2004), wodurch bestehende Populationsstrukturen zusammenbrechen können (Nadler *et al.* 1995). In der vorliegenden Arbeit wurde die Populationsstruktur und Phylogeographie des kürzlich eingeschleppten Schwimmblasen-Parasiten *Anguillicola crassus* im Europäischen Flussaal, *Anguilla anguilla*, charakterisiert. Mittels neu abgeleiteter Mikrosatelliten-Loci (**Kapitel 1**) als auch standardmäßig eingesetzter mitochondrialer Marker konnte dabei ausgeprägter Genfluss zwischen weiträumig entfernten Parasitenpopulationen in Europa gezeigt werden (**Kapitel 3**). Dieses Ergebnis ist ein Beleg für den starken menschlichen Einfluss auf die Populationsstruktur der Nematoden. Jedoch werden diese Verbreitungsrouten in Nord-Süd-Richtung von einer existierenden zoogeographischen Barriere für Invertebraten unterbrochen, wobei die genetische Diversität in eben dieser Richtung kontinuierlich abnimmt. Diese Daten lassen zusammen mit der Topologie des Populations-Distanz-Baums den Schluss zu, dass die Nematoden lediglich einmal von Taiwan aus nach Europa eingeschleppt wurden, und sich desweiteren in Nord-Süd-Richtung ausbreiteten, wodurch die genetische Diversität durch zufällige Drift verloren ging. Die reduzierte genetische Diversität in den invasiven Parasitenpopulationen ist ein typisches Muster für eingeschleppte Arten (Dlugosch & Parker 2008).

Die Klärung der Populationsstruktur und Invasionsgeschichte des Nematoden diente im Folgenden als Grundlage weiterer Untersuchungen. Die über Aalfarmen eingeschleppten Nematoden in Nordamerika trugen beispielsweise nukleäre und mitochondriale genetische Signaturen der in Japan gesammelten Populationen, was auf eine von Europa unabhängige Besiedlung hindeutet (**Kapitel 3**). Im Gegensatz dazu scheint die Verbreitung des Parasiten auf die abgelegene Insel Réunion im Indischen Ozean eng mit der europäischen Kolonisierung verbunden zu sein (**Kapitel 4**). Starkes Indizien hierfür waren die mitochondrialen Signaturen und das Vorkommen nichtheimischer Europäischer Flussaale auf dieser Insel. Insgesamt unterstreichen die beiden Kapitel, dass die Verbreitung des Aalparasiten stark vom Handel mit Lebendaal abhängig ist. Dadurch ist die nachhaltige

Etablierung des Parasiten allein über kontaminiertes Fischtankwasser eher unwahrscheinlich (Kennedy & Fitch 1990).

Einige Aspekte des Parasiten-Wirts-Systems bleiben jedoch nach wie vor offen. So ist es nötig die Beprobung desselben Ortes zeitlich zu wiederholen (Strayer *et al.* 2006), da die Invasion noch sehr jung ist, und sich definitiv noch kein Migrations-Drift-Gleichgewicht eingestellt hat (**Kapitel 3**). Darüberhinaus wurde wiederholt festgestellt, dass die Laichwanderungen befallener Aale durch die Auswirkungen der Schwimmblaseninfektionen stark beeinträchtigt werden könnten (Palstra *et al.* 2007; Lefebvre *et al.* 2007). Es ist zu erwarten, dass ein potentiell erhöhter Selektionsdruck rasche Adaptationen im Aalwirt zur Folge hat. Leider lassen sich anhand neutraler Mikrosatelliten-Marker keine Einsichten zur molekularen Adaptation gewinnen, außer diese wären eng mit unter Selektion stehenden Markern gekoppelt. Ein solcher Marker ist der *MHCIIb*-Locus, der in der molekularen Erkennung von externen Parasiten involviert ist (Janeway *et al.* 2004). Neben dem besseren Verständnis der Immungenetik, wäre ein alternativer Ansatz vielversprechend: unter Anwendung fortgeschrittener Hochdurchsatz-Technologien der zweiten Sequenzier-Generation könnten Erkenntnisse zur Variation im Transkriptom zwischen heimischen und invasiven Populationen des Nematoden offengelegt werden. Diese zweite Strategie könnte mögliche Ziele für eine schnelle Anpassung der Nematoden auf Ebene der Transkriptionsregulierung aufdecken.

Neben einer Reihe potentiell negativer Einflüsse der Parasiten auf seinen neuen Aalwirt, könnte dessen weitreichende Verbreitung in Europa auch nützliches Potential haben. Die abgeleitete Populationsstruktur aus **Kapitel 3** diente mir dabei als genetische Baseline zur Tauglichkeitsprüfung des Parasiten als biologischer Marker für Aal-Wanderung und Aal-Besatz (**Kapitel 5**). Die Verwendung solcher parasitischer Bio-Marker hat eine lange Tradition bei der Untersuchung von Fischbeständen (MacKenzie 2002). Jedoch ist die Anwendung molekularer Marker als Zeiger für Wirts-Wanderungen erst seit kurzem von breiterem Interesse (Wirth *et al.* 2005; Nieberding & Oliveri 2007). Anhand zweier unterschiedlich geführter Aalgewässer konnte gezeigt, dass sich *A. crassus* als biologischer Zeiger für Besatzaßnahmen eignet. Während die Parasiten-Population unter regelmäßigem anthropogenem Aalbesatz (Rhein) eine signifikant starke Abweichung vom Hardy-Weinberg-Gleichgewicht zeigt, trifft der umgekehrte Fall auf ein Gewässer mit natürlicher Glasaal-Rekrutierung zu (Frémur). Das genetische Signal ist dabei für alle vier verwendeten Marker konsistent. Der Nachweis von eingeschleppten Migranten aus anderen Teilen Europas belegt die Besatzmaßnahme in den Rhein. Hinweise für den Befall desselben Aalwirts mit nah-verwandten Parasiten sind für die lokalen Populationen auf Grund von Zufallsverpaarung unerheblich.

Flussaale wurden in vielen Biologielehrbüchern als Parade-Beispiel für Zufallsverpaarung (Panmixie) hervorgehoben. Mehrere unabhängige Studien lieferten jedoch genetische Gegenbeweise für dieses Paradigma (Wirth & Bernatchez 2001; Maes & Volckaert 2002). Bis heute hat jedoch keine Studie explizit den Einfluss von möglicher Hybridisierung auf die genetische Differenzierung der Nordatlantatik-Aale überprüft. In **Kapitel 6** wurden daher bereits vorhandene nukläre Mikrosatelliten- und mitochondriale Sequenzdaten von 1263 Aalen beiderseits des Atlantiks und aus Island statistisch ausgewertet. Mit Hilfe von virtuellen Hybridisierungen wurden in einem Simulationsansatz die Anteile an virtuellen F1-Hybriden schrittweise von Nord- nach Südeuropa erhöht. Mit zunehmender Hybridisierungsstärke stieg auch das Signifikanzniveau des Differenzierungsmusters (IBD), wodurch sich die Ergebnisse aus Wirth & Bernatchez (2001) bestätigen lassen. Die genetische Struktur im Europäischen Flussaal (IBD) ließe sich also am einfachsten auf die introgressive Hybridisierung zwischen den nordatlantischen Aalarten zurückführen. Die unterschiedlichen Informationen zwischen mitochondrialen und nukleären Genen lassen den Schluss zu, dass der Genfluss erst seit kurzem stattfindet, und mit dem Ende der letzten Eiszeit korreliert sein könnte (Vikarianz-Szenario, Avise *et al.* 1990). Dieses Ergebnis steht im Einklang mit den kürzlich abgeleiteten genetischen Isolationsmustern, entweder auf Basis der geographischen (IBD, Wirth & Bernatchez 2001; Maes & Volckaert 2002) oder zeitlichen Distanz (IBT, Maes *et al.* 2006a). Unter der Annahme, dass sich die Überlappungszonen der Laichgründe (Tsukamoto 2006) zwischen den Aal-Arten jährlich verändert, sollte das IBD-Signal in manchen Jahren (bei geringer Überlappung) ab-, und in anderen Jahren zunehmen. Dies würde automatisch zu viel stärkeren IBT-Signalen zwischen verschiedenen Rekrutierungs-Wellen der Aal-Larven führen. Diese Hypothese kann aber nicht erklären, warum nicht auch die mitochondriale Linie zwischen den Kontinenten überlappt. Wir schlagen deshalb vor, dass das Fehlen reiner amerikanischer Aale in Island (siehe auch Albert *et al.* 2006), auf die relativ schnellere ontogenetische Entwicklung und Metamorphose in *A. rostrata* zurückzuführen ist, und deshalb eine erfolgreiche Besiedlung in dieser nördlichen Region unterbleibt (vergleichbar etwa mit einer zu früh hochgehenden "Zeitbombe"). Es bleibt festzuhalten, dass die höheren Überlebensraten von Hybriden in Island (Albert *et al.* 2006) die Hypothese der Introgression mit anschließender Rückkreuzung bestätigt. Dies sollte nicht nur für Island zutreffen, sondern auch für andere nordische Regionen, ein Umstand der den genetischen Vermischungsgradienten zwischen den Atlantik-Aalen in Nord-Süd-Richtung erklären könnte.

Begünstigt durch den hohen Konservierungsgrad der Mikrosatelliten-flankierenden Regionen im Aal (Maes *et al.* 2006b; **Kapitel 2**), wird in **Kapitel 7** die Untersuchung von fünf kommerziell gehandelten Aal-Arten auf Hybridisierung mittels Bayes'scher Cluster-Verfahren vorgestellt. Dabei steht der erstaunlich niedrige genetische Differenzierungsgrad zwischen

dem Japanischen und Europäischen Flussaal im Einklang mit der Detektion eines F1-Hybriden in Frankreich. Dieses Ergebnis deckt sich mit der Einführung Japanischer Aale aus Taiwan nach Nord-Europa (Hartmann & Koops 1989). Desweiteren gibt es Hinweise darauf, dass natürliche Hybridisierung nicht nur zwischen den nordatlantischen Aalen stattfindet (**Kapitel 6**), sondern gelegentlich auch zwischen den nahverwandten australischen Kurzflossen- und Neuseeland-Aalen.

Die abgeleiteten Introgressionssignale sollten lediglich die Spitze des Eisbergs darstellen, da eine nötige Erhöhung der Markerzahl auch gleichzeitig das statistische Vertrauensniveau beim Assignment der Hybridklassen erhöhen würde (Vähä & Primmer 2006). Als direkte Folge des Laichverhaltens im Flussaal und des ineffizienten Bestandsmanagements ist eine nachhaltige genetische Vermischung der Aale als sehr wahrscheinlich anzusehen. Die vorliegenden Daten geben Anlass zur Sorge, dass eine Vermischung bereits nach nur etwa zwei bis drei Generationen langfristige Auswirkungen auf die Spezies-Integrität (Arnold & Hodges 1995), die Reduktion der individuellen Fitness (Dowling & Moore 1985; Rhymer & Simberloff 1996), als auch auf die mögliche komplette Verdrängung von Arten (Hale *et al.* 2004) haben könnte.

EIGENABGRENZUNG

Unless otherwise mentioned, all results in this thesis were obtained by me or under my direct supervision. Results produced by collaborators:

Chapter 4:
Parasitological survey and their statistics were performed by co-authors. The manuscript was initially drafted by Horst Taraschewski, including Fig. 4.1 and Tables 4.1-4.3.

Chapter 5:
Florian Hollandt performed a part of the labwork (Rhine) as part of his Bachelor's Thesis, and contributed Fig. 5.1.

Chapter 6:
Thierry Wirth performed genotyping of the eel specimens, and contributed Fig. 6.5a.

Chapter 7:
Thierry Wirth performed genotyping of the eel specimens, wrote parts of the manuscript, and contributed Fig. S7.1; Appendix 1.

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APPENDIX 1: Supplementary Figures

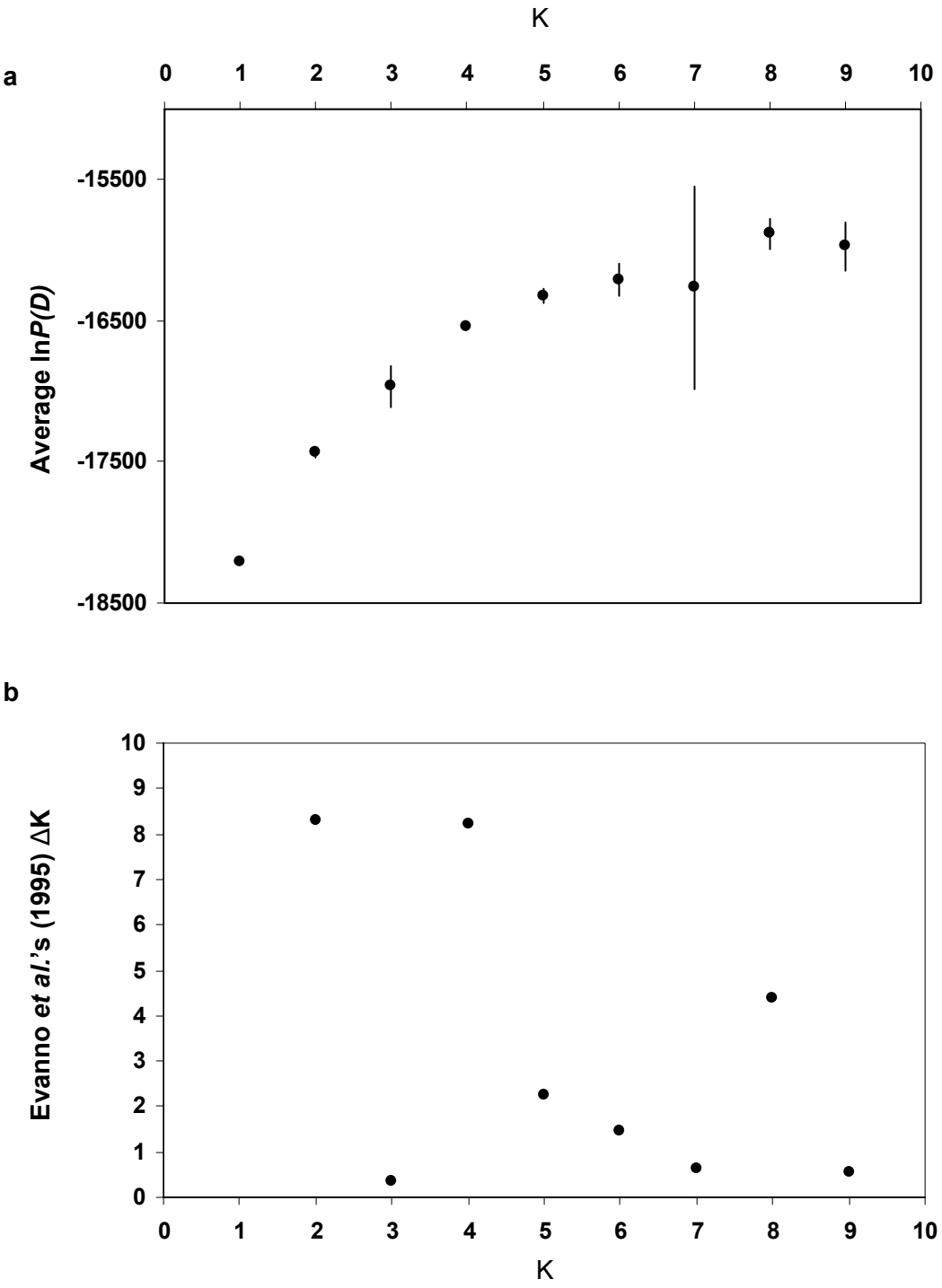

Figure S3.1 **a**) Likelihood probabilities ln*P(D)* representing ten independent runs for each examined number of assumed population clusters (*K*), analysed in STRUCTURE v2.2 (Pritchard *et al.* 2000). Error bars represent SD of arithmetic means. **b**) Subsequent statistical evaluation of the likelihood values for a given number of assumed population clusters (*K*) using the ad-hoc statistic *ΔK* proposed by Evanno *et al.* (2005).

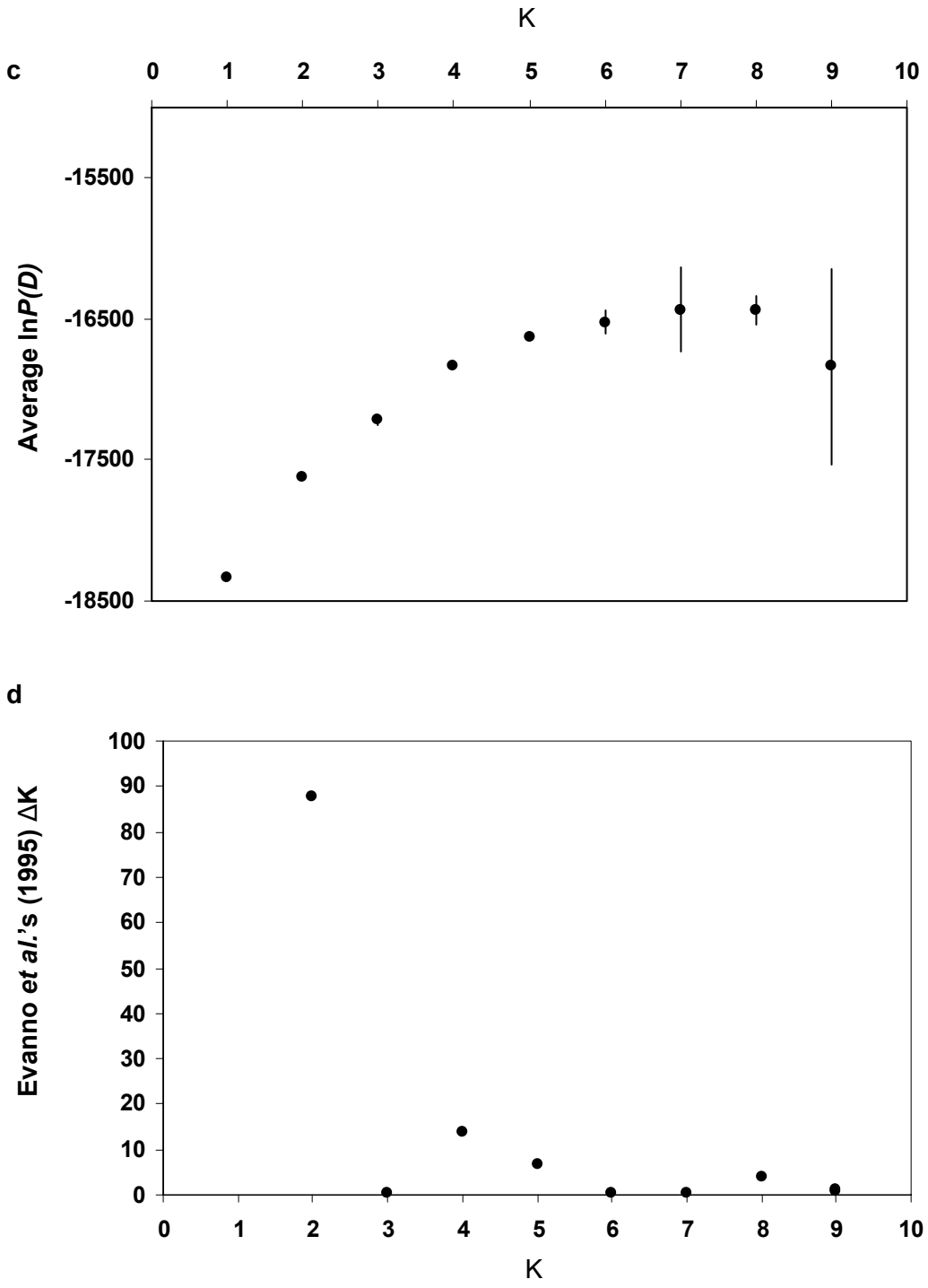

Figure S3.1 (continued) c) & **d)** are analogous to **a)** & **b)** having derived from five independent runs using a null-allele corrected dataset.

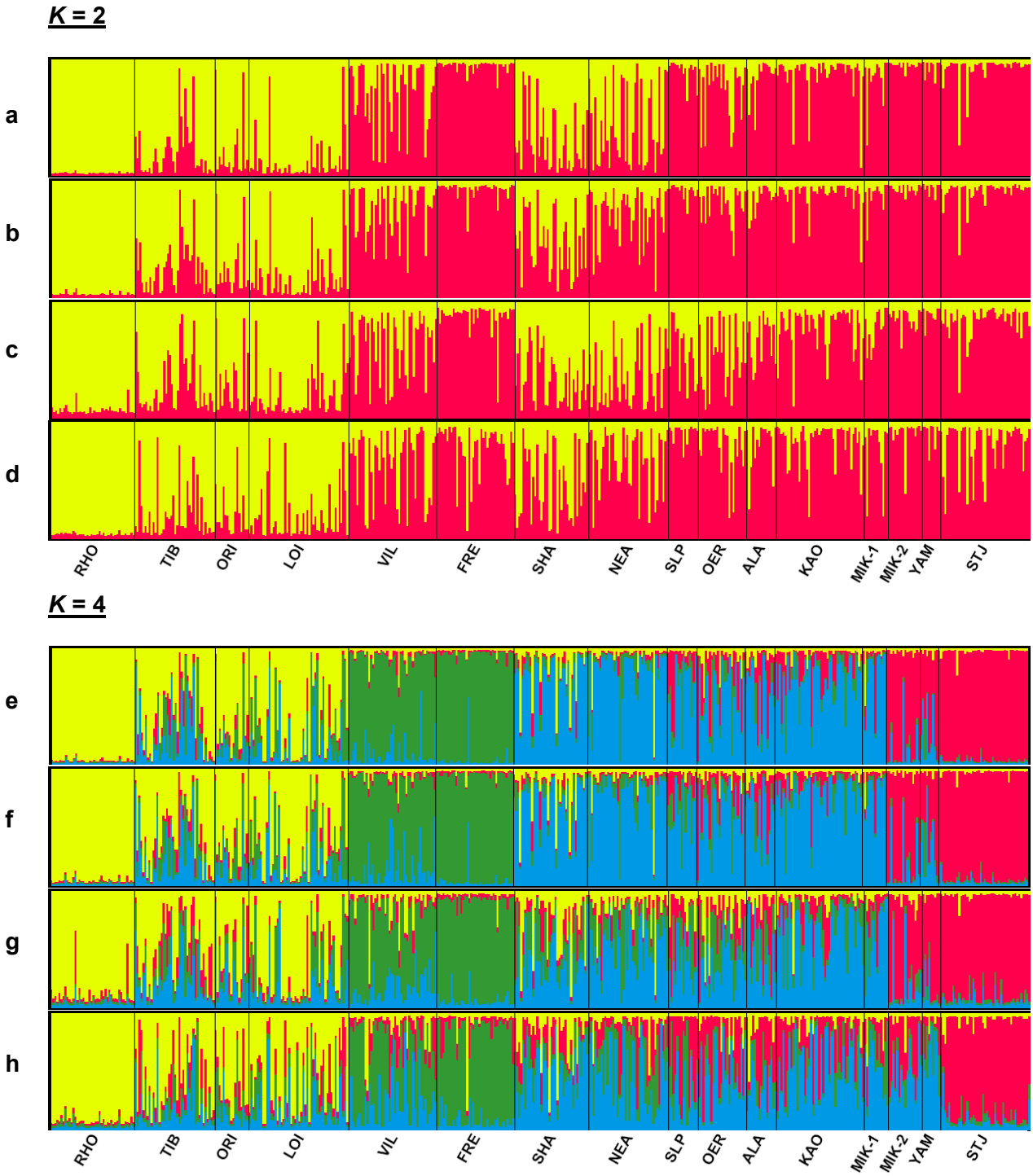

Figure S3.2 Individual-based cluster representation based on Bayesian inference of population structure for two scenarios **a)** – **d)** K = 2 and **e)** – **h)** *K* = 4. Each plot represents different datasets: **a)** & **e)** original, uncorrected dataset for all 7 microsatellite markers; **b)** & **f)** null-allele corrected dataset for all 7 markers; **c)** & **g)** null-allele corrected dataset for the 4 markers deviating from HWE (*AcrCT29, AcrCT53, AcrCT103, AcrCA102*); **d)** & **h)** null-allele corrected dataset for the 3 Markers not deviating from HWE (*AcrCT04, AcrCT27, AcrCT54*). Each color represents one assumed population cluster *K*. Multiply colored bars display an individual's estimated membership proportion in more than one population (q), i.e. admixture. Sampling locations are ordered from Southern to North-Eastern Europe from left to right, followed by Asian and North American samples. The labels indicate sampling location (below; for abbreviations refer to **Table 3.1**) and the region of origin (above).

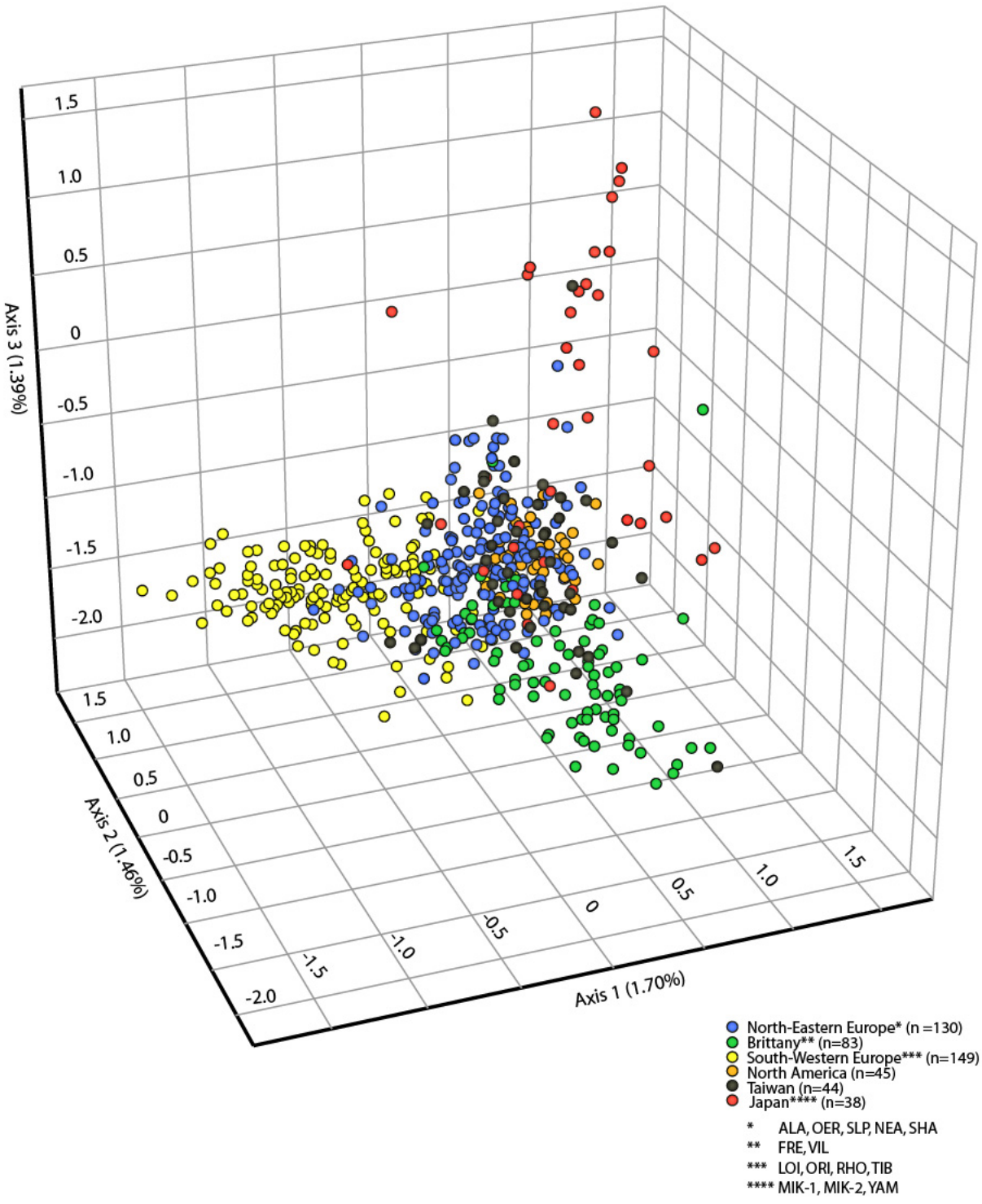

Figure S3.3 Factorial component analysis representing the first three orthogonal axes of variation ranked by informativeness. Circles indicate individual nematodes colored according to their sampling locality. Relative numbers below indicate percent of the total variance explained by the according axis.

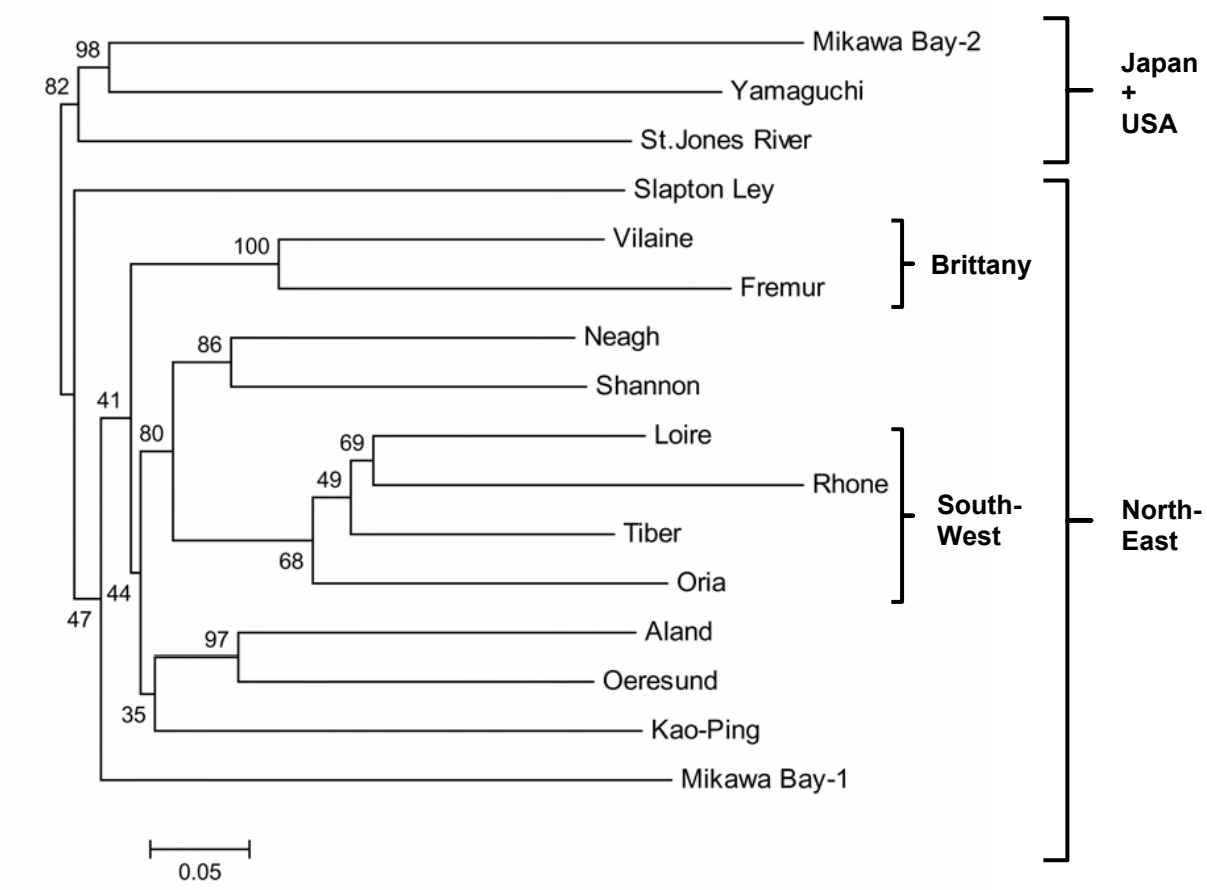

Figure S3.4 *Neighbor-joining* (NJ) phenogram summarizing Cavalli-Sforza & Edwards' (1967) D_{CE} chord distances without null-allele correction among 12 invasive and three native populations. European groupings are highlighted by brackets based on the STRUCTURE tool. The out-group was defined according to the tree's mid-point. Values on the nodes represent the percentage of bootstrap replicates over loci (n = 100). Branch lengths are proportional to the genetic distance between the taxa. The scale bar represents genetic distance D_{CE} of 0.05.

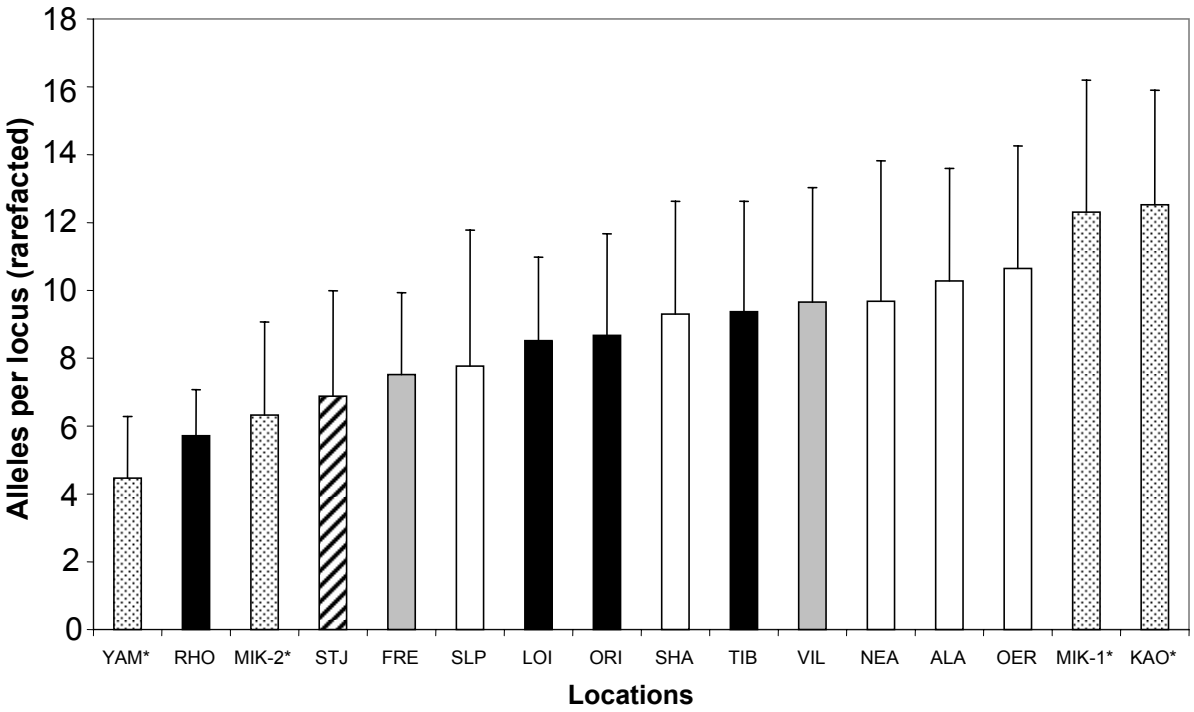

Figure S3.5 Box plot representation of local allelic richness (rarefacted number of alleles per locus) between European, North American and Asian locations. Error bars represent SD of arithmetic means. Bar colors are reminiscent of **Figs. 3.5 a)** and **b)**, location STJ is highlighted in squared black-and-white.

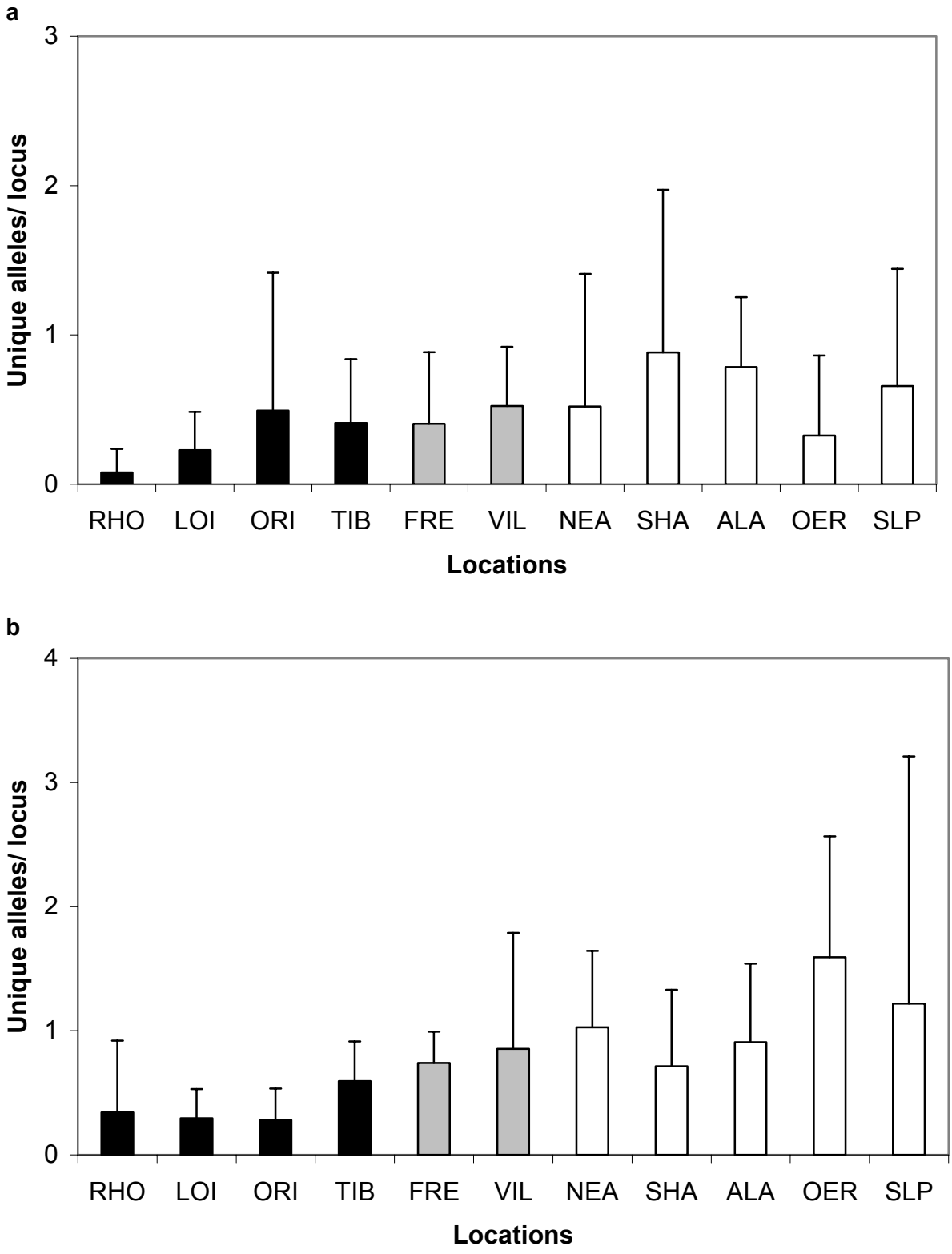

Figure S3.6 Box plot representation of mean allelic uniqueness for **a)** the 4 markers deviating from HWE (*AcrCT29, AcrCT53, AcrCT103, AcrCA102*); and **b)** 3 Markers not deviating from HWE (*AcrCT04, AcrCT27, AcrCT54*). Shared bar colors display shared geographic grouping. Error bars indicate the SD of arithmetic means.

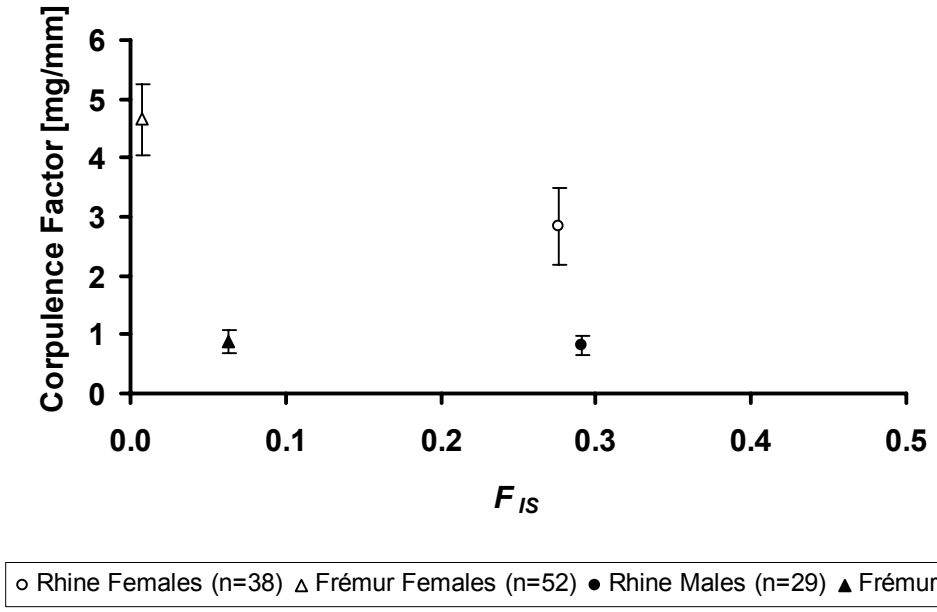

Figure S5.1 Plot of average corpulence factors over average inbreeding indeces F_{IS} highlighted separately for **a)** females, and **b)** and males among sampling localities.

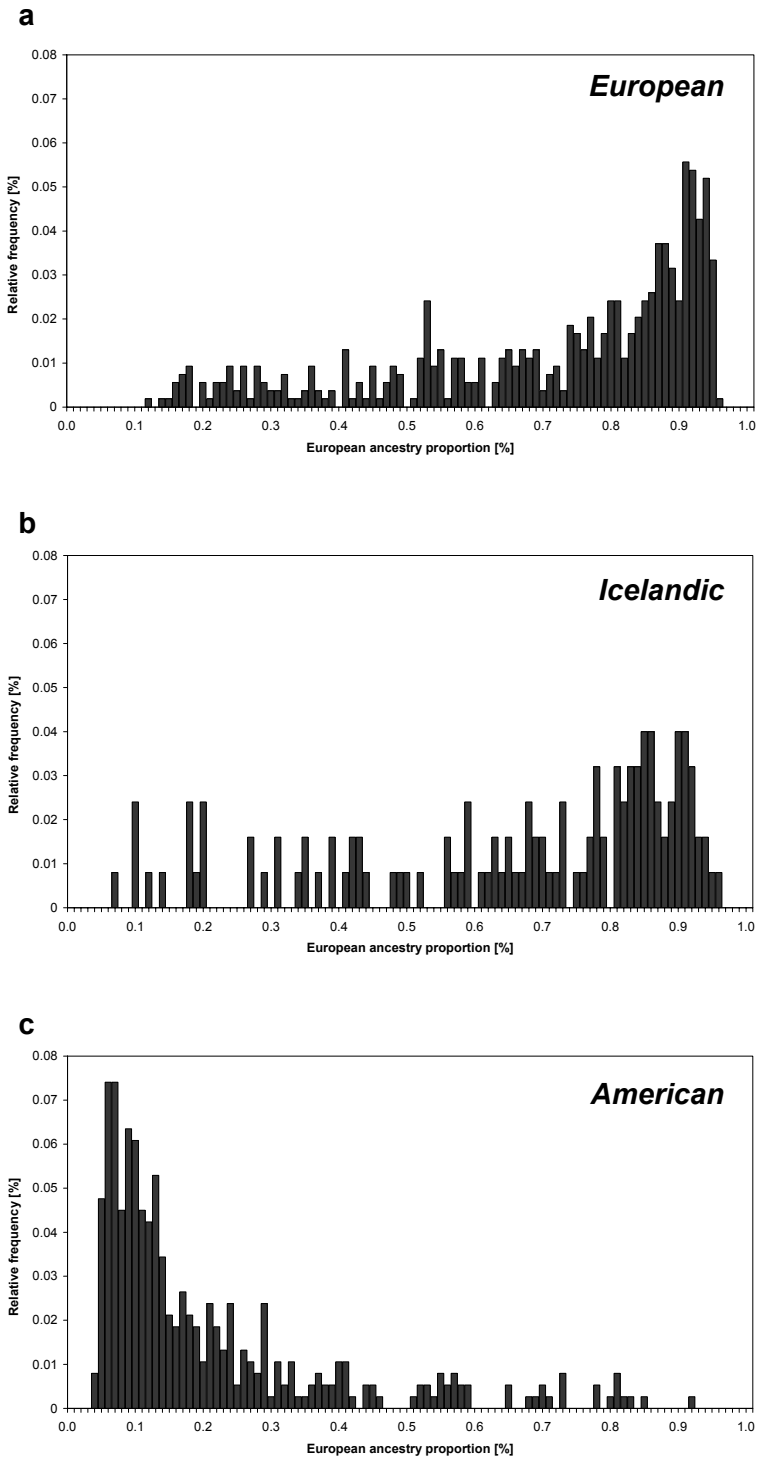

Figure S6.1 Relative frequency distribution of admixture levels depicting overall high levels of admixture North Atlantic eels according to STRUCTURE v2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). Ancestry proportions are illustrated separately for **a)** European continental; **b)** Icelandic; and **c)** American continental eels.

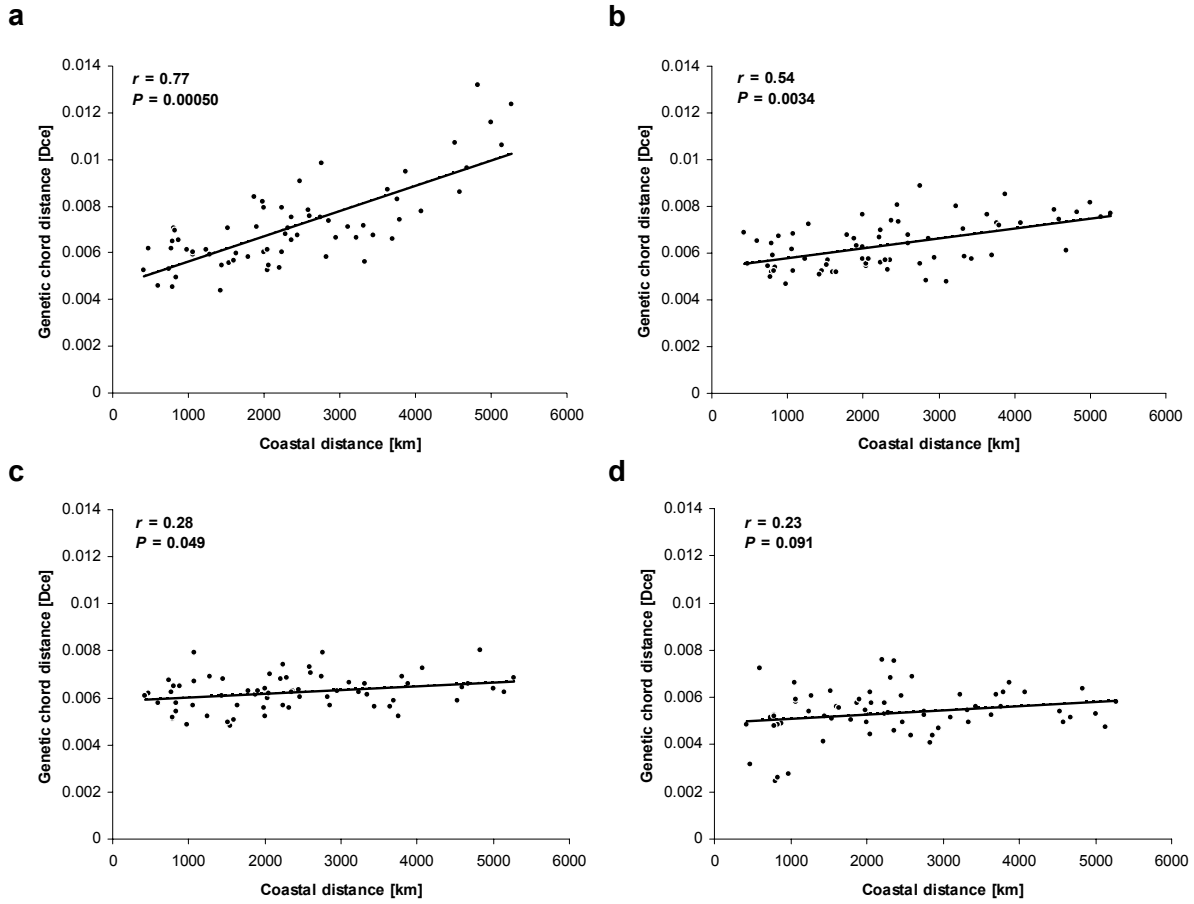

Figure S6.2 Influence of F1 clines on IBD patterns. The purest North Atlantic eel locations (River Minho, (PT), *n* = 43; St. Johns River, Fl. (US), *n* = 35) served as parental gene pools for the first generation crosses. We augmented the proportion of F1 hybrids in a stepwise process by **a)** 4%; **b)** 3%; **c)** 2%; **d)** 1% per population for a total of 12 virtual F1 populations. Significance of IBD was tested using the Mantel statistics for correlated genetic data (Mantel 1967). To test our hypothesis, that IBD patterns can be generated in European eels by increasing levels of gene flow from South to North, the rectangular matrix of pairwise geographical distances from Wirth & Bernatchez (2001) was superimposed on the genetic pairwise D_{CE} distances among the12 virtual populations. Thus, assuming a linear increase of gene flow, we attriburted the Southern-Eastern-most location (River Tiber) the lowest, and the Northern-Western-most locality (Iceland) the highest hybridization rate. Intermediate levels were attributed in ascending orderin the same direction along the European coastline.

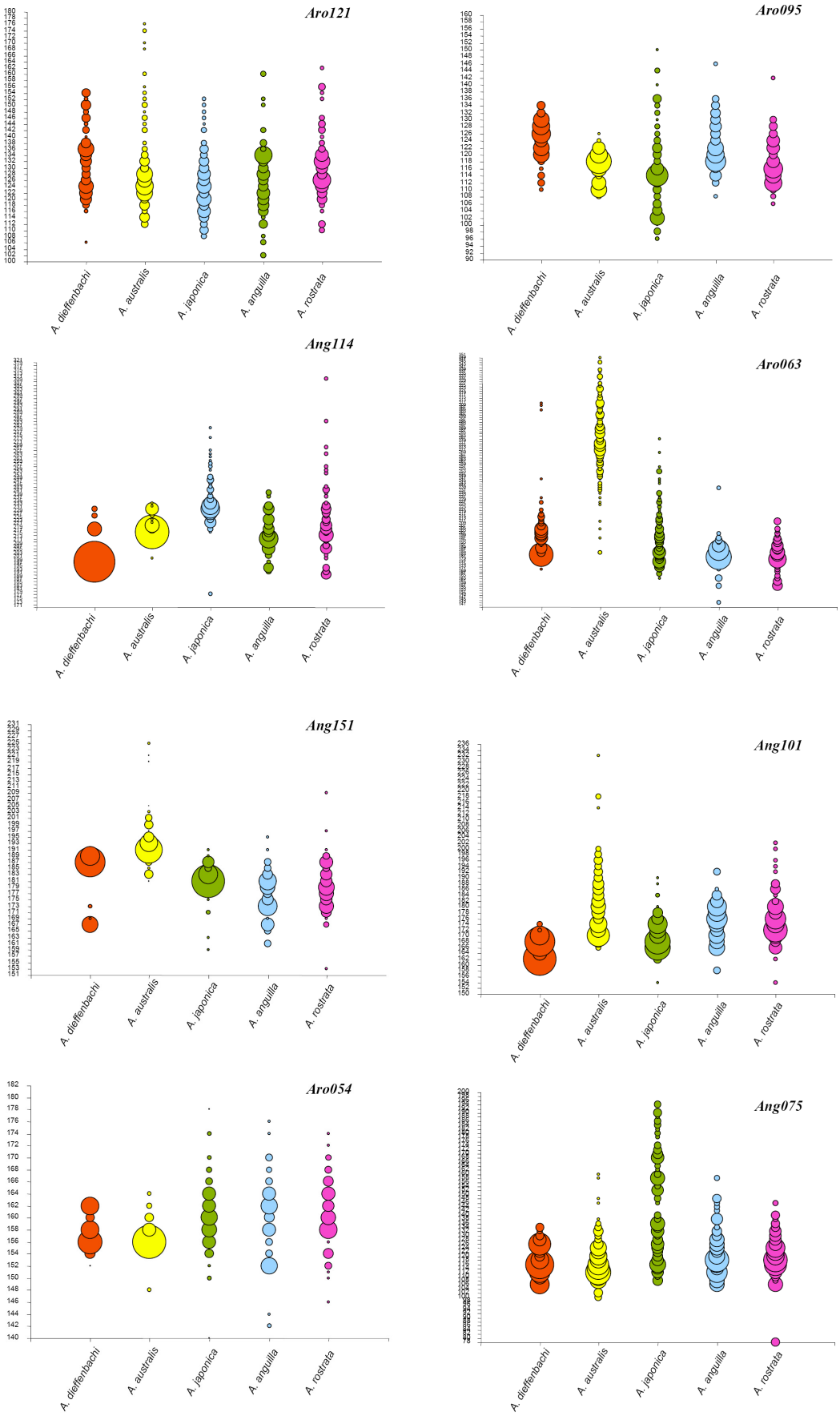

Figure S7.1 Allele frequencies and size distributions of 8 microsatellite loci in five different eel species. The area of the bubbles corresponds to the frequencies of the respective alleles in a given species. The y-axis denotes the allele size in base pairs.

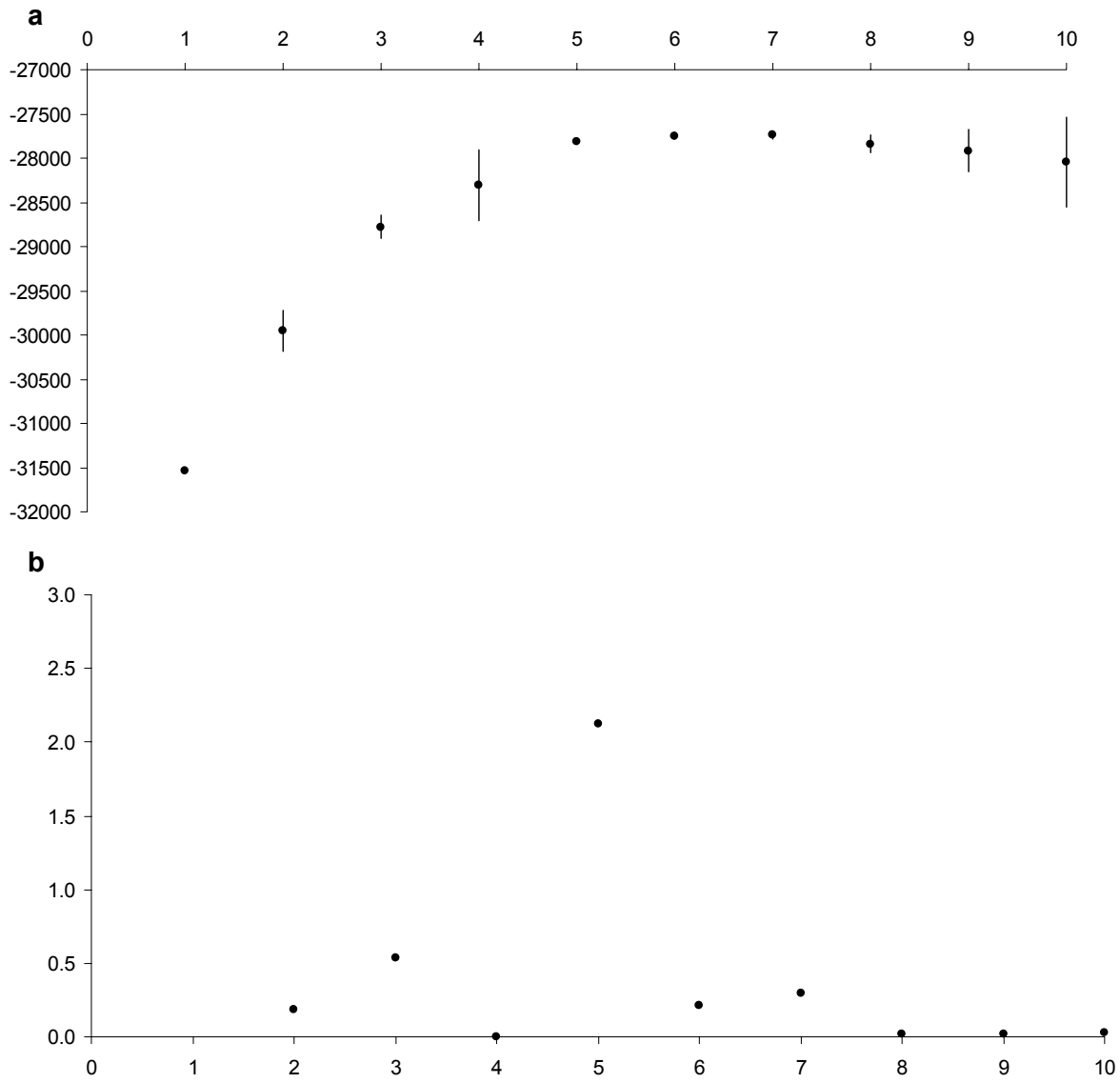

Figure S7.2 a) The number of population clusters represented in our sample estimated by the likelihood of the data, given each of the models of K $\in$ {1, 10} populations for 10 short-run iterations (10,000 Burnins; 20,000 MCMC sampling steps; POPFLAG = 0); **b)** based on this output, Evanno *et al.*'s (2005) ad-hoc statistic ΔK was used to evaluate the most likely number of clusters.

APPENDIX 2: Supplementary Tables

Table S3.1 Allele counts and rarefacted allelic richness and diversity (n = 24 genes) as measured for all microsatellite loci.

Ref	*AcrCT04*			*AcrCT27*			*AcrCT29*			*AcrCT53*			*AcrCT54*			*AcrCT103*			*AcrCA102*			Total		
	A	R	D	A	R	D	A	R	D	A	R	D	A	R	D	A	R	D	A	R	D	A	R	D
ALA	15	13.7	0.47	15	13.3	0.36	8	7.7	1.14	13	11.7	0.22	8	7.3	0.06	14	12.6	0.01	6	5.6	0.80	11.3	10.3	0.44
OER	23	14.4	0.24	19	13.9	0.72	9	7.3	0.02	15	11.9	0.09	10	8.0	0.54	18	13.5	0.97	7	5.6	0.00	14.4	10.6	0.37
SLP	13	12.5	2.44	11	10.6	0.07	4	4.0	0.00	12	11.1	0.97	4	3.9	0.01	10	9.4	0.42	3	3.0	0.00	8.1	7.8	0.56
NEA	28	15.4	1.42	21	12.2	0.43	9	6.3	0.06	21	12.7	1.63	8	5.7	0.00	19	10.8	0.10	5	4.6	0.00	15.9	9.7	0.52
SHA	20	12.4	0.75	18	12.1	0.88	10	7.3	0.35	20	12.7	1.70	8	6.1	0.00	15	9.9	0.00	5	4.5	0.04	13.7	9.3	0.53
FRE	15	9.9	0.65	13	9.9	0.00	5	4.7	0.00	13	10.0	0.13	7	5.7	0.02	11	7.4	0.08	6	5.0	0.54	10.0	7.5	0.20
VIL	20	13.0	1.43	18	12.6	0.53	7	6.4	0.04	22	13.0	0.44	8	6.5	0.01	16	10.5	0.49	7	5.6	0.47	14.0	9.7	0.49
LOI	21	11.3	0.06	16	10.2	0.02	9	6.8	0.25	16	9.7	0.51	10	7.1	0.00	16	10.1	0.00	6	4.4	0.01	13.4	8.5	0.12
ORI	13	10.8	0.27	10	9.1	0.35	6	5.8	0.00	16	13.2	1.45	8	7.0	0.00	12	10.2	0.00	5	4.7	0.00	10.0	8.7	0.30
RHO	8	7.6	0.00	6	5.4	0.00	6	4.2	0.00	9	5.8	0.29	8	6.9	1.00	7	6.2	0.00	5	3.9	0.00	7.0	5.7	0.18
TIB	19	11.7	0.91	19	11.1	0.08	9	6.7	0.14	21	13.0	0.72	10	7.2	0.03	18	11.5	0.26	7	4.3	0.01	14.7	9.4	0.31
KAO	30	16.3	1.72	26	14.4	0.48	14	8.7	1.57	30	16.1	2.85	16	9.3	2.26	22	13.8	1.40	13	9.1	1.89	21.6	12.5	1.74
MIK-1	17	16.1	1.84	15	14.2	0.95	12	11.5	4.68	18	17.2	5.10	10	9.6	0.61	12	11.5	2.86	6	6.0	0.00	16.0	12.3	2.29
MIK-2	8	7.1	0.86	12	10.6	0.09	5	4.4	0.04	11	9.4	2.04	4	3.9	0.09	6	5.0	0.05	4	3.8	0.00	7.0	6.3	0.45
YAM*	14	7.2	2.69	9	5.6	0.37	4	2.3	0.00	10	6.0	0.03	4	3.3	0.02	4	2.9	0.10	4	4.0	0.00	9.6	4.5	0.46
STJ	14	10.0	0.60	13	8.6	0.68	5	4.2	0.00	15	9.7	2.35	6	4.6	0.04	11	8.7	0.00	3	2.3	0.00	11.3	6.9	0.52
Total**	**65**			**37**			**23**			**62**			**23**			**33**			**15**			**36.9**		

Ref, reference name for geographic populations; A, allele count; R, allelic richness (rarefacted); D, allelic distinctiveness (rarefacted);
* rarefaction performed for 18 genes only; ** total number of different alleles for the current microsatellite locus (for n = 946 genes).

Table S3.2 Unbiased expected (H_E) and observed (H_O) heterozygosities for single loci, including respective inbreeding coefficients (F_{IS}) and probabilities (P) of exact tests for Hardy-Weinberg equilibrium for all locations.

Loc/ Ref	ALA	OER	SLP	NEA	SHA	FRE	VIL	LOI	ORI	RHO	TIB	KAO	MIK-1	MIK-2	YAM	STJ
AcrCT04																
H_E	0.940	0.925	0.926	0.953	0.920	0.893	0.936	0.903	0.866	0.868	0.914	0.962	0.960	0.811	0.967	0.906
H_O	1.000	0.875	0.769	0.800	0.946	0.641	0.791	0.918	0.941	0.833	0.821	0.955	0.846	1.000	0.889	0.956
F_{IS}	-0.066	0.055	0.175	0.162	-0.029	0.285	0.157	-0.017	-0.089	0.040	0.103	0.008	0.123	-0.244	0.086	-0.056
P	0.848	0.058	0.214	0.007	0.923	<0.001	0.017	0.150	0.276	0.767	0.525	0.089	0.012	0.551	0.321	0.990
AcrCT27																
H_E	0.938	0.944	0.902	0.900	0.925	0.900	0.933	0.900	0.882	0.785	0.894	0.943	0.929	0.897	0.895	0.856
H_O	0.867	0.958	0.846	0.775	0.892	0.872	0.841	0.940	0.941	0.810	0.725	0.841	0.923	0.938	1.000	0.822
F_{IS}	0.078	-0.015	0.064	0.140	0.036	0.031	0.100	-0.045	-0.069	-0.032	0.191	0.109	0.007	-0.047	-0.125	0.040
P	0.195	0.847	0.460	0.044	0.493	0.005	0.119	0.555	0.827	0.876	0.003	0.072	0.430	0.323	1.000	0.621
AcrCT29																
H_E	0.860	0.820	0.431	0.724	0.778	0.754	0.781	0.794	0.797	0.586	0.779	0.831	0.886	0.629	0.314	0.666
H_O	0.667	0.542	0.071	0.400	0.622	0.385	0.455	0.500	0.529	0.381	0.500	0.682	0.692	0.438	0.333	0.556
F_{IS}	0.231	0.344	0.840	0.451	0.203	0.493	0.421	0.373	0.342	0.353	0.361	0.181	0.226	0.311	-0.067	0.168
P	0.212	0.004	0.002	0.001	0.059	<0.001	<0.001	<0.001	0.052	<0.001	0.003	0.071	0.030	0.159	1.000	0.036
AcrCT53																
H_E	0.917	0.926	0.873	0.912	0.919	0.898	0.931	0.863	0.913	0.782	0.926	0.960	0.975	0.823	0.915	0.878
H_O	0.733	0.583	0.500	0.550	0.486	0.872	0.643	0.820	0.765	0.691	0.725	0.750	0.539	0.875	0.889	0.674
F_{IS}	0.206	0.375	0.437	0.400	0.475	0.030	0.312	0.051	0.166	0.119	0.219	0.221	0.458	-0.066	0.030	0.234
P	0.016	<0.001	<0.001	<0.001	<0.001	0.261	<0.001	0.143	0.019	0.002	0.047	<0.001	<0.001	0.094	0.778	<0.001

Table S3.2 continued:

Loc/ Ref	ALA	OER	SLP	NEA	SHA	FRE	VIL	LOI	ORI	RHO	TIB	KAO	MIK-1	MIK-2	YAM	STJ
AcrCT54																
H_E	0.786	0.844	0.659	0.762	0.808	0.632	0.756	0.810	0.804	0.817	0.809	0.831	0.883	0.629	0.680	0.628
H_O	0.667	0.958	0.714	0.825	0.778	0.641	0.667	0.780	0.706	0.675	0.800	0.864	0.846	0.750	0.778	0.444
F_{IS}	0.157	-0.139	-0.088	-0.084	0.037	-0.014	0.119	0.038	0.125	0.175	0.011	-0.040	0.043	-0.200	-0.155	0.295
P	0.325	0.786	0.398	0.312	0.215	0.397	0.011	0.273	0.584	0.009	0.416	0.074	0.371	0.961	0.881	0.039
AcrCT103																
H_E	0.926	0.943	0.884	0.873	0.883	0.833	0.871	0.890	0.890	0.833	0.916	0.943	0.899	0.625	0.477	0.885
H_O	0.933	0.913	0.929	0.850	0.811	0.769	0.814	0.653	0.824	0.833	0.703	0.909	0.462	0.625	0.333	0.956
F_{IS}	-0.008	0.032	-0.053	0.026	0.082	0.077	0.066	0.269	0.076	-0.001	0.235	0.036	0.497	0.000	0.314	-0.080
P	0.936	0.120	0.298	0.193	0.679	0.068	0.029	<0.001	0.212	0.857	0.017	0.683	<0.001	0.215	0.152	0.203
AcrCA102																
H_E	0.745	0.712	0.611	0.650	0.599	0.703	0.678	0.656	0.781	0.611	0.547	0.872	0.817	0.616	0.821	0.390
H_O	0.800	0.565	0.357	0.700	0.541	0.667	0.590	0.620	0.706	0.500	0.400	0.841	0.444	0.467	0.250	0.289
F_{IS}	-0.077	0.210	0.425	-0.077	0.098	0.053	0.132	0.055	0.099	0.183	0.271	0.036	0.471	0.249	0.727	0.262
P	0.380	0.227	0.051	0.819	0.099	0.796	0.025	0.880	0.100	0.125	0.007	0.359	0.058	0.021	0.029	0.132
Total																
H_E	0.873	0.874	0.755	0.825	0.833	0.802	0.841	0.831	0.848	0.755	0.826	0.906	0.907	0.719	0.724	0.744
H_O	0.810	0.771	0.598	0.700	0.725	0.692	0.686	0.747	0.773	0.675	0.668	0.834	0.679	0.727	0.639	0.671
F_{IS}	0.074	0.123	0.257	0.145	0.129	0.136	0.187	0.103	0.093	0.120	0.199	0.079	0.261	0.000	0.116	0.123

Loc, Locus; Ref, Reference name

Table S3.3 Measures of mitochondrial DNA diversity observed in cytochrome c oxidase subunit I (COI) of *Anguillicola crassus* from 16 locations in Europe, North America and putative source populations from East Asia. Haplotype gene diversity *h* and nucleotide diversity π contain standard deviations of each estimate.

Location	Sample size (n)	Haplotype number	Haplotype diversity (*h*)	Nucleotide diversity (π)
ALA	16	5	0.450 ± 0.151	0.0016 ± 0.0013
OER	30	10	0.846 ± 0.037	0.0043 ± 0.0027
SLP	15	3	0.448 ± 0.135	0.0014 ± 0.0012
NEA	31	7	0.723 ± 0.053	0.0034 ± 0.0022
SHA	30	7	0.749 ± 0.052	0.0035 ± 0.0023
FRE	31	2	0.516 ± 0.024	0.0037 ± 0.0024
VIL	30	5	0.676 ± 0.062	0.0041 ± 0.0026
LOI	32	5	0.599 ± 0.067	0.0029 ± 0.0020
ORI	30	4	0.616 ± 0.072	0.0031 ± 0.0021
RHO	30	3	0.384 ± 0.093	0.0013 ± 0.0011
TIB	30	6	0.653 ± 0.072	0.0029 ± 0.0020
KAO	46	13	0.814 ± 0.043	0.0024 ± 0.0017
MIK-1	12	10	0.970 ± 0.044	0.0152 ± 0.0085
MIK-2	17	3	0.228 ± 0.130	0.0029 ± 0.0020
YAM	7	5	0.905 ± 0.103	0.0076 ± 0.0050
STJ	32	3	0.232 ± 0.094	0.0013 ± 0.0011

Table S6.1 Average genotypic and allelic diversity for North-Atlantic and Pacifc eels, specified for each sampling location.

Species	Location	2n	*A*	*SD*	A_r	*SD*	H_E	*SD*	H_O	*SD*	F_{IS}
A. anguilla	River Tiber (IT)	88	16.8	2.82	13.7	1.99	0.896	0.031	0.782	0.193	0.127
	Lagoon Salse-Leucate (FR)	82	17.7	3.77	14.5	2.74	0.901	0.031	0.771	0.179	0.144
	River Moulouya (MA)	100	18.2	3.56	13.8	1.82	0.901	0.020	0.786	0.139	0.128
	River Minho (PT)	98	17.9	3.95	14.0	2.68	0.898	0.024	0.759	0.199	0.155
	River Adour (FR)	98	19.0	4.18	14.4	2.65	0.903	0.024	0.807	0.129	0.106
	Lake Grand-Lieu (FR)	94	17.4	3.24	13.9	1.96	0.903	0.030	0.782	0.205	0.134
	River Couesnon (FR)	100	18.0	3.12	14.1	2.08	0.900	0.029	0.775	0.190	0.139
	River Severn (GB)	100	17.8	4.24	13.9	2.44	0.908	0.023	0.813	0.155	0.105
	River Elbe (DE)	100	18.2	4.35	14.1	2.91	0.904	0.027	0.810	0.127	0.104
	Lake Arresø (DK)	96	17.6	3.75	13.5	2.77	0.880	0.078	0.811	0.211	0.078
	Lake Vättern (SE)	48	12.8	2.44	12.3	2.23	0.820	0.166	0.723	0.203	0.118
	River Imsa (NO)	74	15.6	3.78	13.4	2.80	0.882	0.075	0.775	0.177	0.121
	River Oelfusa (IS)	250	23.6	5.13	14.2	2.05	0.904	0.026	0.831	0.155	0.081
A. rostrata	River Petite Trinité, QC (CA)	96	17.8	5.78	13.6	4.30	0.870	0.108	0.719	0.208	0.174
	Prince Edwards Island, PE (CA)	86	18.0	5.48	14.8	4.08	0.887	0.104	0.752	0.188	0.152
	River Medomak, ME (US)	92	17.3	5.32	14.2	4.08	0.887	0.096	0.777	0.208	0.124
	Boston Harbor, MA (US)	98	18.3	6.12	14.5	4.56	0.881	0.125	0.766	0.217	0.131
	River Hudson, NJ (US)	92	17.6	5.13	14.2	4.14	0.860	0.175	0.772	0.164	0.102
	River Wye, MD (US)	94	18.0	5.10	14.2	4.18	0.846	0.203	0.762	0.223	0.099
	River South Edisto, SC (US)	98	18.0	6.24	14.0	4.15	0.879	0.087	0.761	0.184	0.134
	River St. Johns, FL (US)	98	19.4	6.62	14.6	4.54	0.880	0.119	0.793	0.173	0.099
A. dieffenbachii*	New Zealand (NZ)	192	13.0	8.85	9.46	5.36	0.758	0.218	0.679	0.249	0.104
A. australis*	Queensland (AU)	220	21.8	16.1	12.9	7.19	0.802	0.181	0.723	0.161	0.099
A. japonica*	Tokyo Bay (JP)	188	25.1	12.3	15.7	6.72	0.878	0.117	0.774	0.132	0.118

* except locus *Aro146;* 2n, gene number*;* A_r*,*, average number of alleles after rarefaction*; SD, Standard deviation; A*, average number alleles; H_e, unbiased average of expected heterozygosity; H_o, average observed heterozygosity; F_{is}, inbreeding coefficient. Sampling locations: IT, Italy; FR, France; MA, Morocco; PT, Portugal; GB, Great Britain; DK, Denmark; SE, Sweden; NO, Norway; IS, Iceland; CA, Canada; QC, Province of Quebec; PE, Province of Prince Edwards Island; US, Unites States of America; ME, State of Maine; MA, State of Maine; NJ, State of New Jersey; MD, State of Maryland; SC, State of South Carolina; FL, State of Florida; NZ, New Zealand; AU, Australia; JP, Japan.

Table S6.2 Pairwise F_{ST} values among North Atlantic, New Zealand, Australian and Japanese eels.

Species	*A. dieffenbachii*	*A. australis*	*A. japonica*	*A. anguilla*	*A. rostrata*
A. dieffenbachii	0				
A. australis	**0.157***	0			
A. japonica	**0.133***	**0.116***	0		
A. anguilla	**0.113***	**0.0874***	**0.0432***	0	
A. rostrata	**0.112***	**0.0870***	**0.0413***	**0.0146***	0

* Statistically significant at the 0.05 level
Bold values indicate statistical significance after Bonferroni correction $\alpha/10 = 0.005$

Table S7.1 Population assignment and inferred ancestry of individual's eels estimated using STRUCTURE.

POP	Samples	Cluster I (*A. dieffenbachii*)	Cluster II (*A. australis*)	Cluster III (*A. japonica*)	Cluster IV (Atlantic eels)
1	*Dief (n = 96)*				
	DiefS.19	0.795	0.000 0.000 0.003	0.000 0.000 0.005	0.000 0.000 0.197
	DiefN.6	0.900	0.000 0.000 0.024	0.000 0.002 0.032	0.000 0.005 0.037
	Aus(n = 110)				
	Aus.12	0.000 0.000 0.041	0.590	0.000 0.023 0.336	0.000 0.001 0.008
	Aus.13	0.000 0.000 0.038	0.810	0.000 0.001 0.129	0.000 0.001 0.021
	AustN.45	0.000 0.000 0.107	0.881	0.000 0.000 0.004	0.000 0.000 0.008
	Japo (n = 94)				
	Japo.4	0.000 0.000 0.001	0.000 0.000 0.001	0.720	0.068 0.078 0.132
	Japo.2-8	0.000 0.000 0.011	0.000 0.000 0.108	0.844	0.000 0.004 0.033
	Japo.2-20	0.000 0.000 0.137	0.000 0.000 0.000	0.859	0.000 0.000 0.004
	Japo.8	0.000 0.005 0.063	0.000 0.000 0.005	0.889	0.000 0.016 0.022
	Atlantic (n = 378)				
	Glieu.25	0.000 0.000 0.002	0.000 0.000 0.001	0.729 0.059 0.025	0.184***
	Glieu.6	0.000 0.000 0.003	0.000 0.000 0.001	0.005 0.410 0.194	0.387*
	Glieu.29	0.000 0.120 0.180	0.000 0.000 0.001	0.000 0.001 0.021	0.677
	Elbe.40	0.000 0.000 0.134	0.000 0.000 0.001	0.000 0.000 0.004	0.861
	Glieu.28	0.000 0.000 0.005	0.000 0.041 0.084	0.000 0.000 0.006	0.863
	Bost.5	0.000 0.000 0.003	0.000 0.048 0.331	0.000 0.001 0.004	0.613
	Bost.1	0.000 0.000 0.009	0.000 0.018 0.211	0.000 0.005 0.012	0.744
	Bost.19	0.000 0.003 0.056	0.000 0.004 0.100	0.001 0.044 0.041	0.751
	Bost.39	0.000 0.000 0.002	0.000 0.000 0.000	0.002 0.144 0.082	0.770
	Med.16	0.000 0.000 0.068	0.000 0.000 0.005	0.000 0.044 0.085	0.797
	Bost.17	0.000 0.000 0.006	0.000 0.000 0.007	0.001 0.068 0.057	0.861
	Johns.6	0.000 0.000 0.002	0.000 0.000 0.013	0.000 0.063 0.054	0.868
	Johns.17	0.000 0.001 0.009	0.000 0.000 0.001	0.000 0.043 0.067	0.879
	Med.20	0.000 0.000 0.017	0.000 0.006 0.083	0.000 0.004 0.011	0.879
	Johns.38	0.000 0.000 0.005	0.000 0.000 0.001	0.001 0.010 0.100	0.884

Note. The probability (q) that each genotype belongs to one of the four clusters was computed. With POP = 1, STRUCTURE estimates the probabilities that each eel has ancestry either in the sampled or first or second past generations (q values computed with prior migration rate = 0.05). Asterisk denotes significance level derived from STRUCTURE; ***, $P < 0.001$; *, $P < 0.05$.