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Genetic signatures in an invasive parasite of *Anguilla anguilla* correlate with differential stock management

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In this article, it is shown that available genetic tools for the omnipresent parasite *Anguillicoloides crassus* in European eels *Anguilla anguilla* are sensitive to different immigration rates into local *A. anguilla* stocks for two separated river systems. Relying on four highly polymorphic microsatellite markers, it was inferred that under natural recruitment, nematode samples meet Hardy–Weinberg expectations for a single panmictic population, while genetic signals show signs for a strong Wahlund effect most likely due to very recent population mixing under frequent restocking of young *A. anguilla*. This was indicated by a low but significant F_{ST} value among within-host populations (infrapopulations) along with high inbreeding indices F_{IS} consistent over all loci. The latter signal is shown to stem from high levels of admixture and the presence of first-generation migrants, and alternative explanations such as marker- and sex-specific biases in the nematode populations could be dismissed. Moreover, the slightly increased degree of relatedness within infrapopulations in the stocked river system cannot explain the excessive inbreeding values found and are most likely a direct consequence of recent influx of already infected fish harbouring parasites with different genetic signatures. Applying a simulation approach using known variables from the nematode's invasion history, only the artificial introduction of a Wahlund effect leads to a close match between simulated and real data, which is a strong argument for using the parasite as a biological tag for detecting and characterizing fish translocation.

Key words: *A. crassus*; biological tag; European eel; fish stocking; parasite; Wahlund effect.

INTRODUCTION

The peculiar life cycle of the European eel *Anguilla anguilla* (L.) comprises a continental resident stage and a long-distance migration loop (Tsukamoto *et al.*, 2002) covering up to 6000 km in the open ocean to reach its spawning grounds in the Sargasso Sea. Since the early 1980s, recruitment levels of *A. anguilla* glass eels have collapsed by 90–99% (Moriarty, 1986, 1996; Dekker, 2000, 2003). This dramatic

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trend was actually preceded by a lack of spawners as depicted by reduced fishing harvest (ICES, 1976; Dekker, 2003), and the reduction in spawner escapement is thought to have led to an ongoing decline of the recruitment numbers representing a downward spiral (Dekker, 2009). Consequently, the European Commission has recently proposed a Council Regulation to establish quantitative management actions for protection and restoration of the *A. anguilla* stocks (COM, 2007). The Regulation mainly aims at increasing the proportion of *A. anguilla* silver eel biomass escaping to sea for a set of 'eel river basins', to be defined by each of the member states, the goal of which is dependent on a range of different quantitative criteria, including an overall reduction of harvest, reservation of a fraction of caught small fish ($<$12 cm) for restocking and reduction of mortality of migrating *A. anguilla*.

According to the multitude of causes proposed for the decline (Feunteun, 2002; Dekker, 2008), different measures must be taken to aid the recovery of *A. anguilla*, which are dependent on the 'eel river basin's' characteristics. For example, concentrated habitat restoration in the most important areas for glass eel recruitment of *A. anguilla* in south-western Europe (Atlantic parts of France, Spain and Portugal) should have the most significance for the global stock (Dekker, 2009). In addition, restocking with small *A. anguilla* was shown to have an overall positive effect on yield in the Baltic Sea regions (Wickström, 2001). Translocation of recruits (stocking), however, represents a considerable increase in distances to the spawning grounds and apparently constrains *A. anguilla* escapement in the Baltic Sea area (Westin, 1990, 2003).

In order to assess the impact of restocking more systematically, fish movement could be monitored by applying external artificial tags, *e.g.* Carlin tags (Westin, 1990) or miniaturized pop-up satellite archival tags (PSAT tags) (Aarestrup *et al.*, 2009). This does not allow for routinely tracking *A. anguilla* on a global scale. On the other hand, first results using immersion marking with the chemical compounds oxytetracycline and azilarin red for *A. anguilla* glass eels before restocking appear promising as mass marking is feasible and growth and mortality of tagged *A. anguilla* are not affected (Simon *et al.*, 2009).

In contrast to artificial tags, genetic markers are ubiquitously available. Due to the very low population genetic structure of *A. anguilla* across the whole distribution range (Lintas *et al.*, 1998; Wirth & Bernatchez, 2001, 2003; Maes & Volckaert, 2002; Dannewitz *et al.*, 2005), the parasite fauna is an attractive surrogate for monitoring *A. anguilla* 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 (Herrington *et al.*, 1939; Mosquera *et al.*, 2000; MacKenzie, 2002). Recently, Criscione *et al.* (2006) demonstrated that the genetic assignment of steelhead trout *Oncorhynchus mykiss* (Walbaum) back to their river of origin is more powerful if the genetics of its freshwater-dependent trematode parasite are used instead of the host's own genetic information. While the application of genetic tools to trace movement of animals by means of their related parasites and pathogens has been proposed repeatedly (Wirth *et al.*, 2005; Nieberding & Olivieri, 2007), this feature remains largely underutilized for aquatic organisms.

One candidate for tracking the *A. anguilla* translocation is the omnipresent rhabditid nematode *Anguillicoloides crassus* (Moravec, 2006). Sampling and classification of nematodes is easy, average prevalence (per cent of hosts infected; Bush *et al.*, 1997) reaches $>$50% with around five nematodes per infected fish

(Wielgoss *et al*., 2008), and the distribution range of *A. crassus* almost matches that of its host (Kirk, 2003), except for Iceland (Kristmundsson & Helgason, 2007). Most importantly, the molecular genetic tools have already been developed (Wielgoss *et al*., 2007) and applied to survey the nematode's population genetic structure in different regions (Sasal *et al*., 2008; Wielgoss *et al*., 2008). The nematode's biology is extensively reviewed by Kirk (2003) and a description of its life cycle is depicted in Fig. 1.

Here, in a pilot study, the sensitivity of four microsatellite markers to frequent stocking of young *A. anguilla* in one river system in central Europe (Rhine) was assessed and contrasted to the genetic and morphometric signatures to a system with natural recruitment close to the sea in Brittany (Frémur). First, paying special attention to parasite structure into infrapopulations, as well as sex bias and marker defects, Hardy–Weinberg expectations (HWE), population differentiation and the degree of infrapopulation-level relatedness were studied. Second, genotype data from previously described locations across Europe are used as baseline (Wielgoss *et al*., 2008) to assess the role of admixture and the presence of first-generation migrants in explaining this pattern.

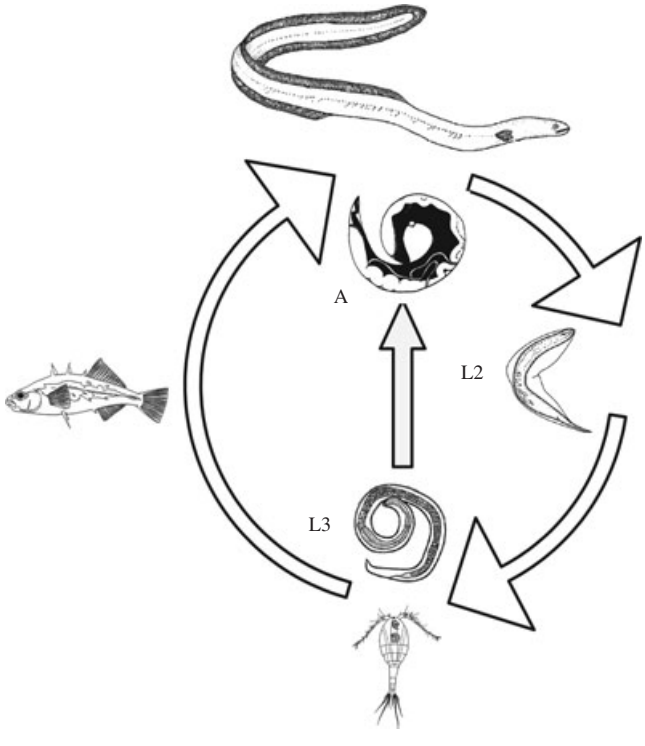

FIG. 1. Schematic life cycle of the invasive nematode parasite *Anguillicoloides crassus.* The adult nematodes (A) reproduce sexually in the swimbladder lumen of their final fish host, *Anguilla anguilla*. L2 larvae are extruded to the aqueous environment over the ductus pneumaticus and the intestine. Copepods and other small crustaceans serve as intermediate hosts (not species specific). *Anguilla anguilla* can get infected by ingesting L3 larvae residing in both crustaceans (obligatory) and prey fishes (facultative). Since the immune defence mechanisms mounted against *A. crassus* are inefficient, most European wild *A. anguilla* harbour several adult nematodes in their swimbladder (Kirk, 2003; Wielgoss *et al*., 2008).

MATERIALS AND METHODS

SAMPLING MATERIAL

All samples were collected with fyke nets in October 2006. The first sample derived from a small side arm along the River Rhine in Karlsruhe, Germany, called Rußheimer Altrhein (RHI; 49·212° N; 8·398° E). A total of $n = 62$ *A. anguilla* were collected with a nematode prevalence of 0·32, and an infection intensity (Bush *et al*., 1997) of 5·6 adult nematodes per infected fish. The second locality was sampled in France upstream a dam system called Bois Joli in the River Frémur (FRE; 48·56° N; 2·08° W). A total of $n = 70$ *A. anguilla* were sampled, with a nematode prevalence of 0·57, and an infection intensity of 6·3 adult nematodes per infected fish. While the Rußheimer Altrhein is strongly influenced by annual restocking with 1000–2000 bootlace *A. anguilla* of total body length, $L_T = 10–15$ cm ordered from various unspecified sources (mainly traders in northern Germany; F. Hartmann, pers. comm.), *A. anguilla* recruit naturally in the Bois Joli, which is situated only 6 km upstream of the sea, connected by the River Frémur. This river system is equipped with fish ladders and lifts, which are frequently surveyed by the company FISHPASS in Rennes, France. Captured *A. anguilla* were gutted and viscera removed and processed in the laboratory. All adult nematode parasites were assigned a label indicating their respective host infrapopulation (*e.g.* RHI21a, RHI21b, for two individual parasites sampled in the same host) and singly stored in screw-cap tubes in 70% ethanol until further investigation. For both localities, the largest within-host infrapopulations of the parasites (Bush *et al*., 1997) were investigated with $n = 8$ (RHI) and $n = 7$ (FRE). These harboured a total of $n = 76$ (RHI) and $n = 112$ (FRE) adult nematodes, respectively.

MORPHOMETRICS AND SEXING

Each nematode was classified and sexed according to Moravec & Taraschewski (1988), photographed and total wet body mass (M_T) was determined to the first decimal of the mg-scale on a calibrated fine balance. Total length (L_T) was approximated from the photographs using the CAD-programme AB Viewer version 6.3 (Softgold Ltd; www.cadsofttools.com). 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 measurements along the nematodes' central lines were highly concordant with the measurements of the respective nematodes' circumferences ($n = 62$; $r^2 = 0·997$), the former measurement was used for all nematodes. Male nematodes below the balance's scale (<0·1 mg) were excluded from the morphometric analyses, as were some female specimens due to body rupture (RHI: two females, six males; FRE: eight females, one male). Data were inspected for normal distribution (Shapiro–Wilks tests) and homoskedasticity (Breusch–Pagan tests) using R version 2.10.1 (www.r-project.org). Where necessary, data were normalized using transformation techniques as stated in the text. The nematodes nested within hosts, which in turn have been sampled randomly at two sites *a priori* chosen due to differences in stocking management (fixed). The following two linear mixed models were formulated for the tests:

$$Y_{ijkl} = \mu + \alpha_i + B_{ij} + \gamma_{ijk} + \varepsilon_{ijkl} \quad (1)$$

$$Y_{ijl} = \mu + \alpha_i + B_{ij} + \varepsilon_{ijl} \quad (2)$$

where $Y_{ij(k)l}$ = response variable, μ = overall mean, $\alpha_i = i$th location (fixed), $B_{ij} = j$th infrapopulation in ith location (random), $\gamma_{ijk} = k$th sex in jth infrapopulation in ith location (fixed) and $\varepsilon_{ij(k)l}$ = random residual error.

Nested analyses of variance (nANOVA) were performed using the aov-function in R version 2.10.1. Alternatively, F-values and probabilities were calculated for fixed factors, given 'Infrapopulation' is random using the REML technique under the lme-function, as supplied in the *nlme*-package. Moreover, to account for unequal sampling sizes among *A. anguilla* infrapopulations, the Satterthwaite approximation (Gaylor & Hopper, 1969) was calculated by hand as outlined in Sokal & Rohlf (1995) and P values compared with the uncorrected calculations. In a first analysis, a three-level nANOVA (equation 1) was performed for both

L_T and M_T following the procedure presented in Sokal & Rohlf (1995). The hierarchy consisted of single nematodes of different sexes nested within hosts, host infrapopulations and local populations. In a second test, two-level nANOVAs (equation 2) were carried out focusing on both sexes separately to check for gender-specific variation. To simplify the models, interaction terms were removed, as neither was significant at the 5% level.

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*, following a multiplex polymerase chain reaction (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 3130xl genetic analyser, a total of 32 previously scored individuals were rerun on the new system to account for consistent size calling in GeneMapper.

POPULATION-BASED MICROSATELLITE ANALYSES

Microsatellite loci were tested for linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) 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, as well as the jackknifing 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). An analysis of molecular variance (AMOVA) was performed 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). Finally, pair-wise relatedness r_{xy} (Queller & Goodnight, 1989) was calculated at the level of infrapopulations using SPAGeDi version 1.2g (Hardy & Vekemans, 2002). For this purpose, r_{xy} measurements were exclusively calculated for infrapopulations within sampling localities. The observed total infrapopulation averages were compared with normally distributed samples of randomly reassigned values among hosts within locations (1000 draws). A right-tailed test for significance at the 5% level was performed to test for significant departure from the null hypothesis of 'no difference in average relatedness among infrapopulations'. Finally, the presence of a Wahlund effect was simulated by generating data in Easypop version 1.7 (Balloux, 2001). Based on the inferred recent invasion history of *A. crassus* (Wielgoss *et al.*, 2008), the simulation started from a single introduced population of effective size $N_e = 1000$, split into 10 populations of size 100 and run for 100 generations and for four independent loci. It was assumed that genetic diversity was maximal with 50 alleles per locus and migration rates were kept at one instance per population and generation ($m < 0{\cdot}01$; island model). After 100 generations, *F*-statistics were inferred for both separate and lumped data sets of size 1000 using Fstat version 2.9.3 (Goudet, 1995).

INDIVIDUAL-BASED MICROSATELLITE ANALYSES

Two different Bayesian clustering 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 Génétix 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 redrawn in Matlab version 7.8 (Release R2009a; The MathWorks Inc.; www.mathworks.com) for improved graphical representation. Second, genetic admixture in either population was assessed in Structure version 2.2 (Pritchard *et al.*, 2000; Falush *et al.*, 2003, 2007). The data set was complemented by previously genotyped samples from southern France ($n = 41$; Camargue). Given the Rhine's genetic affinity towards north-eastern European populations and Frémur's derived 'Brittany' signature, the known number of European

populations in the data set, $K = 3$ (Wielgoss *et al.*, 2008), was highly supported from sampling 200 000 Markov chain Monte Carlo (MCMC) repeats after discarding the first 50 000 steps (burn-ins). These analyses were performed under the admixture model, without prior information on sampling localities, and choosing correlated allele frequencies among populations. Hence, these settings were used to infer average individual population membership coefficients (Q) and CI ($P > 0{\cdot}05$) assuming $K = 3$ populations. To identify first-generation migrants in the data set, Rannala & Mountain's (1997) Bayesian method was used as implemented in Geneclass version 2.0h (Piry *et al.*, 2004). In brief, the programme derived the likelihood statistics L_{home} and $\Lambda = (L_{home} - L_{max})$ (Paetkau *et al.*, 2004) for individuals of either sampling site to be first-generation immigrants from a known baseline data set comprising 362 individuals derived from 11 broadly distributed European localities (Wielgoss *et al.*, 2008). Subsequently, the probabilities for being a resident were derived for 10 000 simulated individuals. The cut-off criterion was set to $P = 0{\cdot}05$. Individuals below this value were listed as potential immigrants from the proposed region.

RESULTS

MORPHOMETRIC DIFFERENTIATION: REGIONS AND SEXES

Sex ratios (R_S) are slightly skewed towards females in both sampling locations, with $R_{SRHI} = 1{\cdot}23$ and $R_{SFRE} = 1{\cdot}38$. Heteroskedasticity could be refuted in all cases using Breusch–Pagan tests considering the linear models, equations (1) and (2) ($P > 0{\cdot}05$, each), however, except for female L_T ($P > 0{\cdot}05$), neither measurement was normally distributed according to a Shapiro–Wilks test for normality ($P < 0{\cdot}01$, each). Since total data sets for M_T and L_T (Table I) including both sexes could

TABLE I. Sample sizes (n), total masses (M_T) and total length (L_T) of adult parasitic nematodes, *Anguillicoloides crassus*, listed separately for sampling locality, infrapopulation and sex

	Females			Males		
Infrapopulation	n	M_T (mg)	L_T (mm)	n	M_T (mg)	L_T (mm)
River Rhine (RHI)						
RHI04	2	82·4	25·7	3	8·17	16·3
RHI06	4	38·1	24·3	2	3·75	13·3
RHI11	5	72·7	27·3	3	25·10	32·7
RHI12	3	44·0	24·1	3	18·30	20·0
RHI21	5	40·0	25·5	6	14·20	21·1
RHI25	10	156·0	34·7	5	36·10	26·1
RHI40	5	247·0	40·5	5	31·90	24·2
RHI42	4	55·2	29·9	2	7·50	29·5
Total and mean	38	100·0	30·1	29	20·80	23·1
River Frémur (FRE)						
FRE04	5	105·2	27·6	3	11·00	14·0
FRE17	8	111·0	28·0	2	17·80	20·1
FRE18	7	86·5	24·4	4	9·69	15·8
FRE26	5	132·0	29·2	5	19·30	20·5
FRE45	15	136·0	25·5	13	30·70	21·8
FRE55	9	69·2	27·6	8	16·30	20·5
FRE56	3	88·8	21·9	4	8·00	16·2
Total and mean	52	108·0	26·5	39	19·60	19·5

not be fit to a normal distribution by parametric transformation, rank transformation (RT-1; Conover & Iman, 1981) was chosen instead. On the other hand, square root transformation considerably increased the fit for the separate male and female data sets as inspected by Q-Q plots, and performing Shapiro–Wilks tests normality could no longer be rejected ($P > 0{\cdot}05$, each).

As expected, there was a marked sexual dimorphism in *A. crassus* with females growing significantly larger ($F_{S1,140} = 27{\cdot}1$, $P < 0{\cdot}001$) and heavier ($F_{S1,140} = 115{\cdot}4$, $P < 0{\cdot}001$) than males, whereas neither test among host infrapopulation nor sampling localities revealed significant contributions to overall sample variance in the three-level nested ANOVA at the 5% level (Appendices 1 and 2). When dividing for sexes *a priori*, only the body mass of females showed marginally significant differentiation among infrapopulations within locations ($F_{S13,73} = 1{\cdot}80$, $P > 0{\cdot}05$), represented in two-level ANOVA tables (Appendices 3 to 6). The unbalanced sampling did not influence the statistical values, thus, though Satterthwaite's correction did alter the d.f. and mean square values, no significant test was inferred.

TEST FOR LINKAGE DISEQUILIBRIUM AND HARDY–WEINBERG EQUILIBRIUM

According to an exact test for linkage disequilibrium among markers, neither comparison indicated significant deviations within sampling localities ($P > 0{\cdot}05$, each). While the FRE sample is in agreement with HWE at all four loci, neither marker matched HWE in the RHI, according to Fisher's exact test ($P < 0{\cdot}001$, each). The F_{IS} values were consistent among marker loci (Table II), while they were not sex specific (Table III). Heterozygote deficits in the Rhine are evenly distributed over all microsatellite size classes at each locus, according to the Microchecker

TABLE II. Measurements of genetic diversity for *Anguillicoloides crassus* listed separately for sampling locality and microsatellite marker

Location	$H_{E(n.b.)}$	H_O	F_{IS}	*A*	AR	s.d.	95% CI
River Rhine ($n = 67$)							
AcrCT27	0·9453	0·7308*	0·2269	30·0	25·00		
AcrCT53	0·9610	0·7273*	0·2432	39·0	36·20		
AcrCT54	0·7608	0·5636*	0·2592	11·0	9·83		
AcrCA102	0·6272	0·4483*	0·2852	9·0	8·69		
Total	0·8249	0·6175*	0·2514	22·3	19·90	13·10	12·90
River Frémur ($n = 91$)							
AcrCT27	0·8912	0·8839	0·0082	14·0	12·90		
AcrCT53	0·8979	0·8929	0·0056	19·0	16·60		
AcrCT54	0·7241	0·6786	0·0628	7·0	7·00		
AcrCA102	0·7717	0·7411	0·0397	9·0	7·62		
Total	0·8221	0·7991	0·0280	12·3	11·00	4·54	4·45

n, number; $H_{E(n.b.)}$, Nei's unbiased estimate of the expected heterozygosity; F_{IS}, inbreeding coefficient calculated as $(H_{E(n.b.)} - H_O)/H_{E(n.b.)}$; *, significant deviation from Hardy–Weinberg expectations for $P = 0{\cdot}05$; A, number of alleles; AR, allelic richness after rarefaction ($2n = 104$ genes).

TABLE III. Single and averaged F_{IS} values of *Anguillicoloides crassus* listed separately for sampling locality, marker and sex

Location	Sex	*n*	Single locus F_{IS} *AcrCT27*	*AcrCT53*	*AcrCT54*	*AcrCA102*	Mean ± S.D.	95% CI
River Rhine	Female	38	0·316	0·283	0·203	0·301	0·276 ± 0·050	0·049
	Male	29	0·131	0·309	0·260	0·462	0·291 ± 0·137	0·134
River Frémur	Female	52	0·025	0·004	−0·026	0·003	0·011* ± 0·012	0·014
	Male	39	0·048	0·040	0·080	0·085	0·063 ± 0·023	0·022

n, number; F_{IS}, inbreeding coefficient calculated as $(H_{E(n.b.)} - H_O)/H_{E(n.b.)}$.
*, negative F_{IS}-value excluded from calculating the mean.

TABLE IV. Excluding one microsatellite marker at a time, a jackknifing procedure depicts stable inbreeding and differentiation indices among infrapopulations within localities

Jackknifing	F_{IS}	F_{IT}	F_{ST}
River Rhine ($n = 67$)			
Without *AcrCT53*	0·268	0·279	0·0156
Without *AcrCT54*	0·271	0·286	0·0198
Without *AcrCT27*	0·283	0·297	0·0194
Without *AcrCA102*	0·255	0·257	0·00270
Mean ± S.D.	0·267 ± 0·018	0·278 ± 0·025	$0·0133 \pm 0·0120^{s,5\%}$
River Frémur ($n = 91$)			
Without *AcrCT53*	0·0348	0·0368	0·00209
Without *AcrCT54*	0·0149	0·0187	0·00391
Without *AcrCT27*	0·0387	0·0349	−0·00394
Without *AcrCA102*	0·0197	0·0237	0·00411
Mean ± S.D.	0·0259 ± 0·0173	0·0276 ± 0·0131	$0·00183 \pm 0·00565^{NS,5\%}$

s, significantly different under the null model of 'zero' differentiation among infrapopulations; NS, not significantly different from 'zero'.

programme (van Oosterhout *et al.*, 2004). Moreover, when applying a jackknifing procedure, removing one locus at a time, all loci contributed similarly to the given F_{IS} (Table IV). Thus, the deviations from HWE are independent of null alleles and other marker defects but are connected to a population-level effect.

PAIR-WISE RELATEDNESS WITHIN INFRAPOPULATIONS

When assessing the pair-wise relatedness (r_{xy}) within infrapopulations for each locality, the samples from the Rhine were significantly different from random expectations, with a slightly positive infrapopulations average of 0·025, which was situated in the extreme right-hand tail of the distribution [$P < 0·001$; Fig. 2(a)]. On the contrary, the observed value from the Frémur was slightly negative, but not significantly distinct from the median of −0·010 ('no pair-wise relatedness') and clustered within the distribution [$P > 0·05$; Fig. 2(b)].

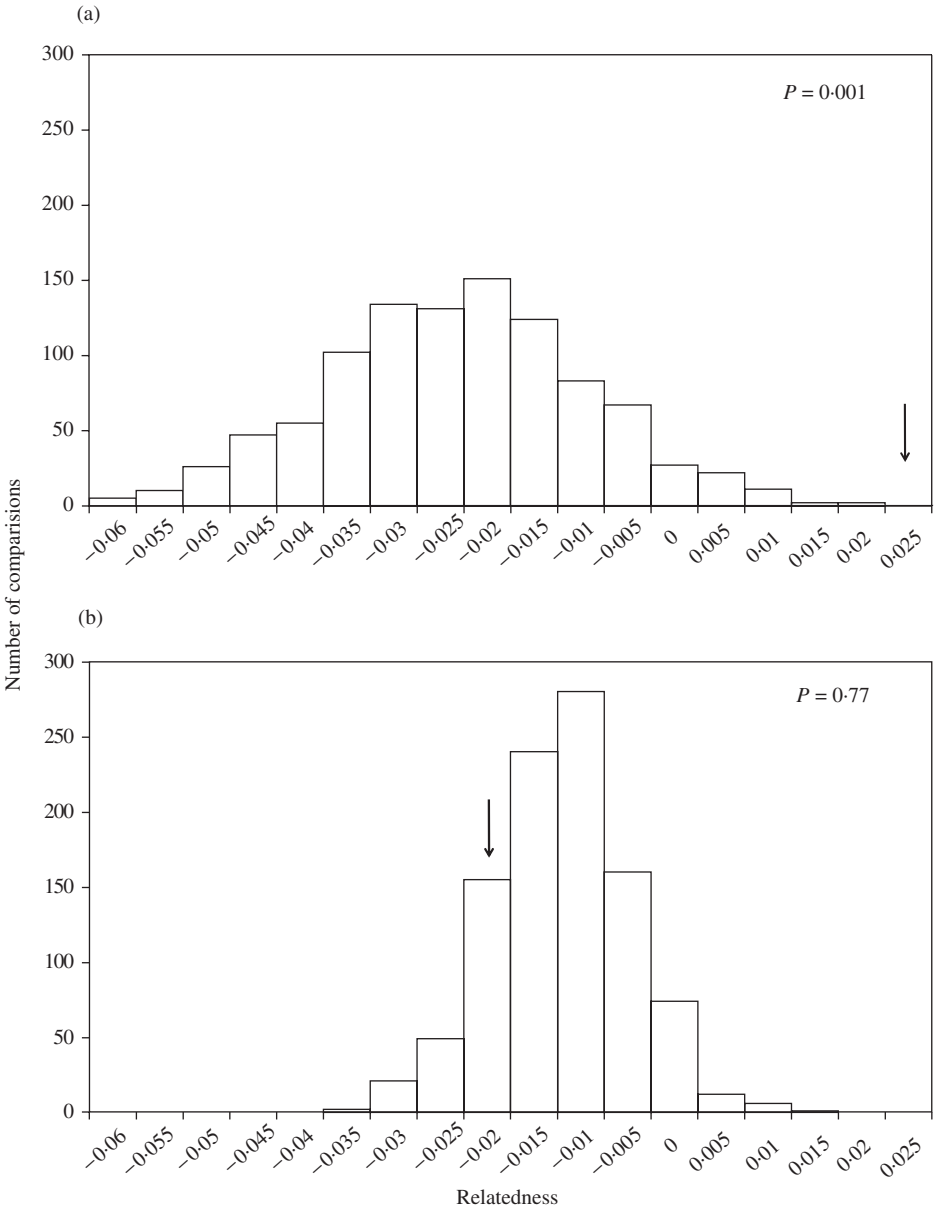

FIG. 2. Comparing simulated and observed (↓) measurements of pair-wise relatedness r_{xy} (Queller & Goodnight, 1989) using SPAGeDi version 1.2g (Hardy & Vekemans, 2002). Means of infrapopulation relatedness for (a) the Rhine River ($n = 67$; $P < 0{\cdot}001$) and (b) Frémur River ($n = 91$; $P > 0{\cdot}05$), respectively.

HIERARCHICAL *F*-STATISTICS

A locus-by-locus AMOVA indicated that the component adding to the overall genetic variance least was the variance among infrapopulations within regions (0·58%), whereas 4·66% of the variance is confined among regions. The highest

values of genetic variation were found at the individual level, where genetic variance was confined within the individuals (84·7%) and among individuals within infrapopulations (10·0%). The average fixation indices over all loci accumulated to the following values $\Phi_{IS} = 0{\cdot}1058$ ($P < 0{\cdot}001$); $\Phi_{SC} = 0{\cdot}0061$ ($P > 0{\cdot}05$); $\Phi_{CT} = 0{\cdot}0466$ ($P < 0{\cdot}001$); $\Phi_{IT} = 0{\cdot}1527$ ($P < 0{\cdot}001$), where P values $<0{\cdot}05$ depict significant differentiation at the given hierarchical level. From these values, it is clear that all but fixation index Φ_{SC} and its respective variance component differ highly significantly from a randomly generated distribution given the data. A further analysis of among infrapopulations within localities revealed a low but significant $F_{ST} = 0{\cdot}014$ ($P < 0{\cdot}05$) in the Rhine. On the contrary, the Frémur sample was not differentiated among infrapopulations ($F_{ST} = 0{\cdot}0007$, $P > 0{\cdot}05$).

TESTS FOR IMMIGRATION AND SIMULATION OF THE WAHLUND EFFECT

According to a FCA, the only split of the data set occurs along the first axis, separating both localities (Fig. 3). While most samples cluster within close range of their respective group members, parts of the Rhine samples scatter widely in variance space. Consequently, assuming population structure, a high proportion of admixed individuals in the Rhine River is apparent (Fig. 4). Using Q estimates in Structure, only one third of individuals appear to have a pure genetic background (Fig. 5). Several single individuals appear to have been introduced as first-generation migrants into the Rhine River. This hypothesis could be verified using Geneclass.

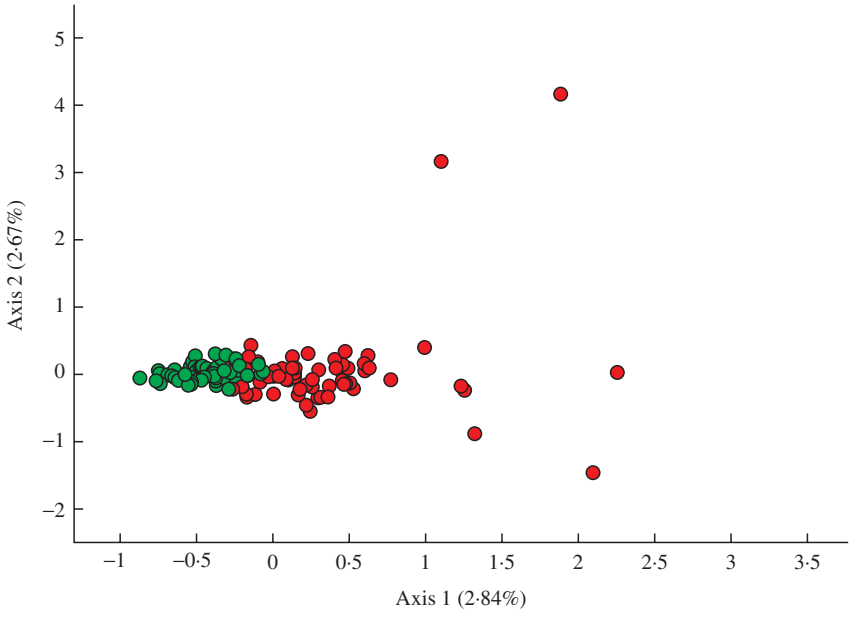

FIG. 3. Factorial component analysis highlighting individual clustering of specimens of *Anguillicoloides crassus* for the first two dimensions of variance. The only split of the data is apparent among the two sampling localities [Frémur (●) and Rhine (●) Rivers]. While most individuals cluster in close vicinity, several outliers indicate differentiation within the Rhine sample.

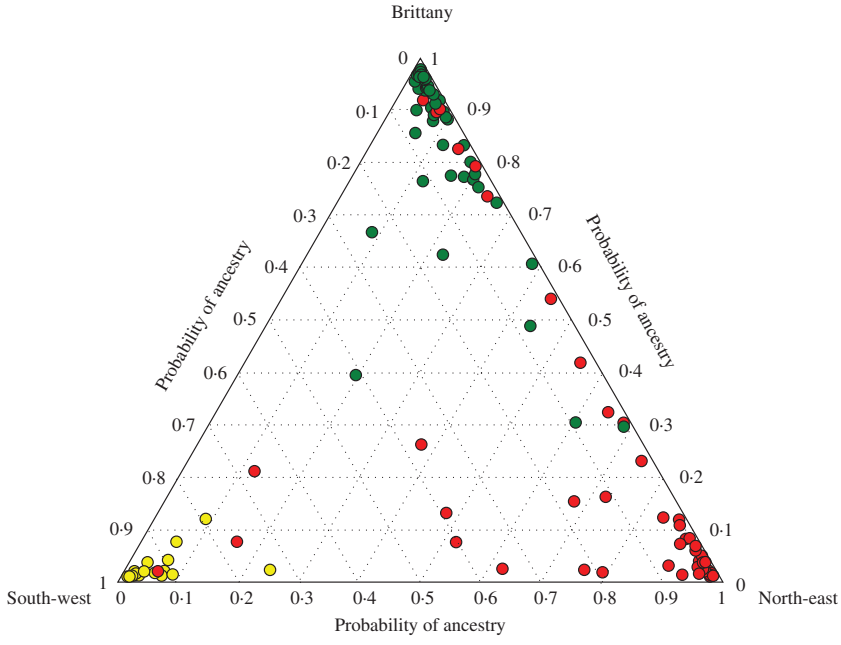

FIG. 4. Levels of admixture of *Anguillicoloides crassus* specimens within and among European sampling localities [Frémur (●) and Rhine (●) Rivers and Camargue (●)] depicted by a ternary plot of ancestry proportions (Q) according to previously detected population clusters in Europe (Wielgoss *et al*., 2008). Pure ancestry is indicated for corner positions, whereas admixed states are present at intermediate ranges.

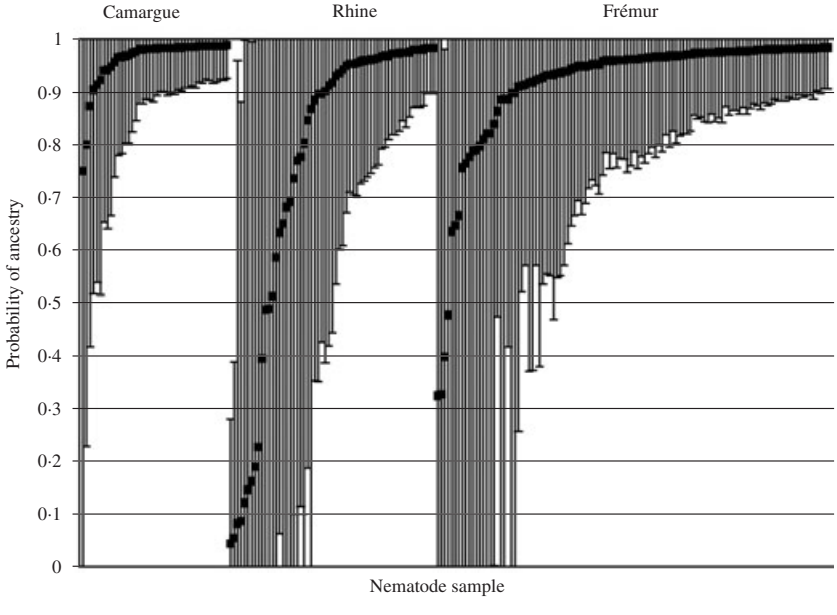

FIG. 5. Representation of individual admixture proportions Q of specimens of *Anguillicoloides crassus* within European sampling localities derived from the programme Structure version 2.2 (Pritchard *et al*., 2000). The maximum ($Q_{max} = 1{\cdot}0$) on the y-axis represents pure origin for each population sample, *i.e.* Camargue, Rhine River and the Frémur River. The minimum value $Q_{min} = 0$ highlights expatriates. Error bars are S.D.

While there is no single instance of immigration in the Frémur River, the presence of three first-generation migrants is supported for the Rhine River (Table V). Applying a simulation, the impact of an artificially introduced Wahlund effect was inspected. The settings were chosen according to knowledge of recent invasion history. After simulating allelic changes in 10 populations of 100 individuals for 100 generations, the genetic diversity dropped continuously to final levels in the range of 0·49–0·86, when starting with 50 alleles per locus. This finding is consistent with real data for microsatellite markers (Wielgoss *et al*., 2008). In the case of separate populations, F_{IS} values were not much different from 0 in either population and deviated by maximally 0·01. On the other hand, the F_{ST} value among populations approached 0·20 for single markers. On the contrary, lumping of the separate populations into one big mixed population of 1000 individuals, *i.e.* introducing a Wahlund effect, leads to F_{IS} values of the same range as found in the real data, 0·150–0·221, which is correlated with a small increase in F_{ST}, and highly increased genetic diversity between 0·932 and 0·940.

DISCUSSION

EVIDENCE FOR THE WAHLUND EFFECT AND FIRST-GENERATION MIGRANTS

In this study, it is shown that available genetic tools for the parasite *A. crassus* (Wielgoss *et al*., 2007) are sensitive to different immigration rates into local *A. anguilla* stocks for two separated river systems. While random mating of nematodes is apparent in naturally recruiting hosts retrieved from the River Frémur, the Rhine sample shows strong deviation from HWE consistent over all loci and between sexes. As there is no sign for marker-specific issues to explain this observation, several independent measures give reasonable evidence for the detection of a Wahlund effect (Hartl & Clark, 1997), most likely as a direct consequence of annual restocking of infected *A. anguilla*. First, the highly polymorphic microsatellite markers show consistent patterns of high heterozygote deficiency (average $F_{IS} = 0{\cdot}25$), and markers contributing most to the overall significant F_{ST} are also the ones showing the highest F_{IS} values ($r = 0{\cdot}953$). This pattern is absent in the Frémur River ($r = -0{\cdot}855$). Second, genetic differentiation among infrapopulations in the Rhine River is markedly higher than between samples on opposite sides of the Baltic Sea (Wielgoss *et al*., 2008), while allelic richness corrected for sample size is double the number found in the Frémur sample. Third, the presence of first-generation migrants in the Rhine River is highly supported using both individual clustering and assignment approaches based on Bayesian statistics and marks the recent influx of genetic signatures mainly from south-western parts of the parasite's distribution area, paralleling the known distribution path of captured small *A. anguilla* for restocking purposes in Europe. As a side note, both statistics, L_{home} and Λ, 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 in Wielgoss *et al*. (2008). Fourth, an artificially introduced Wahlund effect using simulated data from Easypop version 1.7 (Balloux, 2001) revealed patterns already

TABLE V. Detection of first-generation migrants from a baseline data set comprising 362 *Anguillicoloides crassus* individuals of 11 European sampling localities (Wielgoss *et al.*, 2008). Rannala & Mountain's (1997) Bayesian computation method was used, and two different likelihood statistics: $-\log_{10}(L_{home})$ and Λ (Paetkau *et al.*, 2004) as given in Geneclass version 2.0h (Piry *et al.*, 2004) were computed. Probabilities of being a resident were performed using the sampling method of Paetkau *et al.* (2004), comparing observed genotypes with 10 000 simulated genotypes

Individual	Λ	P	Assigned to	$-\log_{10}(L_{home})$	P	Assigned to
Rhine04c	0·239	<0·05	North Baltic Sea (ALA)	12·176	<0·05	North Baltic Sea (ALA)
Rhine04d	1·780	<0·01	North Irish Sea (SHA)	12·333	<0·01	North Irish Sea (SHA)
Rhine06e	0·809	>0·05	North Irish Sea (NEA)	7·531	>0·05	North Irish Sea (NEA)
Rhine12h	0·332	>0·05	West Breton Sea (VIL)	10·521	>0·05	West Breton Sea (VIL)
Rhine21f	1·569	>0·05	North Irish Sea (NEA)	8·129	>0·05	North Irish Sea (NEA)
Rhine25n	1·604	<0·05	North Irish Sea (SHA)	10·342	<0·05	North Irish Sea (SHA)
Rhine40h	0·535	>0·05	South Mediterranean Sea (TIB)	9·218	>0·05	South Mediterranean Sea (TIB)

$\Lambda = -\log_{10}/L_{home} - \log_{10} L_{max}$; ALA, Åland Island (Finland); SHA, Shannon (Ireland); NEA, Neagh (Ireland); VIL, Vilaine (France); TIB, Tiber (Italy).

observed in the real data set, above all, 1) rapidly increasing F_{IS} values over generations and consistent over loci and 2) inflated allelic richness. This finding is in line with the present results and immigration of infected *A. anguilla* are probably reflected in the parasite's genetic signals.

A similar pattern of HWE deviation has previously been detected in a parasite host system comprising a marine anguilliform species, *Conger conger* (L.), and its trematode parasite, *Lecithochirium fusiforme*. Based on six 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 *C. conger*, 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).

INTERPRETATION OF HIERARCHICAL ANALYSES

The nesting of nematodes within hosts allowed for statistical analyses of both morphometric and genetic measurements in a hierarchical manner. As a drawback, the missing replication at the highest level (sampling localities) did not allow for statistically determining if nematodes responded to stocking in general or only in the specific case of the Rhine samples. First, an AMOVA revealed a significant differentiation of the two sampling localities of $\Phi_{CT} = 0{\cdot}0466$ ($P < 0{\cdot}001$). The value is intermediate to the analogous and also highly significant θ_{ST} measures reported in Wielgoss *et al*. (2008) for Brittany ($\theta_{ST} = 0{\cdot}042$) and the easternmost Baltic Sea samples ($\theta_{ST} = 0{\cdot}068–0{\cdot}077$). This pattern is in line with the Rhine's intermediate position among these regions and could be even lower than expected due to the presence of individuals with Brittany signatures. The elevated inbreeding index of $\Phi_{IS} = 0{\cdot}11$ ($P < 0{\cdot}001$) is probably derived from the Rhine's high inbreeding index, whereas the non-significant differentiation at the level 'among infrapopulations within regions' ($\Phi_{SC} = 0{\cdot}0061$; $P > 0{\cdot}05$) is contrasted by the presence of higher than expected pairs of related parasites within hosts in the Rhine River: here, the average pair-wise relatedness is positive and the null model of 'all unrelated' individuals within infrapopulations can be refuted with high confidence ($P < 0{\cdot}001$).

This is a strong argument for initial non-random mixing of related nematodes in the same infrapopulations due to the translocation event of infected hosts into a new habitat, in which further infections are acquired. On the contrary, there is no good evidence for frequent inbreeding, non-sexual propagation or clumped transmission of individual nematodes into single hosts, because the average relatedness is only marginally higher than zero ($r_{xy} = 0{\cdot}02$) and under natural recruitment (Frémur River), random mating is observed within hosts. Above all, the mixing of nematodes is expected to be random, given the host's indirect life cycle. If infrequent non-random mixing explains the pattern, one possibility could be the transmission of related larval aggregates into the intermediate hosts, such as is known for parasite–host systems comprising sheep and frog final hosts (Boag *et al*., 1989; Zelmer *et al*., 1999). This would also require joint proper development and survival until reaching a novel final host (Fig. 1).

Most of the variance in both both L_T and M_T was explained by the sexual dimorphism among males and females. In contrast, there is no or only weak support for

differentiation on other hierarchical levels such as among 'host infrapopulations', or 'local populations', either including or separating for both sexes in the nANOVA. Interestingly, despite the ineffective immunological response against the parasite (Knopf & Lucius, 2008; Knopf *et al*., 2008), the females' total body masses differ marginally significantly among hosts at the $\alpha = 10\%$ level with $P = 0{\cdot}060$. This trend appears to be relevant as body measurements among males are not differentiated either among hosts or regions, and female lengths are also not affected. Earlier reports on density-dependent control of the number of gravid females within infrapopulations might be responsible for the observed pattern (Ashworth & Kennedy, 1999). In this case, the number of gravid females might be biased in response to co-inhabitants or nutritional status of the host.

CRITICAL REMARKS TO SAMPLING DESIGN AND APPLICABILITY

Using co-dominant genetic markers, the two differently managed systems clearly revealed a measurable genetic difference by means of the parasite tag of the fish. Thus, the sensitivity of the marker system appears suitable to indicate restocking and fish translocation. The lack of replication of the two treatments (restocking *v*. natural recruitment), however, did not allow for general conclusions in this study by means of its parasite, *A. crassus*. Moreover, it was not possible to retrieve direct information on the actual origin of the small *A. anguilla* used for restocking, and thus it remains to be tested whether farms or natural habitats of glass eels and elvers of *A. anguilla* are the major source of primary infection.

Since the detection of stocking is unnecessary if strictly regulated, and stock managers must rely on quantitative measures for making decisions (Dekker, 2009), the direct application of the present method is most suitable for cases in which, 1) previously uninfected areas are invaded (such as in the case of the Island of Réunion; Sasal *et al*., 2008), 2) an unknown source population is screened for its most likely region of origin (such as in the case of the European invasion history; Wielgoss *et al*., 2008) and 3) when explicit stocking events went unrecognized. The screening of escaped migrant *A. anguilla* in the open ocean represents another, highly interesting field but is hampered by the fact that migrants have never been observed free in the open ocean (Dekker, 2009).

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APPENDIX 1. Table of a mixed three-level nested ANOVA of total body mass including both sexes in adult nematodes, *Anguillicoloides crassus*, based on rank-transformed data (RT-1; Conover & Iman, 1981)

Source of variation	Effect attribute	d.f.	SS	MS	F_S	P	F'_S	P'	F_{lme}	P_{lme}
Locations	Fixed	1	1047	1047	0·29	0·60	0·26	0·62	0·49	0·50
Infrapopulations	Random	13	46 839	3603	0·03	1·00	0·36	0·88		
Sex	Fixed	1	125 898	125 898	115·4	<0·0001			118·5	<0·0001
Residuals	Random	142	154 889	1091						
Total		157	328 673							

SS, sum of squares; MS, mean squares; F_S, F-statistic; F'_S, derived F-statistic using Satterthwaite's approximation, as devised by Sokal & Rohlf (1995), for which all criteria were met; F_{lme}, F-value inferred from a linear mixed effect model using REML technique; P, probability given F_S; P', probability given derived F'_S; P_{lme}, probability given F_{lme}.

APPENDIX 2. Table of a mixed three-level nested ANOVA of total lengths including both sexes in adult nematodes, *Anguillicoloides crassus*, based on rank-transformed data (RT-1; Conover & Iman, 1981)

Source of variation	Effect attribute	d.f.	SS	MS	F_S	P	F'_S	P'	F_{lme}	P_{lme}
Locations	Fixed	1	12 177	12 177	3·49	0·085	3·13	0·11	2·74	0·12
Infrapopulations	Random	13	45 391	3492	0·08	1·00	0·76	0·73		
Sex	Fixed	1	43 495	43 495	27·10	<0·0001			29·10	<0·0001
Residuals	Random	142	227 616	1603						
Total		157	328 679							

SS, sum of squares; MS, mean squares; F_S, F-statistic; F'_S, derived F-statistic using Satterthwaite's approximation, as devised by Sokal & Rohlf (1995), for which all criteria were met; F_{lme}, F-value inferred from a linear mixed effect model using REML technique; P, probability given F_S; P', probability given derived F'_S; P_{lme}, probability given F_{lme}.

APPENDIX 3. Table of a mixed two-level nested ANOVA of total body mass of female adult nematodes, *Anguillicoloides crassus*, based on square root-transformed data

Source of variation	Effect attribute	d.f.	SS	MS	F_S	P	F'_S	P'	F_{lme}	P_{lme}
Locations	Fixed	1	12·96	13	0·45	>0·05	0·40	0·54	0·75	>0·05
Infrapopulations	Random	13	374·7	29	1·8	>0·05				
Residuals	Random	75	1203·1	16						
Total		89	1590·8							

SS, sum of squares; MS, mean squares; F_S, F-statistic; F'_S, derived F-statistic using Satterthwaite's approximation, as devised by Sokal & Rohlf (1995), for which all criteria were met; F_{lme}, F-value inferred from a linear mixed effect model using REML technique; P, probability given F_S; P', probability given derived F'_S; P_{lme}, probability given F_{lme}.

APPENDIX 4. Table of a mixed two-level nested ANOVA (Sokal & Rohlf, 1995) depicting hierarchical distribution of variance of total lengths in adult female nematodes, *Anguillicoloides crassus*, based on untransformed data

Source of variation	Effect attribute	d.f.	SS	MS	F_S	P	F'_S	P'	F_{lme}	P_{lme}
Locations	Fixed	1	285·1	285	3·06	>0·05	2·98	0·12	2·88	>0·05
Infrapopulations	Random	13	93·2	93·2	1·11	>0·05				
Residuals	Random	75	6303·3	84						
Total		89	7800·2							

SS, sum of squares; MS, mean squares; F_S, F-statistic; F'_S, derived F-statistic using Satterthwaite's approximation, as devised by Sokal & Rohlf (1995), for which all criteria were met; F_{lme}, F-value inferred from a linear mixed effect model using REML technique; P, probability given F_S; P', probability given derived F'_S; P_{lme}, probability given F_{lme}.

APPENDIX 5. Table of a mixed two-level nested ANOVA of total body mass of male adult nematodes, *Anguillicoloides crassus*, based on square root-transformed data

Source of variation	Effect attribute	d.f.	SS	MS	F_S	P	F'_S	P'	F_{lme}	P_{lme}
Locations	Fixed	1	0·365	0·370	0·06	>0·05	0·06	0·82	0·13	>0·05
Infrapopulations	Random	13	73·229	5·63	1·64	>0·05				
Residuals	Random	53	182·39	3·44						
Total		67	255·984							

SS, sum of squares; MS, mean squares; F_S, F-statistic; F'_S, derived F-statistic using Satterthwaite's approximation, as devised by Sokal & Rohlf (1995), for which all criteria were met; F_{lme}, F-value inferred from a linear mixed effect model using REML technique; P, probability given F_S; P', probability given derived F'_S; P_{lme}, probability given F_{lme}.

APPENDIX 6. Table of a mixed two-level nested ANOVA of total lengths of male adult nematodes, *Anguillicoloides crassus*, based on square root-transformed data

Source of variation	Effect attribute	d.f.	SS	MS	F_S	P	F'_S	P'	F_{lme}	P_{lme}
Locations	Fixed	1	2·23	2·23	2·51	>0·05	2·24	0·17	3·16	>0·05
Infrapopulations	Random	13	11·55	0·89	1·59	>0·05				
Residuals	Random	53	29·60	0·56						
Total		67	43·37							

SS, sum of squares; MS, mean squares; F_S, F-statistic; F'_S, derived F-statistic using Satterthwaite's approximation, as devised by Sokal & Rohlf (1995), for which all criteria were met; F_{lme}, F-value inferred from a linear mixed effect model using REML technique; P, probability given F_S; P', probability given derived F'_S; P_{lme}, probability given F_{lme}.