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No evidence for host specialization or host-race formation in the European bitterling (*Rhodeus amarus*), a fish that parasitizes freshwater mussels

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Abstract

Coevolutionary relationships between parasites and hosts can elevate the rate of evolutionary changes owing to reciprocal adaptations between coevolving partners. Such relationships can result in the evolution of host specificity. Recent methodological advances have permitted the recognition of cryptic lineages, with important consequences for our understanding of biological diversity. We used the European bitterling (*Rhodeus amarus*), a freshwater fish that parasitizes unionid mussels, to investigate host specialization across regions of recent and ancient sympatry between coevolving partners. We combined genetic data (12 microsatellite and 2 mitochondrial markers) from five populations with experimental data for possible mechanisms of host species recognition (imprinting and conditioning). We found no strong evidence for the existence of cryptic lineages in *R. amarus*, though a small proportion of variation among individuals in an area of recent bitterling–mussel association was statistically significant in explaining host specificity. No other measures supported the existence of host-specific lineages. Behavioural data revealed a weak effect of conditioning that biased behavioural preferences towards specific host species. Host imprinting had no effect on oviposition behaviour. Overall, we established that populations of *R. amarus* show limited potential for specialization, manifested as weak effects of host conditioning and genetic within-population structure. *Rhodeus amarus* is the only species of mussel-parasitizing fish in Europe, which contrasts with the species-rich communities of bitterling in eastern Asia where several host-specific bitterling occur. We discuss costs and constraints on the evolution of host-specific lineages in our study system and more generally.

Keywords: coevolution, cuckoo, host–parasite relationship, speciation, symbiosis

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Introduction

Brood parasitism is a reproductive strategy that allows parasitic species to avoid costs associated with brood care by parasitizing hosts that act as foster parents for developing offspring. Brood parasitism is known from a range of lineages of both vertebrate and invertebrate taxa and may be associated with host specialization in some cases, but in other cases, parasites remain generalists and exploit a range of potential hosts (Sorenson & Payne 2002). Given that each host species may evolve different counter-adaptations to parasitism, it is often adaptive for parasites to specialize, resulting in the speciation of host-specific lineages (Malausa *et al.* 2005) or the formation of host gentes (female specialist lineages that crossbreed with nonspecialist males) (Gibbs *et al.* 2000). However, specialization may carry costs, for example, associated with locating appropriate specific hosts. Hence, phenotypic plasticity in host use may often be adaptive, with generalist parasites opportunistically exploiting several host species (Poulin 2007; Nyman *et al.* 2010).

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Important insights into the evolution of host specialization have been obtained from studies on avian brood parasitism and herbivorous arthropods. In both groups, there is substantial variability in host specificity. In avian brood parasites, there are examples of strict host specificity associated with host–parasite cospeciation (African indigobirds, *Vidua* spp.) (Sorenson *et al.* 2003), female host-specific lineages (gentes in the European cuckoo, *Cuculus canorus*) (Brooke & Davies 1988), and more relaxed host specificity with opportunistic host use (brown-headed cowbird, *Molothrus ater*) (Sorenson & Payne 2002). The application of new research techniques to studies of host–parasite coevolution has demonstrated a complex structure of previously undetected races and species. For example, the existence of gentes in pallid cuckoos, *Cuculus pallidus*, was recently revealed through reflectance spectrophotometry (Starling *et al.* 2006), and the existence of cryptic species in fig wasps was detected using high-resolution genetic markers (Molbo *et al.* 2003). Surprisingly, little is known on brood parasitism in fishes, despite its occurrence in several lineages of cyprinids (Smith *et al.* 2004; Zhang *et al.* 2008; Yamane *et al.* 2009), mochokid catfishes (Kolbmüller *et al.* 2006), gasterosteoids, cottids, liparids and other freshwater and marine families (reviewed in Akagawa *et al.* 2008).

The extent to which host specialization is affected by genetic background and imprinting is much debated (Madden & Davies 2006). Juvenile imprinting of specific characteristics of a host, the host's nest or typical habitat is central to most hypotheses of cuckoo gentes (Honza *et al.* 2001; Skjelseth *et al.* 2004), speciation in indigobirds (Payne *et al.* 2000) and coral-dwelling fishes (Munday *et al.* 2004) or host-race formation in herbivorous insects (Linn *et al.* 2004). Specialization through imprinting of host-specific calls, odours or habitats permits an easier switch between hosts over microevolutionary timescales, while the rate of errors by ovipositing females (either unintentionally or as an adaptive bet-hedging strategy) influences the likelihood of specialization, with high error rates hindering and low error rates reinforcing host-race formation.

Here, we combined genetic data from wild populations with behavioural experimental data to investigate host specialization in the European bitterling (*Rhodeus amarus*), a freshwater fish that parasitizes unionid mussels by laying its eggs in mussel gill cavities. Male bitterling lead females to mussels for spawning. Females inspect mussels by sampling water emerging from the mussel's exhalant siphon and, if they choose to oviposit, use a long ovipositor to place their eggs deep inside the mussel gill cavity. Eggs and embryo development inside the mussel lasts approximately 4 weeks (Aldridge 1999; Konečná *et al.* 2010). Bitterling embryos are costly to a mussel (Reichard *et al.* 2006), because they disrupt mussel filtration, decrease feeding efficiency and gas exchange and damage the ciliated epithelium of the gills. In response, mussels eject bitterling eggs and embryos, and bitterling have evolved counter-adaptations to avoid ejections (Smith *et al.* 2001, 2004). Notably, mussel species differ in gill anatomy (Liu *et al.* 2006), which probably affects ejection rates.

Our primary prediction on the existence of host-specific races was based on the premise that host specialization may be beneficial to a parasite because it permits the evolution of fine-scale adaptations to exploit a specific host. The current study was prompted by evidence for an overall host preference among bitterling populations in accordance with the relative proportion of mussel species available for oviposition (Smith *et al.* 2000b, 2004; Mills & Reynolds 2002a). Further, specialist bitterling species are common in Eastern Asia (Liu *et al.* 2006; Kitamura 2007; Reichard *et al.* 2007b), where the bitterling–mussel association is more ancient than in Europe (at least 16 Myr compared to <3 Myr) (Tomoda *et al.* 1977; Bohlen *et al.* 2006). *Rhodeus amarus* is currently widely distributed throughout Europe and its occurrence in the area around the Black and Aegean Sea basins (referred to as Mediterranean henceforth) is dated to the Pliocene. However, its distribution in Central and Western Europe is relatively recent, following the expansion from the Black Sea region following the last deglaciation (Bohlen *et al.* 2006; Van Damme *et al.* 2007; Bryja *et al.* 2010). Hence, the coevolutionary association between *R. amarus* and unionid mussels in Central Europe is significantly shorter than the association in the Mediterranean region (Bohlen *et al.* 2006; Reichard *et al.* 2007a; Van Damme *et al.* 2007).

In a previous study on reciprocity in the relationship between European bitterling and unionid mussels (Reichard *et al.* 2010), we established that host species preferences at the population level were largely congruent among bitterling populations from areas of recent and ancient association, though mussels had evolved strong responses to bitterling parasitism in a region of ancient sympatry. Further, a low level of individual consistency in host preference was found, suggesting host generalism (Reichard *et al.* 2010). However, the experimental set-up controlled microspatial host distribution by testing host preference under aquarium conditions, while habitat specificity is fundamental for resource specialization in birds, fish and herbivorous insect (Payne *et al.* 2000; Honza *et al.* 2001; Linn *et al.* 2004; Munday *et al.* 2004; Skjelseth *et al.* 2004). Second, Reichard *et al.* (2010) tested wild fish of unknown origin with respect to host species and hence inevitably combined males and females originating from different host species in behavioural trials. These haphazard pairings may have resulted in ovipositions that did not match the prefer-

ences of one or both partners, thereby eroding the strength of conclusions on host choice consistency. Finally, reproductive isolation of related, sympatric species often relies on subtle spatial and temporal differences in habitat and resource use, which may be undermined under experimental conditions (e.g. Raeymaekers *et al.* 2010). Consequently, we designed a study to directly test the existence of host-specific races.

In the present study, we used five bitterling populations to test whether *R. amarus* form host-specific races. We tested for the existence of significant differences in nuclear and mitochondrial markers between putative host races (indicating the existence of cryptic species), differences in mitochondrial but not in nuclear markers (possible existence of female gentes) or no difference in any genetic marker between samples from different hosts (no specialization). The existence of female-specific gentes in bitterling is plausible, because females appear to exert the final decision to oviposit in bitterling. We investigated specificity at the host species and genus level. We predicted a lower rate of specialization in an area of recent (Central Europe) than ancient (Mediterranean region) association if the recent expansion of the bitterling's range has been associated with a relaxation in host specificity as bitterling encountered hosts that were evolutionarily naive with respect to bitterling parasitism (Reichard *et al.* 2007a).

Finally, we tested whether embryo imprinting and juvenile/adult conditioning to specific host mussel species are affected by host preferences in adult fish using laboratory-raised pairs of the same host species origin. This prediction was based on the finding that host preferences may vary among bitterling populations and, at the population level, bias preference towards the dominant mussel species. Specialization through imprinting or conditioning to host-specific odour is possible, because bitterling oviposition choices are strongly dependent on olfactory cues (Heschl 1989; Agbali *et al.* 2010). We used a set of 12 microsatellite markers and two mitochondrial sequences to investigate genetic differences between fish embryos dissected from sympatric host species in five sites in areas of ancient and recent bitterling–mussel association. We further used full two-factorial experimental designs to rear bitterling embryos (imprinting) and juveniles/adults (conditioning) with particular host species and tested their oviposition preferences at sexual maturity.

Material and methods

Study system

The European bitterling inhabits lowland lakes and rivers, and these habitats may support one to four host mussel species (Smith *et al.* 2004). The species of unionid mussels that commonly cooccur with bitterling in Europe are *Anodonta cygnea*, *Anodonta anatina*, *Unio pictorum* and *Unio tumidus*. In Asia Minor, *Unio crassus* replaces *U. tumidus*. All these mussel species serve as bitterling hosts, but bitterling males and females are able to differentiate between them and make sophisticated oviposition choices (Smith *et al.* 2000b; Mills & Reynolds 2002a).

Host ejections constitute an important source of bitterling embryo mortality (Smith *et al.* 2004). In a recently introduced bitterling population in England, 20–80% of eggs and embryos were ejected during the first week of incubation (Mills & Reynolds 2002a), and this figure was comparable to bitterling embryo mortality in Central Europe (Smith *et al.* 2000b). In Turkey (ancient bitterling–mussel association), almost 50% of all eggs may be ejected immediately after oviposition (Reichard *et al.* 2010). In bitterling species from East Asia, overall ejection rates may exceed 90% (Reichard *et al.* 2007b). There are clear interspecific differences between host mussels in complexity of gill structure (Liu *et al.* 2006), width of gill lamellae (Mills *et al.* 2005), oxygen consumption (Smith *et al.* 2001), respiration (Reichard *et al.* 2007b) and ventilation rate (Mills & Reynolds 2002b), and they are likely to affect ejection rates from host gills (Mills & Reynolds 2002a; Reichard *et al.* 2007b). Adaptations of bitterling embryos for remaining wedged in mussel gills include egg size and shape, presence of scaly tubercles on the egg and yolk sac and yolk sac extensions (Smith *et al.* 2004). In response to variation in key host traits, the existence of host-specific lineages was hypothesized in a Japanese bitterling species, *Acheilognathus tabira* (Kawanabe *et al.* 2001; Oshiumi & Kitamura 2009), though their distribution is allopatric and different lineages appear specialized to locally abundant host species (J. Kitamura, personal communication).

Study area

Five sites were chosen for the genetic study of host differentiation, one of which (Lake Sapanca) was used in the study by Reichard *et al.* (2010). Two sites were in the area of ancient sympatry (Mediterranean region). The River Vardar (40°37′N, 22°43′E) in Greece represented a river site; only two *Unio* species were found in the study stretch of the river. Bitterling from the River Vardar represent an ancient endemic lineage that may have inhabited the region since the Pliocene (Bohlen *et al.* 2006; Bryja *et al.* 2010). The collection site was in a downstream reach, near the city of Thessaloniki and about 13 km from the river mouth. Lake Sapanca (40°42′N, 30°15′E) in Western Anatolia, Turkey, repre-

sents an ancient lacustrine bitterling population with access to high mussel density and diversity; the mussel community includes two *Unio* and two *Anodonta* species. The lake is of tectonic origin, with a surface of 47 km^2 and a maximum depth of 52 m. In the region of recent sympatry, bitterling embryos were sampled from the River Morava (48°44′N, 17°02′E) and two lakes within its floodplain, Lake Dedava (48°38′N, 16°58′E) and Lake Hvezda (48°39′N, 16°56′E) in the Czech Republic. Sites were selected based on the availability of unionid mussels, with sites containing mussels from both host genera targeted. Based on sampling effort estimates during mussel collection, mussel density in these sites was similar to that in the River Vardar but lower than in Lake Sapanca. Bitterling density was high at all sites. There is no direct relationship between mussel and bitterling abundance (Smith *et al.* 2000a).

Genetic study: bitterling genetic variation within and among different hosts

The study sites supported from two to four species of mussels (Table 1). A diver collected mussels by hand and nondestructively examined them for the presence of bitterling embryos using a mussel-opening device (Kitamura 2005). When a mussel containing bitterling embryos was found, its valves were gently prised open and a single embryo (to avoid sampling siblings) was collected using a pipette. Embryos were immediately fixed in 96% ethanol. We aimed to collect 12–20 embryos per host species, though we recovered only 7 embryos from *U. tumidus* in the River Morava and only 10 embryos from *A. anatina* in Lake Hvezda.

At all study sites, the distribution of all host species overlapped, except for Lake Sapanca. In Lake Sapanca, host species were spatially segregated, and we were unable to locate a site with a complete overlap of all mussel species. Hence, while *A. anatina* and *U. pictorum* were collected at the same site (depth 1.2–3.5 m), *A. cygnea* occurred at a depth of 3–7 m on the opposite bank of the same bay (a distance of approximately 1400 m). The last species, *U. crassus*, was collected in the lower section of a small stream that debouches into the lake (approximately 700 m from the lake and 1600 m from the *A. cygnea* site). The embryos in Lake Sapanca were collected in 2 years. In 2008, we only collected embryos from sympatric *U. pictorum* and *A. anatina*. In 2009, we collected embryos from all four host species with the risk that divergence among host races in the lake may be affected by microallopatry and consequent differences in habitat conditions (e.g. depth, temperature, bottom substrate and current).

A total of 231 embryos were genotyped on a set of 12 nuclear microsatellite markers, with subsets of 118 embryos for partial (692 bp) cytochrome *b* sequences and 204 embryos analysed for a 316-bp section of the control region. DNA extraction, PCR conditions and microsatellite and mtDNA (i.e. control region and cytochrome *b* sequences) genotyping followed our own established protocols (Reichard *et al.* 2008; Zaki *et al.* 2008; Bryja *et al.* 2010). Details on individual genetic markers (i.e. linkage equilibrium, allelic richness, het-

Table 1 Pairwise F_{ST} values between groups of embryos recovered from particular host species at sampling sites based on 12 microsatellite loci. Estimates based on nine loci are in parentheses. The numbers of embryos recovered are shown in parentheses for each host at each site

	F_{ST}		
Lake Sapanca 2009	*Unio pictorum* (12)	*Anodonta anatina*	*Anodonta cygnea*
Unio crassus (12)	0.0027 (0.0091)	−0.0067 (−0.0047)	−0.0071 (−0.0101)
A. cygnea (15)	−0.0016 (−0.0100)	−0.0100 (−0.0193)	
A. anatina (12)	0.0028 (0.0049)		
Lake Sapanca 2008	*U. pictorum* (20)		
A. anatina (20)	−0.0060 (−0.0045)		
Lake Sapanca (pooled)	*U. pictorum* (32)	*A. anatina*	*A. cygnea*
U. crassus (12)	−0.0015 (0.0008)	−0.0051 (−0.0028)	−0.0071 (−0.0101)
A. cygnea (15)	0.0006 (0.0059)	0.0015 (−0.0045)	
A. anatina (32)	−0.0029 (−0.0046)		
River Vardar	*U. pictorum* (15)		
Unio tumidus (14)	0.0011 (−0.0015)		
River Morava	*U. pictorum* (12)	*A. anatina*	
U. tumidus (7)	−0.0066 (−0.0108)	−0.0065 (0.0046)	
A. anatina (17)	0.0136 (0.0181)		
Lake Dedava	*U. pictorum*	*A. anatina* (15)	
U. tumidus (15)	0.0075 (−0.0128)	0.0146 (−0.0003)	
U. pictorum (15)		−0.0033 (−0.0014)	
Lake Hvezda	*U. pictorum* (20)		
A. anatina (10)	0.0111 (0.0223)		

erozygosity, occurrence of null alleles) are presented in the study of Bryja *et al.* (2010).

Genetic differentiation between embryos from different host species (or genera, respectively) was quantified by computing pairwise estimators of F_{ST} according to Weir & Cockerham (1984); their significance was tested by 1000 permutations in GENETIX 4.05 (Belkhir *et al.* 1996–2004). These microsatellite analyses were performed for the data set containing all 12 loci and for the reduced data set excluding three problematic loci, which were difficult to read in some individuals or showed increased frequencies of null alleles (see Bryja *et al.* 2010 for more details). For three spatially close localities in the Czech Republic (referred to as Morava basin henceforth), we also performed hierarchical AMOVA using ARLEQUIN 3.11 (Excoffier *et al.* 2005) to analyse the distribution of molecular variation among groups of individuals (grouped according to localities and to host species/genus nested within localities).

The haplotypes of cytochrome *b* and control region and their frequencies were identified using DNASP v. 5 (Librado & Rozas 2009). Median-joining (MJ) networks (Bandelt *et al.* 1999) were constructed in NETWORK 4.6.0.0 (http://www.fluxus-engineering.com). The difference in distribution of specific haplotypes between embryos from particular host species (or genera, respectively) was tested by contingency tables.

Behavioural experiment

A large stock of adults (over 250 fish) was collected from a site with an abundant population of *A. anatina* and *U. tumidus* and a low frequency of *U. pictorum* (River Morava basin). These fish were kept in a large artificial pool (12 × 6 m) containing both test host species (*A. anatina* and *U. tumidus*) in high abundance. Mussels were collected from the same site as adult fish. After 2 weeks of spawning, mussels of each species containing bitterling eggs were separately transferred into two large water-filled fibreglass tubs (1400 L) in which the spawned eggs were allowed to complete development and the juvenile fish to emerge naturally from the mussels. In mid-summer, juvenile fish from each mussel host species were transferred to a different fibreglass tub with either their natal mussels or the second mussel species. Fish were conditioned to the host species by raising them exclusively with the respective mussels until adulthood when the fish were tested. This resulted in four treatments: imprinted on *A. anatina* and conditioned with *A. anatina*; imprinted on *A. anatina* and conditioned with *U. tumidus*; imprinted on *U. tumidus* and conditioned with *U. tumidus*; and imprinted on *U. tumidus* and conditioned with *A. anatina*. All groups overwintered under identical conditions in breeding facilities of the Institute of Vertebrate Biology where fish experienced natural variation in ambient temperature and daylight. In April, when fish achieved maturity, pairs from each experimental group were tested for spawning preferences for the two host species.

Experiments were conducted in aquaria measuring 75 × 40 × 40 cm. Experimental aquaria were isolated using opaque barriers so that fish in adjacent aquaria could not interact. Each aquarium contained a layer of sand, artificial vegetation in rear corners and two sand-filled plastic boxes placed in the centre of the tank, 25 cm apart. Each box contained a single mussel, *A. anatina* and *U. tumidus*, with their position (left or right) randomly determined. Experimental mussels were haphazardly selected from a stock of similar-sized mussels and contained no bitterling eggs or embryos prior to the experiment. Light regime and water temperature matched natural variations.

Haphazardly selected males from each treatment were placed in an individual experimental aquarium with both mussels 1 day prior to the trial to allow them to establish territoriality. Females were monitored each morning, and those in spawning condition (with their ovipositor fully extended) were gently captured using a hand net. Females were individually placed into a glass box positioned between the mussels in the experimental tank with a territorial male from their treatment to settle, and after 20 min, the female was released from the glass box and behavioural recording started. Reproductive behaviour directed towards mussels was recorded as follows: *Male and female inspection of mussel siphons*; serving as mussel quality assessment before oviposition; Smith *et al.* (2001)). *Male leading*; the male guides a female towards a particular mussel while courting, indicating male preference for an oviposition site (Smith *et al.* 2002). *Sperm release*; male ejaculates over the inhalant siphon of mussel. *Female skimming*; female makes contact with the mussel exhalant siphon with the base of her ovipositor but without inserting her ovipositor into the mussel. This behaviour serves in mate attraction (Smith & Reichard 2005) and may indicate female preference for an oviposition site (Candolin & Reynolds 2001). *Spawning*; female inserts the base of ovipositor into the exhalant siphon and the eggs are deposited in the mussel gill. This behaviour is the ultimate measure of female oviposition choice. Fish behaviour was observed for 40 min or until oviposition, whichever occurred first. Following behavioural observations, test fish and mussels were left in the aquarium for at least 3 h to enable further oviposition. Fish and mussels were then removed from the aquarium, and the number of bitterling eggs on the gills of the test mussels was counted.

Behaviours were expressed as rates per hour, and behavioural preference for a particular host species was tested using MANOVA because fish behaviours were not independent. A difference between behaviours directed towards either *A. anatina* or *U. tumidus* was calculated for each preference measure (male leading, sperm release, male and female inspections of mussel siphon and female skimming) and subjected to factorial two-way MANOVA, with imprinted and conditioned host species as factors. Interactions were excluded from the final model, because they were nonsignificant (Engqvist 2005). A total of 35 replicates were completed. However, owing to the failure of many females to become reproductively active over the course of the experiment, 12 females were used twice in the experiment, though always with a different male. This resulted in some level of pseudoreplication, and a mixed model analysis could not be used as exact female identity could not be traced between the first and second trial (marks for used fish were not unique). Therefore, we performed all tests with the full set of replicates ($n = 35$) and also with a conservative subset of 23 replicates.

In addition to behavioural data, direct oviposition preference was calculated on the basis of data from mussel dissections. The effects of imprinting and conditioning on oviposition choice were analysed using Fisher's exact tests. The interaction between imprinting and conditioning on oviposition choice was tested by three-dimensional contingency tables. Differences in the proportion of ovipositions between the two host species were analysed by comparison of goodness of fit between the models with and without an imprinting × conditioning × spawning preference interaction (chi-squared test) (Crawley 2007). Overall host preference was tested using a Fisher's exact test. The analyses were performed with both (full and conservative) data sets.

Results

Genetic data

Embryos collected from sympatric host species were not genetically differentiated at either microsatellite loci (Table 1) or mitochondrial sequences (Tables 2 and 3). None of the within-site microsatellite-based pairwise F_{ST} comparisons between embryos from particular host species was significant (Table 1), and this outcome was robust even when embryos were pooled across host genera to account for a possibility of lower precision in host specialization (Lake Sapanca: $F_{ST} = -0.003$ and -0.004; River Morava: $F_{ST} = 0.008$ and 0.016; Lake Hvezda: $F_{ST} = 0.011$ and 0.023; Lake Dedava: $F_{ST} = 0.005$ and 0.004 for genus-specific estimates based on 12 and 9 loci, respectively, all $P > 0.05$).

Nested AMOVA on microsatellite data from the three Morava basin populations demonstrated significant geographical variation among populations, which explained 4.1% of variability in the data set ($F_{CT} = 0.041$, $P < 0.001$) despite the close proximity of the three sites. No differences were found between host species nested within populations ($F_{SC} = 0.006$, $P = 0.074$). At the genus level, however, the host species effect explained a statistically significant proportion (0.79%) of variance in the data set ($F_{SC} = 0.008$, $P = 0.043$). There was also a significant geographical variation among populations ($F_{CT} = 0.039$, $P < 0.001$).

Haplotype diversity was high at cytochrome *b* sequences (see Table S1 for alignment of sequences, Supporting information), with a clear geographical structure among bitterling populations (Fig. 1). The locally dominant haplotype for each site was shared by about 45–50% embryos regardless of their host origin in ancient populations and 80–85% in the recent populations

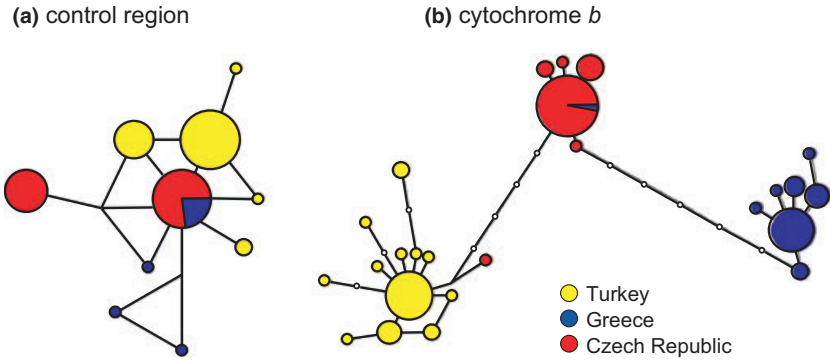

Fig. 1 Median-joining network of 204 sequences of 316 bp of control region (a) and 118 sequences of 692 bp of cytochrome *b* (b). Each coloured circle represents one haplotype, the size corresponds to its relative frequency, and the colours indicate geographical origin of samples: yellow – Lake Sapanca in Turkey, blue – River Vardar in Greece and red – three geographically close sites in the River Morava basin, Czech Republic. The small open circles represent number of nucleotide differences between the haplotypes.

Table 2 Total numbers of embryos from each host-possessing control region and cytochrome *b* haplotypes

Control region				
Lake Sapanca*	*Anodonta anatina*	*Unio pictorum*	*Unio crassus*	*Anodonta cygnea*
h5	3	4	0	3
h6	24	20	11	10
h7	1	0	0	1
h9	1	0	0	0
h10	0	1	0	0
River Vardar		*U. pictorum*	*Unio tumidus*	
h1		1	0	
h2		11	12	
h3		1	0	
h4		1	0	
River Morava	*A. anatina*	*U. pictorum*	*U. tumidus*	
h2	14	11	7	
h8	1	0	0	
Lake Dedava	*A. anatina*	*U. pictorum*	*U. tumidus*	
h2	10	12	8	
h8	4	2	6	
Lake Hvezda	*A. anatina*	*U. pictorum*		
h2	9	15		
Cytochrome *b*				
Lake Sapanca	*A. anatina*	*U. pictorum*		
h1	5	10		
h2	1	1		
h3	1	0		
h4	2	2		
h5	1	1		
h6	1	0		
h7	1	0		
h8	1	0		
h9	0	1		
h10	0	1		
h11	0	1		
h12	0	1		
River Vardar		*U. pictorum*	*U. tumidus*	
h13		3	0	
h14		6	7	
h15		1	0	
h16		1	3	
h17		1	0	
h18		1	0	
h19		1	1	
h20		0	1	
River Morava	*A. anatina*	*U. pictorum*	*U. tumidus*	
h18	13	11	6	
h21	3	1	0	
h25	1	0	0	
Lake Hvezda	*A. anatina*	*U. pictorum*		
h18	9	12		
h21	0	1		
h22	0	1		
h23	0	1		
h24	0	2		

*Pooled across years.

(Fig. 2). Haplotype diversity was lower in populations from the Morava basin (6 haplotypes among 53 embryos) than in the River Vardar (8 haplotypes among 26 embryos) and Lake Sapanca (12 haplotypes among 31 embryos). For the control region (Table S2, Supporting information), the most common haplotypes were found

Table 3 Results of χ^2 goodness-of-fit contingency tables used to test the random distribution of control region (a) and cytochrome *b* (b) haplotypes among bitterling embryos from particular host species and genera. Only a single control region haplotype was found in Lake Hvezda

	χ^2	d.f.	*P*
(a) Control region			
Species level			
Lake Sapanca	9.29	12	0.678
River Vardar	2.91	3	0.406
River Morava	1.24	2	0.539
Lake Dedava	2.80	2	0.247
Genus level			
Lake Sapanca	3.95	4	0.413
River Morava	1.24	1	0.226
Lake Dedava	0.00	1	1.000
(b) Cytochrome *b*			
Species level			
Lake Sapanca	9.10	11	0.387
River Vardar	7.97	7	0.335
River Morava	2.78	4	0.595
Lake Hvezda	3.28	4	0.513
Genus level			
River Morava	0.64	2	0.725

in more than 70% of embryos at each site (Fig. 3). Only two haplotypes were found among three Morava basin populations combined (total of 101 embryos), five haplotypes in Lake Sapanca (31 embryos) and four haplotypes in the River Vardar (26 embryos). The proportion of individual haplotypes among embryos from particular host species or genera did not differ from a random distribution (Table 3). MJ networks illustrating among-hosts genetic structure on mitochondrial markers are shown in Figs 2 and 3.

Behavioural data

The full data set showed that behavioural preference was significantly affected by prior conditioning. Fish conditioned to *Anodonta anatina* had a relatively higher preference score for *A. anatina* than fish conditioned to *Unio tumidus* (Wilk's lambda = 0.68, $F_{5,28} = 2.63$, $P = 0.045$). No effect of host imprinting was found (Wilk's lambda = 0.79, $F_{5,28} = 1.47$, $P = 0.232$). The interaction was not significant (Wilk's lambda = 0.85, $F_{5,27} = 0.96$, $P = 0.459$ in full model). The conservative subset of replicates indicated that fish behavioural preference was not affected by host conditioning (Wilk's lambda = 0.65, $F_{5,16} = 1.74$, $P = 0.183$) or host imprinting (Wilk's lambda = 0.65, $F_{5,16} = 1.73$, $P = 0.185$). The interaction between imprinting and conditioning was not significant (Wilk's lambda = 0.93, $F_{5,15} = 0.24$, $P = 0.940$ in full model).

Fish oviposited into a single mussel in 30 replicates and into both mussels in 3 replicates. Two females did not oviposit. There was no effect of host imprinting and host conditioning on oviposition choice (Fisher's exact tests, $P > 0.42$ in both cases), neither was there a combined effect of host imprinting and host conditioning on the probability of oviposition in a particular host species (Contingency tables, deviance = −0.517, $P = 0.470$). The outcome was consistent between both data sets. Overall, fish from all treatments preferred *U. tumidus* over *A. anatina* for oviposition (chi-squared test, $\chi^2 = 8.8$, d.f. = 1, $P = 0.003$ for $n = 33$ ovipositions, and $\chi^2 = 8.9$, d.f. = 1, $P = 0.003$ for a conservative subset of $n = 22$ ovipositions).

Discussion

We combined a genetic and behavioural approach to investigate host specialization in a parasitic fish. We did not find evidence that European bitterling are differentiated into host-specific lineages, irrespective of their history of association with host mussels. Behavioural conditioning moderately influenced the pattern of oviposition between host species, but this effect was unlikely to be sufficient to maintain host-specific lineages. Hierarchical AMOVA demonstrated that a statistically significant component of variation in microsatellite markers was explained by differences between hosts in the Morava basin, but the effect was probably biologically negligible, with only 0.79% of variation ascribed to differences between host genera. This finding is corroborated by nonsignificant differences in pairwise F_{ST} values for all within-site comparisons. The results are consistent with the outcome of a separate study which demonstrated that bitterling from two populations with a dominance of *Anodonta anatina* paid significantly more attention to *A. anatina* than bitterling from another two populations that had access to a diverse mussel community, though a preference for *A. anatina* was not consistent among and within individuals (Reichard *et al.* 2010). That study also revealed that bitterling from sites with a high density of hosts made more unambiguous oviposition choices than bitterling from populations with low mussel density, further suggesting that conditioning with respect to the status of a host community can influence bitterling oviposition behaviour (Reichard *et al.* 2010).

We predicted that bitterling would be more likely to specialize in areas of ancient association with hosts. In Central Europe, where the association is relatively recent (Van Damme *et al.* 2007; Bryja *et al.* 2010), bitterling exploit evolutionarily naive hosts (Reichard *et al.* 2007a) that have a limited capacity to eject bitterling eggs and embryos (Reichard *et al.* 2005, 2010). This 'evolutionary lag' in host response means that adapta-

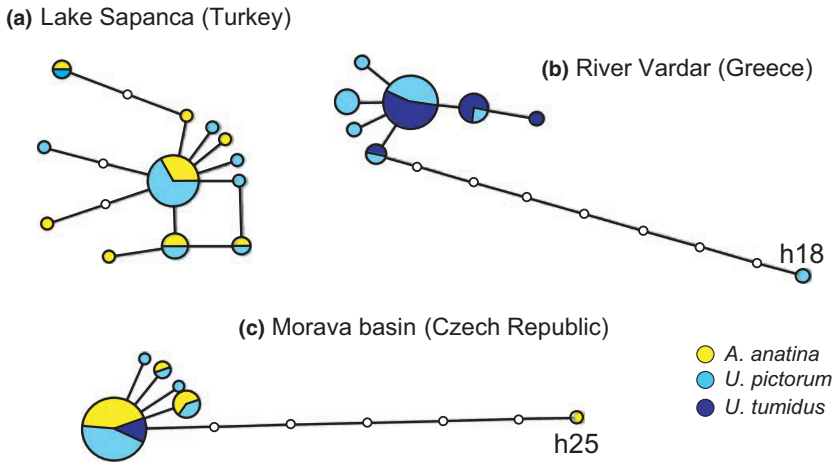

Fig. 2 Median-joining network of cytochrome *b* (692 bp) haplotypes from Lake Sapanca (a), River Vardar (b) and Morava basin (c). Each coloured circle represents one haplotype, the size corresponds to its relative frequency, and the colours indicate host origin of samples: yellow – *Anodonta anatina*, light blue – *Unio pictorum*, dark blue – *Unio tumidus*. The small open circles represent number of nucleotide differences between the haplotypes.

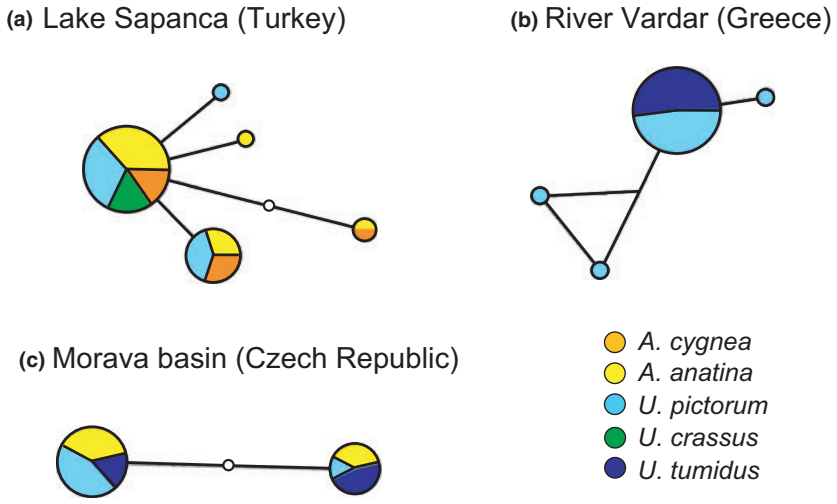

Fig. 3 Median-joining network of control region (316 bp) haplotypes from Lake Sapanca (a), River Vardar (b) and Morava basin (c). Each coloured circle represents one haplotype, the size corresponds to its relative frequency, and the colours indicate host origin of samples: yellow – *Anodonta anatina*, light blue – *Unio pictorum*, dark blue – *Unio tumidus*. The small open circles represent number of nucleotide differences between the haplotypes.

tions for parasite egg ejection may be limited. In ancient Lake Sapanca, host species distributions were partially microallopatric, a situation that is predicted to further increase the likelihood of the evolution of specialization (Brown & Pavlovic 1992). Despite this segregation, no evidence was found for host specialization by bitterling, and marginally significant results were obtained from the areas of recent rather than ancient association. We believe that our failure to detect reliable differences in mitochondrial and nuclear markers between hosts was not attributable to small sample size or low resolution of markers and correctly described the existing situation. For example, the F_{ST} values reported in support of significant differentiation between cuckoo host races using a comparable approach and the same number of genetic markers and sample size were typically several times higher than F_{ST} values among host groups in our study (Fossøy *et al.* 2011). In general, our sample size was similar or superior to related studies (e.g. Gibbs *et al.* 2000; Payne *et al.* 2000; Sorenson *et al.* 2003; Fossøy *et al.* 2011), which showed clear differences between host-specific races.

Cost and constraints of host specificity

Host specialization may carry costs to a parasite, thereby constraining the evolution of host-specific lineages. First, different host species may vary in their population dynamics, and specialist parasites are more likely to fail to locate an appropriate host (Jaenike 1990; Thompson 1994; Stokke *et al.* 2007; Soler *et al.* 2009). European bitterling inhabit lakes and rivers, including their floodplains. Bitterling from river populations are dispersed downstream and across floodplains after their emergence from host mussels (Reichard *et al.* 2002) and may encounter different host species in floodplain habitats (Smith *et al.* 2000a). Such heterogeneous and dynamic habitats, combined with significant dispersal are predicted to constrain fine-tuned adaptations owing to regional rather than local selection (Brown & Pavlovic 1992).

Second, hosts differ in their quality, measured in terms of mortality rate of bitterling embryos, both among and within species. *Anodonta cygnea* is a host species with the lowest survival of bitterling embryos, while there is no overall significant difference in survival in *A. anatina*, *Unio tumidus* and *Unio pictorum* hosts when measured at the population level (Smith *et al.* 2000a). However, bitterling discriminate host quality within host species and prefer to oviposit in mussels that contain few other embryos (Smith *et al.* 2000a). This choice is adaptive because embryo mortality in mussels is strongly density-dependent (Smith *et al.* 2000b) and unparasitized mussels are superior hosts. By retaining generalism, bitterling have a broader range of potential hosts, which may be adaptive if costs associated with oviposition in a mussel with a large number of developing embryos are higher than costs arising from suboptimal adaptations to avoid ejection.

The evolution of host-specific lineages may also be constrained by the occasional use of alternative host species when the preferred host is not available or by errors in host identification. The magnitude of the error rate in locating appropriate hosts varies widely among parasitic species. Approximately 7–8% of female European cuckoos were found to lay their eggs in the nests of two host species (Davies & Brooke 1998; Fossøy *et al.* 2011). Even for a highly specialized African indigobird, the error rate was about 0.8% (Sorenson *et al.* 2003). Nonzero fitness in alternative habitats (or hosts) effectively hinders the evolution of specialism (Coyne & Orr 2004). However, the overall effect of oviposition errors depends on host response to such ovipositions and is diminished when host response to those ovipositions unambiguously leads to ejections. This is the case in a strict single-host specialist bitterling, the Chinese bitterling (*Rhodeus sinensis*). In this species, females oviposited in alternative host mussels when their preferred hosts contained many eggs from previous ovipositions, but none of the eggs laid in nonpreferred hosts developed successfully (Reichard *et al.* 2007b).

It is not clear why some parasite lineages are host-specific, while other lineages are broad generalists and whether specialism or generalism is an evolutionarily derived state (Poulin 2007; Nyman 2010). Among teleost fish, other parasitic fish lineages are typically generalists, but at least one parasitic lineage codiversified with its hosts. An endemic parasitic catfish from Lake Tanganyika, *Synodontis multipunctatus*, has apparently diversified in parallel with its mouthbrooding cichlid hosts, which serve as foster parents for their offspring (Kolbmüller *et al.* 2006). Several other species of parasitic *Synodontis* catfishes from Lake Tanganyika are also reported to parasitize cichlids, and aquarium observations suggest that catfish can parasitize mouthbrooding cichlids from other African lakes (Day *et al.* 2009). Similarly, *Pungtungia hertzi* (Cyprinidae), which lay eggs in the nests of other fishes, can use phylogenetically distant hosts (Yamane *et al.* 2009). In cowbirds (Lanyon 1992) and feather lice (Johnson *et al.* 2009), an evolutionary trajectory from specialism to generalism is observed, while broad generalism is observed in the black-headed duck, *Heteronetta atricapilla*, a single obligate brood parasitic duck in the Oxyurinae (Sorenson & Payne 2002). Thus, it appears that generalism is prevalent in cases when a single species within a clade is a brood parasite and specialism may be the basal state in parasitic lineages that have speciated or, alternatively, ancient host specialization led to speciation. In the case of the European bitterling, this species is unusual in that it is the sole representative of these fishes in Europe and Asia Minor. In the absence of competition from other bitterling species, the retention of generalism in host use appears adaptive. It is notable that in East Asia, where there are at least 40 bitterling species (Arai & Akai 1988) and where different species often occur sympatrically, several appear to be host specialists. The evolution of host specialism might thus be a response to interspecific competition for oviposition sites in the form of niche truncation (Kitamura 2007). Our finding that oviposition choice can be slightly affected by fish experience and a certain level of host specificity detected on microsatellite markers lends support to the possibility that host-specific lineages might evolve under certain ecological scenarios (e.g. interspecific competition) and that capacity has been retained in European bitterling.

Understanding the evolution of host specificity can yield valuable insights into the evolution of biological diversity and the role of interspecific interactions in evolutionary processes (Kempf *et al.* 2009; Nyman *et al.*

2010). The recent development of new methodologies has revealed host specificity in several lineages resulting in an upward correction of estimates of global biodiversity (Smith *et al*. 2007). However, host specialization is clearly not an inevitable outcome of coevolution, because significant costs may be associated with specialization. Phenotypic plasticity in host use may be a successful strategy if the cost of specialization outweighs the benefits of specialization (Thompson 1994), and this appears to be the case in the association between European bitterling and unionid mussels.

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Author contributions

M.R. and C.S. conceived and designed the study, M.R. and M.P. collected samples, J.B. conducted genetic analyses, M.P. collected behavioural data, and M.R. analysed data and drafted the manuscript.

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Data accessibility

Variable positions of DNA sequences: Tables S1 and S2 (Supporting information). Microsatellite genotypes and experimental behavioural data: DRYAD entry doi:10.5061/dryad.v52mq.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Table S1 Variable positions of the cytochrome *b* haplotypes.

Table S2 Variable positions of the control region haplotypes.

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