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Evidence of Host Specificity and Congruence between Phylogenies of Bitterling and Freshwater Mussels

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Huan-Zhang Liu, Yu-Rong Zhu, Carl Smith, and Martin Reichard (2006) Evidence of host specificity and congruence between phylogenies of bitterling and freshwater mussels. *Zoological Studies* **45**(3): 428-434. Bitterling (Cyprinidae: Acheilognathinae) are freshwater fishes with a unique spawning relationship with freshwater mussels on whose gills they lay their eggs. During the breeding season of bitterling fishes, we collected 843 mussels belonging to 16 species from Lake Qinglan, central China and examined their gill chambers for the presence of bitterling larvae. Three species of bitterling larvae were identified; *Acheilognathus tonkinensis*, *Ach*. cf. *meridianus*, and *Ach*. *barbatulus*, in 3 species of mussel: *Unio douglasiae*, *Lamprotula caveata*, and *L. tortuosa*, suggesting host specialization. Using our own and other published data, we compared the respective phylogenies of bitterling and mussels, but failed to show clear congruence. However, broad specializations are evident, with *Acheilognathus* and *Tanakia* showing preferences for mussels with a relatively simple gill structure (Ableminae), and *Rhodeus* spp. showing preferences for mussels of the Anodontinae and Unioninae, which have more-complex gill structures. http://zoolstud.sinica.edu.tw/Journals/45.3/428.pdf

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In coevolution, we see the reciprocal evolution of adaptations between interacting species, i.e., adaptations that are shaped by coevolutionary relationships in one species are mirrored by those in their coevolving partner (Thompson 2002). Coevolution may play an important role in almost every aspect of adaptation and speciation (Thompson 2002), and there has been considerable interest in understanding coevolutionary adaptations in host-parasite (Futuyma and Slatkin 1983) and mutualistic relationships (Bronstein 2001).

The bitterling-mussel relationship may provide a further model for understanding coevolution. Bitterling (Cyprinidae: Acheilognathinae) are freshwater fishes with a unique spawning relationship with unionid freshwater mussels (Bivalvia: Unionidae). Bitterling spawn on the gills of freshwater mussels; female bitterling use long ovipositors to place their eggs on the gills of a mussel through the mussel's exhalant siphon. Males fertilize the eggs by releasing sperm into the mussel's inhalant siphon. Bitterling embryos develop inside the mussel gill chamber for approximately 1 mo, ultimately leaving the mussel as actively swimming larvae (Smith et al. 2004). Freshwater mussels themselves, whose larvae are termed glochidia, are obligate ectoparasites of fish, so bitterling risk infection by associating with larval mussels. However, bitterling appear to be parasites of mussels (Mills and Reynolds 2003); significantly reducing mussel growth while avoiding acting as hosts for glochidia (Reichard et al. unpublished data).

The bitterling group comprises approximately 40 species in Europe and Asia (Arai 1988), while

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there are approximately 670 unionid mussels worldwide (Graf 2001). Basic phylogenetic relationships for these 2 groups have been proposed (Okazaki et al. 2001, Huang et al. 2002). However, it is still uncertain whether a coevolutionary relationship exists between bitterling and mussels (Smith et al. 2000 2001 2004, Mills and Reynolds 2003).

Much recent research has concentrated on the European bitterling *Rhodeus sericeus* (Pallas), which has a restricted number of hosts (reviewed by Smith et al. 2004). European bitterling actively discriminate among mussel species in making oviposition decisions. A study by Smith et al. (2000) examined the preferences of European bitterling for the 4 most-common species of mussel in Western Europe, *Anodonta anatina*, *A. cygnea*, *Unio pictorum*, and *U. tumidus*, each with 2 levels (high and low) of fullness of embryos. This study was conducted in the Czech Republic under natural conditions. The results showed that *R. sericeus* avoided spawning in mussels that already contained high numbers of embryos and altogether avoided 1 species, *A. cygnea*. A laboratory study demonstrated that these choices were adaptive; embryo mortality in mussels was strongly density dependent and the strength of the density dependence was significantly higher in *A. cygnea* (Smith et al. 2000). Replication of this study has consistently demonstrated the same results (Smith et al. 2001). The oxygen content of the water leaving the exhalant siphon is the most likely proximate cue for oviposition choice by European bitterling (Smith et al. 2001, Mills and Reynolds 2002). Together these results have been taken to indicate that oviposition choice in *R. sericeus* depends on variations in host quality that females can discriminate (Smith et al. 2000 2004, Mills and Reynolds 2003). However, these studies are impossible to interpret in a coevolutionary context because the association of *R. sericeus* and its mussel hosts in the western portion of its distribution is probably recent.

In East Asia, bitterling have a wider range of potential host species and a long evolutionary history of association with them (Arai 1988). Here, however, the basis for host choice by bitterling is unclear (Kondo et al. 1984, Fukuhara et al. 1998). The aims of this study were, first, to measure the degree of host specialization in a range of bitterling species, and, second, to examine the phylogenetic relationships of mussels and bitterling for congruence, thereby providing support for their coevolution (Poulin 1998, Thompson 1999).

MATERIALS AND METHODS

Mussel and bitterling collection

Mussels were collected from Lake Qinglan, Jiangxi Province, China. This lake is within the catchment of the middle reaches of the Yangtze River. Several mussel and bitterling species are abundant in this lake (Liu unpublished data). Mussels are commercially exploited in the lake as a food resource.

Mussels were collected on 3 dates in 2004 (Mar. 27, Apr. 18, and May 10) which correspond to the spawning season of bitterling (Shen 2000, Zhang and Li 2002). Mussels were collected by commercial mussel fisherman using a bottom dredge. The dredge was hauled behind a powered fishing vessel for distances of 200-300 m in a water depth of 2.0-2.5 m. Fishermen targeted areas of the lake where mussels were most likely to be abundant, but within these areas, sites for dredging were haphazardly selected. After collection, mussels were identified to species on the deck of the fishing boat and immediately dissected to check for the presence of bitterling larvae. If larvae were found on the gills of a mussel, the mussel was fixed in alcohol together with the bitterling larvae. The shells of all mussels that did not contain bitterling were retained to confirm identification. In our sampling, mussel and bitterling species occurred together throughout the sampling areas and did not show evident preferences for distinct microhabitats.

Identification of bitterling larvae

Because keys for larval bitterling do not exist, we used molecular methods to identify bitterling larvae. Methods for DNA extraction, PCR, and sequencing followed Liu (2002). The complete mtDNA cytochrome (cyt) *b* gene of each larva was sequenced and compared with sequence data for adult bitterling (Liu unpublished data) to identify species. We sequenced the mtDNA cyt *b* gene of 127 larvae collected from mussels.

Statistical analysis

To test for an association between bitterling embryos and mussels, data among sampling dates were pooled, and a X^2 contingency test of independence was used to test for a significant deviation from the expected.

RESULTS

During sampling on Mar. 27, 457 mussels belonging to 15 species were collected. The dominant species were *Lamprotula caveata*, *Cristaria plicata*, and *Unio douglasiae*. Only 2 species of mussels, *U. douglasiae* and *L. caveata*, were found to contain bitterling larvae. Sequence data unambiguously showed the bitterling larvae to be *Acheilognathus tonkinensis* (Vaillant) in *U. douglasiae*, and *Ach.* cf. *meridianus* (Wu) in *L. caveata*.

In the sample collected on Apr. 18, 183 mussels belonging to 10 species were collected. The dominant species were *L. caveata* and *U. douglasiae*. On this occasion *L. caveata* and *L. tortuosa* were found to contain bitterling larvae. Sequence data unambiguously showed that the bitterling larvae were all *Ach. barbatulus* Günther.

On the final sampling date on May 10, 194 mussels belonging to 11 species were collected. Again, the dominant species were *L. caveata* and *U. douglasiae*. Only one species of mussel, *L. caveata*, was found to contain bitterling larvae; sequence data showed these to be *Ach. barbatulus*.

The species composition of the mussel samples, and those containing bitterling larvae are summarized in table 1. The association of bitterling and mussel genera is summarized in table 2. For all sampling dates combined, the distribution of embryos between mussel genera used by bitterling deviated significantly from expected if mussels were considered independently of genus (X^2 = 45.0, *df* = 2, $p < 0.001$). Note that this test is valid with low sample sizes if the average expected frequency is at least 6 (Zar 1999). In the case of the present test the mean expected frequency was 7.5.

DISCUSSION

Host preference of bitterling in Qinglan Lake

Table 1. The number of each mussel species collected on each sampling date. Number of mussels containing bitterling larvae in parentheses

Mussel species	Mar. 27	Apr. 18	May 10
Acuticosta chinensis	2 (0)	0 (0)	29 (0)
Anodonta globosula	6 (0)	0 (0)	1 (0)
Anodonta woodiana	10 (0)	20 (0)	1 (0)
Arconaia lanceolata	6 (0)	1 (0)	0 (0)
Cristaria plicata	66 (0)	0 (0)	0 (0)
Cuneopsis capitata	1 (0)	5 (0)	0 (0)
Cuneopsis heudei	4 (0)	0 (0)	1 (0)
Cuneopsis pisciculus	6 (0)	3 (0)	2 (0)
Hyriopsis cumingii	6 (0)	5 (0)	0 (0)
Lamprotula caveata	147 (2)	54 (10)	57 (13)
Lamprotula leai	10 (0)	0 (0)	1 (0)
Lamprotula tortuosa	0 (0)	1 (1)	0 (0)
Lanceolaria gladiola	6 (0)	19 (0)	5 (0)
Lanceolaria grayana	28 (0)	0 (0)	16 (0)
Schistodesmus lampreyanus	3 (0)	22 (0)	10 (0)
Unio douglasiae	156 (19)	53 (0)	71 (0)

Table 2. Association of bitterling larvae with mussels. Numbers refer to number of mussels containing a given bitterling species among all sampling dates

	Bitterling species		
Mussel genus	*Acheilognathus barbatulus*	*Ach.* cf. *meridianus*	*Ach. tonkinensis*
Lamprotula spp.	24	2	0
Unio sp.	0	0	19

Our study lasted approximately 8 wk during the peak period of bitterling spawning (Shen 2000, Zhang and Li 2002). Our results show a strong pattern of association between bitterling and mussel species. We examined the gills of 834 mussels belonging to 16 species, among which 45 individuals contained bitterling embryos. Embryos of *Ach*. *tonkinensis* were found exclusively on the gills of *U*. *douglasiae*, while embryos of *Ach*. cf. *meridianus* were found only on *L*. *caveata*. *Acheilognathus barbatulus* embryos were found in the gill cavities of *L*. *caveata* and *L*. *tortuosa*. Previous work has shown that *Ach*. *barbatulus* spawns exclusively on *L*. *caveata* (Liu unpublished data). As far as we are aware, all the potential mussel hosts were equally available to bitterling; all mussels overlapped in their spatial distributions within the lake, and bitterling were abundant and ubiquitous. No evident preferences of mussels for distinct microhabitats were found. Further, although the 2 most-common mussel species (*L*. *caveata* and *U*. *douglasiae*) were used most frequently by bitterling, significantly more embryos were found in these 2 than expected. Thus, our study suggests host specificity by bitterling in Qinglan Lake.

Although there are few comparable studies to our own for Asian bitterling, previous research demonstrated similar findings. Kondo et al. (1984) investigated the spawning preferences of 5 bitterling species in a small creek in Japan and showed that *Tanakia lanceolata* (Temminck *et* Schlegel) had a preference for *Inversidens japanensis* and *I*. *yanagawensis*; *Ach*. *tabira* (Jordan *et* Thompson) showed a preference for *Pseudodon omiensis*; while *Ach*. *rhombeus* (Temminck *et* Schlegel) preferred *U*. *douglasiae*. Fukuhara et al. (1998) investigated the use of freshwater mussels for oviposition by bitterling in 3 creeks in Japan. They also found that bitterling preferred particular mussel species, with *Ach*. *tabira* using *P*. *omiensis*, while *Rhodeus ocellatus* (Kner) embryos were found most frequently on *Anodonta woodiana*. The preference of *R*. *ocellatus* for spawning on the gills of *Anodonta* was also reported by Honda (1982), Nagata (1985), and Kondo et al. (1987), and experimentally by us (Reichard et al. unpublished data).

Coevolution between bitterling and mussels

Although several studies have shown clear host preferences by bitterling, it is uncertain whether mussels and bitterling have coevolved (Mills and Reynolds 2003, Smith et al. 2004). Ultimately, to confirm coevolution, it is necessary to demonstrate reciprocal genetic changes between coevolving partners (Thompson 2002). However, congruence between the phylogenies of coevolving groups can be used to infer coevolution (Poulin 1998).

Phylogenetic relationships of bitterling and mussels were proposed by Okazaki et al. (2001) and Huang et al. (2002), respectively. Bitterling are grouped into 3 genera: *Acheilognathus*, *Tanakia*, and *Rhodeus* (Okazaki et al. 2001). The East Asian mussels are grouped into 3 subfamilies: the Ableminae, Anodontinae, and Unioninae (Huang et al. 2002). Although based on relatively few studies of host specificity, it is clear that most *Rhodeus* bitterling show a preference for mussels belonging to the Anodontinae and Unioninae, while *Acheilognathus* and *Tanakia* bitterling show broad preferences for the Ableminae (Fig. 1). We have not tested the significance of the congruence of the phylogenetic trees, since our knowledge of the host specificity of bitterling species is still limited. Also, there are some exceptions to this overall pattern; some *Acheilognathus* spp. also exploit unionine mussels (Fig. 1). These exceptions may represent examples of host switching, like that seen in avian malaria parasites (Ricklefs and Fallon 2002) and parasitic hymenoptera (Dowton 2001). Alternatively, some species could be spawning site generalists. Such species may have a preference for particular mussel species when available, but are able to use other mussel species if the preferred species are not available. Behavioral experiments have demonstrated that in order to identify the host specificity of a bitterling species, it is necessary to allow bitterling the choice of different mussel species (Liu unpublished data). Alternatively, the broad congruence of bitterling and mussel phylogenies may be illusory, arising through sequential colonization of mussels by different bitterling species, or through independent duplications and loss of associations across the trees (Poulin 1998).

Host preference by bitterling is possibly determined by the anatomical structure of the host mussel gill. Wu (1998) categorized mussels on the basis of their gill structure, identifying 4 broad groups with increasing gill complexity: those without true water tubes or septa, those with water tubes and perforated septa, those with water tubes and non-perforated septa, and those with tripartite water tubes and non-perforated septa. The simplest gill type is found in the Ableminae, with the other gill types found in both the Anodontinae and Unioninae.

Because gill structures vary to such a marked degree, bitterling embryos may be adapted to particular gill types. Bitterling embryos display unique features that enable them to survive in a mussel gill chamber and which vary among genera. Bitterling embryos of the genus *Rhodeus* have 2 wing-like yolk projections (Suzuki et al. 1986, Suzuki and Jeon 1987 1988, Aldridge 1999), while species of *Acheilognathus* and *Tanakia* have none. Also, all species of *Rhodeus*, and some of *Acheilognathus* and *Tanakia* possess scaly tubercles on their yolk-sac (Fukuhara et al. 1982), which may play a role in helping the embryo remain lodged in the gills of its mussel host. The reproductive anatomy of adults may also be tailored to particular mussel species or groups. For example, ovipositor length varies among species (Reichard et al. unpublished data).

In summary, we show mussel host preferences in 3 species of bitterling. These preferences are congruent with those of previous studies, which together provide support for host specializa-

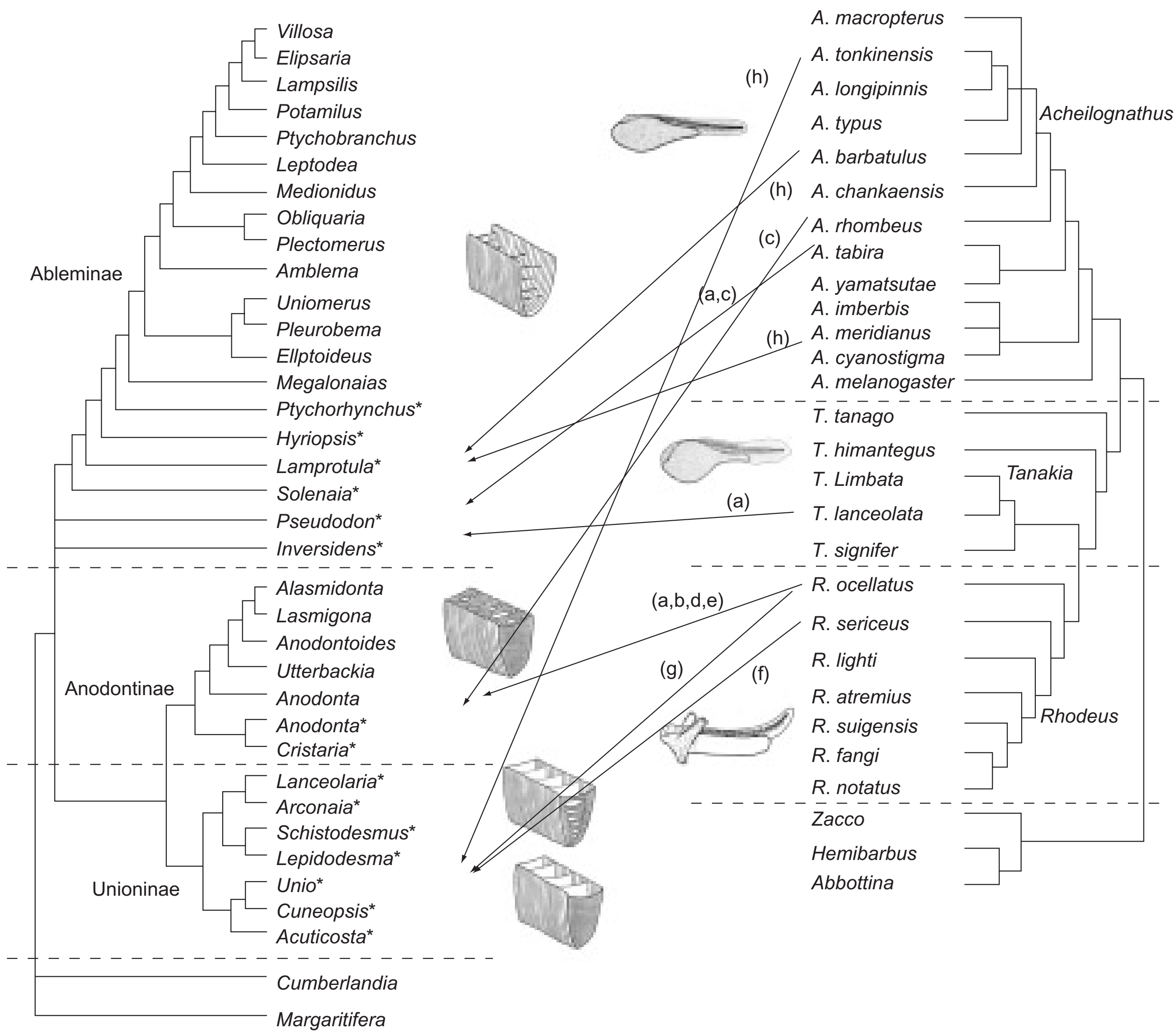

Fig. 1. Congruence of the phylogenetic relationships between bitterling fishes and East Asian freshwater mussels. Asterisks denote mussel species sympatric with bitterling. Phylogenetic trees of mussels and bitterling are from Huang et al. (2002) and Okazaki et al. (2001), respectively, with modifications. Types of mussel gills are from Wu (1998). Types of bitterling larvae are from Suzuki and Jeon (1987 1988) and Suzuki et al. (1986). Data on host preferences are from (a) Fukuhara et al. (1998), (b) Honda (1982), (c) Kondo et al. (1984), (d) Kondo et al (1987), (e) Nagata (1985), (f) Smith et al. (2000), (g) Reichard et al. unpublished data, and (h) the present study.

tion in bitterling. Comparison of the phylogenetic trees of bitterling and mussels failed to show clear congruence, although broad specializations are evident, with *Acheilognathus* and *Tanakia* showing preferences for mussels with a relatively simple gill structure (Ableminae), and *Rhodeus* spp. showing preferences for the Anodontinae and Unioninae, with more-complex gill structures. At present, there are too few empirical data on mussel host preferences of bitterling species for a comprehensive evaluation of the congruence between the phylogenetic trees for these 2 groups. However, we hope that this study will stimulate further studies. Ongoing research we are conducting will explore the morphological, physiological, and behavioral adaptations of bitterling for spawning and the responses of mussels to exploitation by bitterling.

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