Invisible pair bonds detected by molecular analyses

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A focus on pair bonds between males and females is fundamental to study the evolution of social organization. Because pair bonds are generally identified from direct observations of pairs that maintain physical proximity, pair bonds may have been overlooked in animals that do not exhibit such visible pairs. The Lake Tanganyika cichlid fish Xenotilapia rotundiventralis forms schools that consist of mouthbrooding and non-brooding adults in mid-water, and visible pairs are not recognized. A previous study suggested that mouthbrooding females transfer fractions of the young to males when the young become large. However, it remains a mystery whether the mating pairs maintain pair bonds so that the females can transfer the young to their mates. To answer this question, we conducted a parentage analysis using 10 microsatellite markers. The analysis showed that the mouthbrooding adults were most likely genetic fathers and mothers of the young in their mouths. This finding suggests that the female-to-male shift of young takes place between mating partners, and thus the mating pairs maintain pair bonds at least until the shift of young. The present study is the first to detect pair bonds in animals in which physical proximity has not been observed.

Keywords: cichlid; Tanganyika; microsatellite; parentage relationships; schools

1. INTRODUCTION

A focus on pair bonds between males and females is critical for the study of the evolution of social organization [1,2], although pair bonds do not necessarily reflect the genetic mating system [3,4]. Pair bonds are generally identified from direct observations of pairs that maintain physical proximity between males and females [2]. However, in this identification method, pair bonds may be overlooked when physical proximity is not recognized. For example, in fishes that form schools, it is hard to continuously record the spatial distances between particular individuals. Molecular assays may be useful to uncover such overlooked, invisible pair bonds.

Xenotilapia rotundiventralis is a small mouthbrooding cichlid fish from Lake Tanganyika, Africa. There are no sexual differences in body coloration or body shape [5], and adult males (51 mm standard length (SL) on average) are only a little larger than adult females (49 mm SL on average) (note that Yanagisawa et al. [6] called this species Microdonotochromis sp.). This zooplanktivorous fish forms schools composed of about 500–2500 individuals of mouthbrooding and non-brooding adults in mid-water with no pairs recognized by eye [6] (figure 1, also see the electronic supplementary material). Yanagisawa et al. [6] found that females brood offspring that vary in developmental stage from egg to large young (less than 15 mm SL); the developmental stage of offspring is almost the same in a female’s mouth, but differs greatly among brooding females); the number of young in a female’s mouth decreases when the young are 6–9 mm SL; and males brood young larger than 4.8 mm SL only (usually larger than 9 mm SL). These facts strongly suggest that the females solely brood eggs and small young in their mouths, and subsequently transfer fractions of the young to males when the young become large [6]. However, because pairs are not recognized in the schools, the question arises of whether the mating pairs maintain pair bonds so that the females can transfer the young to their mates. To answer this question, we conducted parentage analysis using microsatellite markers.

2. MATERIAL AND METHODS

(a)Study sites and fish

Fish were collected at the southern coast of Nkumbula Island near Mbuyu, Zambia, at the southern end of Lake Tanganyika (8°46′ S, 31°06′ E) with a screen net in September 2009. In this locality, *X. rotundiventralis* forms a school 1–3 m above the rocky bottom at 8–9 m water depth. This school is about 3–5 m in diameter, and consists of mouthbrooding and non-brooding males and females. There were three schools in this area in 1991 [6], but only one school was found during the period of our sampling. Fish were collected with transparent plastic bags (24 × 34 cm) immediately after they were caught in order not to mix young between adults. Fish were killed in a solution of anesthetics FA 100 (Takeda Pharmaceutical Co. Ltd.). The right pectoral fin of the adult fish and the whole bodies of young in the mouth, if any, were fixed in 100% ethanol for DNA examinations. The sex of the adult fish was determined from the shape of the genital papilla. Out of 35 adults, 14 males (MO1–M14) and nine females (F01–F09) were brooding young (one to five young (7.5–15.7 mm SL) in males’ mouths, two to seven young (5–15.6 mm SL) in females’ mouths), and eight males (M15–M22) and four females (F10–F13) were not brooding. Population allele frequencies of the microsatellite markers were estimated from these 35 adults plus 60 additional adult samples (43 males, M23–M65, and 17 females, F14–F30) that were collected at the southern coast of Nkumbula Island in October 2009 and August 2010. We used these 23 mouthbrooding adults, 72 young in their mouths, 12 non-brooding adults and 60 additional adults for the parentage analysis.

(b) Analyses of microsatellite data

Ten microsatellite loci were used for genotyping (see the electronic supplementary material for the methods of DNA extraction and amplification). Departure from Hardy–Weinberg (HW) equilibrium for every microsatellite locus and linkage disequilibrium for all pairs of loci were tested within the 95 adults using ARLEQUIN v. 3.11 [7] (10000 Markov chain steps, 1000 dememorization steps in the HW test; 10000 permutations in the linkage disequilibrium test). Critical significance levels were corrected following the sequential Bonferroni procedure [8].

The parentage relationships between the 72 young and the 95 candidate parents (65 candidate fathers and 30 candidate mothers) were reconstructed using a maximum-likelihood method implemented by COLONY v. 2.0 [9,10]. In the option of this program, we set mating system as ‘female polygamy’ and ‘male polygamy’, length of...
run as ‘very long’, analysis method as ‘full-likelihood’, and likelihood precision as ‘high’. We did not allow allele frequency to update during calculation; did not assume sibship size a priori; did not assume genotyping errors or mutations; used the outbreeding model; set number of known paternal/maternal sibships, number of offspring with excluded fathers/mothers, and number of excluded paternal/maternal sibships as zero; and set probability a father/mother included in the candidate parents as 0.1. The population allele frequencies estimated from the 95 adults were loaded.

3. RESULTS

No linkage disequilibrium was found in any possible pairs among the markers examined (likelihood ratio tests: $p > 0.05$ in 43 tests after sequential Bonferroni correction) except for the pair of Abur44 and Ttem9 and the pair of Abur120 and Abur139, which showed marginal linkage disequilibrium ($p > 0.01$). No departure from HW equilibrium was found for any microsatellite markers (table 1).

The maximum-likelihood analysis showed that the mouthbrooding adults were most likely the genetic fathers and mothers of the young in their mouths ($p \geq 0.998$ in 71 dyads, $p = 0.495$ in one dyad; see the electronic supplementary material for the robustness of this parentage analysis). M38 was most likely the genetic father of the two young brooded by F06 ($p = 1.00$ in both dyads). This male was collected 8 days after F06 and her young were collected, and was not brooding offspring in his mouth at that time. No genetic parent–child relationships were found in the other dyads between the 95 adults and young brooded by the other adults. In the 23 mouthbrooding adults, all young in a clutch were most likely full-sibs ($p \geq 0.835$ in 95 dyads, $p = 0.496$ in one dyad). No full-sib or half-sib relationships were found between young from different adults.

### Table 1. Details of microsatellite loci of the 95 adults that are genotyped in the present study ($H_e$: observed heterozygosity, $H_s$: expected heterozygosity, $\alpha = p > 0.05$ in a test of departure from Hardy–Weinberg equilibrium after a sequential Bonferroni correction).

<table>
<thead>
<tr>
<th>locus</th>
<th>no. of alleles</th>
<th>allele frequencies</th>
<th>$H_s$</th>
<th>$H_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzeb4</td>
<td>95 6</td>
<td>0.005–0.511</td>
<td>0.653</td>
<td>0.623</td>
</tr>
<tr>
<td>GM264</td>
<td>95 21</td>
<td>0.005–0.158</td>
<td>0.937</td>
<td>0.929</td>
</tr>
<tr>
<td>Abur44</td>
<td>95 23</td>
<td>0.005–0.147</td>
<td>0.905</td>
<td>0.940</td>
</tr>
<tr>
<td>Abur46</td>
<td>93 7</td>
<td>0.022–0.468</td>
<td>0.720</td>
<td>0.716</td>
</tr>
<tr>
<td>Abur120</td>
<td>95 10</td>
<td>0.005–0.437</td>
<td>0.737</td>
<td>0.758</td>
</tr>
<tr>
<td>Abur132</td>
<td>95 9</td>
<td>0.005–0.321</td>
<td>0.789</td>
<td>0.793</td>
</tr>
<tr>
<td>Abur139</td>
<td>92 16</td>
<td>0.005–0.147</td>
<td>0.946</td>
<td>0.912</td>
</tr>
<tr>
<td>Abur209</td>
<td>95 13</td>
<td>0.005–0.184</td>
<td>0.884</td>
<td>0.885</td>
</tr>
<tr>
<td>Ttem8</td>
<td>95 6</td>
<td>0.016–0.316</td>
<td>0.758</td>
<td>0.743</td>
</tr>
<tr>
<td>Ttem9</td>
<td>95 14</td>
<td>0.005–0.216</td>
<td>0.842</td>
<td>0.897</td>
</tr>
</tbody>
</table>

4. DISCUSSION

The present genetic analysis revealed that the mouthbrooding adults of *X. rotundiventralis* were most likely the genetic mothers and fathers of the young in their mouths. This result suggests that each female broods offspring that she has laid and each male receives the young that he has fertilized from his mate. This finding strongly suggests that the female-to-male shift of young takes place between mating partners. Therefore, the mating pairs most likely maintain the pair bonds at least until the female-to-male shift of young occurs. There are two possible explanations of why these pair bonds are not recognized by eye: one explanation is that the pairs maintain physical proximity, but mingle with other conspecific individuals in schools, and the other explanation is that the pairs do not maintain physical proximity most of the time. At present, there is no information regarding which explanation is more likely. One adult male (M38) was most likely the genetic father of the two young brooded by a female (F06). These adult male and female may have been a mating pair.

The fact that males of *X. rotundiventralis* brood only young implies that females probably transfer offspring after eggs hatch in their mouths. In the mouthbrooding cichlid fish from Lake Tanganyika, eggs hatch 3–6 days after spawning [11,12], suggesting that the pairs of *X. rotundiventralis* maintain pair bonds during at least 3 days (from spawning to the shift of young), although this estimate may be too conservative (young shift occurs 9.4 ± 0.5 days after spawning in a congener, *Xenotilapia flavipinnis* [11]).

The pair bonds of *X. rotundiventralis* may improve the survival rate of the young. Maintenance of the
pair bond allows the females to transfer fractions of the young to their mates. Yanagisawa et al. [6] suggested that division of the young between the mother’s and the father’s mouths, which doubles the brooding space, would enable the young to grow larger. A female-to-male shift of young has also been reported in some other cichlid fish from Lake Tanganyika, i.e. Xenotilapia boulergeri, X. flavipinnis, Xenotilapia longispinis, Eretmodus cyanostictus and Tanganicodus irsacae [11–13], but its evolutionary significance may be different from that in X. rotundiventralis. The females of these species transfer their entire broods to their mates. Yanagisawa [11] suggested that the release of females from the mouthbrooding task would accelerate the females’ feeding and gonadal recovery.

Monogamous fish have been reported from 23 teleost families, and these species maintain pairs in territories on substrata [14]. Maintenance of pair bonds in schools in mid-water has not been reported so far except for X. rotundiventralis. Schooling of fish is generally thought to reduce the risk of being eaten [15] and/or to increase the efficiency of foraging [16]. More studies will be needed to reveal the benefit of the schooling of X. rotundiventralis and the mechanism by which they distinguish their mating partners from the other individuals in the schools.

The present analysis showed that young in a clutch were most likely full-sibs, suggesting genetic monogamy, which is an exclusive mating relationship between a male and a female [17]. However, the present analysis provides no information on the social mating system in this species. Social monogamy can be identified by paired males and females that spend extensive periods of time together [17], but it is not known whether the mating pairs of X. rotundiventralis maintain physical proximity.

In summary, we have presented molecular evidence that the mating pairs of X. rotundiventralis maintain pair bonds for a prolonged period in schools. The present study is the first to identify pair bonds in animals in which physical proximity of the pair members has not been observed.

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