

Invisible pair bonds detected by molecular analyses

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Short title: Invisible pair bonds

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 mouth-brooding and non-brooding adults in mid-water, and visible pairs are not recognized. A previous study suggested that mouth-brooding 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 ten microsatellite markers. The analysis showed that the mouth-brooding 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.

Key words: 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 [e.g. 3,4]. Pair bonds are generally identified from direct observations of pairs that maintain physical proximity between males and females [e.g. 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 mouth-brooding 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) [6] (note that Yanagisawa *et al.* [6] called this species *Microdontochromis* sp.). This zooplanktivorous fish forms schools composed of about 500 to 2500 individuals of mouth-brooding 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 (< 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 strongly decreases when the young are 6–9 mm SL; and males brood young larger than 4.8 mm SL only (usually larger than 9.0 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 Mpulungu, 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 mouth-brooding 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.

Collected fish were put in transparent plastic bags (24 cm x 34 cm) immediately after they were caught in order not to mix young between adults. Fish were killed in a solution of anaesthesia 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 (M01 to M 14) and 9 females (F01 to F09) were brooding young [1 to 5 young (7.5–15.7 mm SL) in males' mouths, 2 to 7 young (5.0–15.6 mm SL) in females' mouths], and 8 males (M15 to M22) and 4 females (F10 to 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 to M65, and 17 females, F14 to F30)

that were collected at the southern coast of Nkumbula Island in October 2009 and August 2010. We used these 23 mouth-brooding 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 version 3.11 [7] (100 000 Markov chain steps, 1000 dememorization steps in the HW test; 10 000 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 version 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 mouth-brooding 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 ($p = 1.000$ 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 mouth-brooding 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.

4. DISCUSSION

The present genetic analysis revealed that the mouth-brooding adults of *X. rotundiventralis* were most likely the genetic mothers and fathers of the young in their mouths. This result suggests that each

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

10
11 The fact that males of *X. rotundiventralis* brood only young implies that females probably transfer
12 offspring after eggs hatch in their mouths. In the mouth-brooding cichlid fish from Lake Tanganyika,
13 eggs hatch 3 to 6 days after spawning [e.g. 11,12], suggesting that the pairs of *X. rotundiventralis*
14 maintain pair bonds during at least 3 days (from spawning to the shift of young), although this estimate
15 may be too conservative (young shift occurs 9.4 ± 0.5 days after spawning in a congener, *X. flavipinnis*
16 [11]).

17
18 The pair bonds of *X. rotundiventralis* may improve the survival rate of the young. Maintenance of the
19 pair bond allows the females to transfer fractions of the young to their mates. Yanagisawa *et al.* [6]
20 suggested that division of the young between the mother's and the father's mouths, which doubles the
21 brooding space, would enable the young to grow larger. A female-to-male shift of young has also been
22 reported in some other cichlid fish from Lake Tanganyika, i.e. *X. boulengeri*, *X. flavipinnis*, *X.*

1 *longispinis*, *Eretmodus cyanostictus* and *Tanganicodus irsacae* [11–13], but its evolutionary
2 significance may be different from that in *X. rotundiventralis*. The females of these species transfer
3 their entire broods to their mates. Yanagisawa [11] suggested that the release of females from the
4 mouth-brooding task would accelerate the females' feeding and gonadal recovery.

5
6 Monogamous fish have been reported from 23 teleost families, and these species maintain pairs in
7 territories on substrata [14]. Maintenance of pair bonds in schools in mid-water has not been reported
8 so far except for *X. rotundiventralis*. Schooling of fish is generally thought to reduce the risk of being
9 eaten [e.g. 15] and/or to increase the efficiency of foraging [e.g. 16]. More studies will be needed to
10 reveal the benefit of the schooling of *X. rotundiventralis* and the mechanism by which they distinguish
11 their mating partners from the other individuals in the schools.

12
13 The present analysis showed that young in a clutch were most likely full-sibs, suggesting genetic
14 monogamy, which is an exclusive mating relationship between a male and a female [17]. However, the
15 present analysis provides no information on the social mating system in this species. Social monogamy
16 can be identified by paired males and females that spend extensive periods of time together [17], but it
17 is not known whether the mating pairs of *X. rotundiventralis* maintain physical proximity.

18
19 In summary, we have presented molecular evidence that the mating pairs of *X. rotundiventralis*
20 maintain pair bonds for a prolonged period in schools. The present study is the first to identify pair
21 bonds in animals in which physical proximity of the pair members has not been observed.

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3

- 1 **Figure 1.**
- 2 *Xenotilapia rotundiventralis* at Nkumbula Island, near Mpulungu, Zambia. (a) The upper left fish
- 3 appears to be a non-brooding adult, and the right fish appears to be a mouth-brooding adult. Sexes
- 4 cannot be identified from the photograph. (b) A school of adult fish.

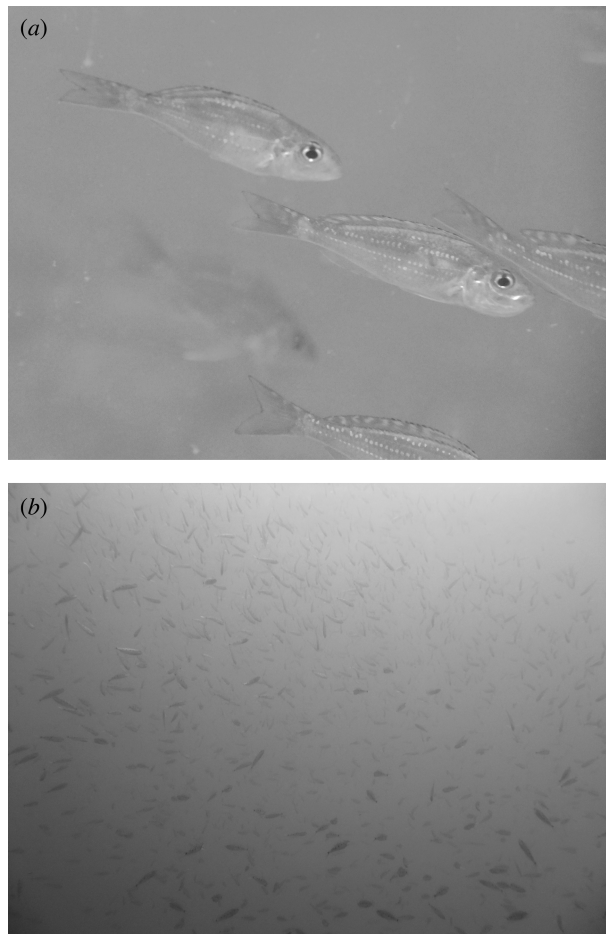


Figure 1

Table 1.

Details of microsatellite loci of the 95 adults that are genotyped in the present study (H_o : observed heterozygosity, H_e : expected heterozygosity, ^{NS} $p \geq 0.05$ in a test of departure from Hardy-Weinberg equilibrium after a sequential Bonferroni correction).

locus	n	no. of alleles	allele freq.	H_o	H_e
Pzeb4	95	6	0.005–0.511	0.653 ^{NS}	0.623
GM264	95	21	0.005–0.158	0.937 ^{NS}	0.929
Abur44	95	23	0.005–0.147	0.905 ^{NS}	0.940
Abur46	93	7	0.022–0.468	0.720 ^{NS}	0.716
Abur120	95	10	0.005–0.437	0.737 ^{NS}	0.758
Abur132	95	9	0.005–0.321	0.789 ^{NS}	0.793
Abur139	92	16	0.005–0.147	0.946 ^{NS}	0.912
Abur209	95	13	0.005–0.184	0.884 ^{NS}	0.885
Ttem8	95	6	0.016–0.316	0.758 ^{NS}	0.743
Ttem9'	95	14	0.005–0.216	0.842 ^{NS}	0.897

SUPPLEMENTARY MATERIALS

Behaviours of Xenotilapia rotundiventralis

Yanagisawa *et al.* [1] reported that pairs were not identified in *X. rotundiventralis*. Indeed, in our behavioural observations of the school, which were conducted for 29 hours 45 min in total in November and December of 1991 and September to December of 2009, neither mouth-brooding nor non-brooding individuals exhibited either physical proximity between mates or any other behaviours associated with reproduction except for mouth-brooding. They continuously performed picking actions in the daytime (5:00–18:00). In our brief observations at night, these individuals stayed motionless on the bottom, and their arrangements appeared to be random. Adults were not found outside of the school, whereas free-swimming young were found invariably in schools of young of two other cichlids, *Lepidiolamprologus elongatus* and *Perissodus microlepis* [1,2]. The timing and location of mating and young transfer is not known for *X. rotundiventralis*.

DNA extraction and amplification

Total DNA was extracted using an AquaPure Genomic DNA Kit (Bio-Rad). Polymerase chain reaction (PCR) was conducted using a PC 818 Program Temp Control System (Astec) for the microsatellite loci using the following programme: one cycle of 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s; and one cycle of 72 °C for 7 min. Ten microsatellite loci were used for genotyping: Pzeb4 [3]; GM264 [4]; Abur44, Abur46, Abur120, Abur132, Abur139 and Abur209 [5]; and Ttem8 and Ttem9' [6]. The forward and/or reverse primers were redesigned for four microsatellite loci using the Primer 3 program (<http://frodo.wi.mit.edu/primer3/>), i.e. Abur44-F:

5'-CCCCAAATCCATCACCTAATC-3', Abur46-F: 5'-GTGTGCGACAGTTTGAATGC-3', Abur46-R: 5'-CTTACTTCTGGCCTGCTTGC-3', Abur120-F: 5'-AGTGTATGTACCGGGTGTTCG-3', Abur120-R: 5'-ATTCAGCAATGTCAGCAACG-3', and Abur132-R: 5'-ATCAGTGGTTGGAGGGAAAC-3'. Forward primers were labelled with fluorescent dye 6-FAM (Ttem8 and Ttem9'), HEX (Pzeb4) or NED (GM264), or the M13 method for fluorescent labelling of PCR products [7] was used (six Abur markers). The microsatellite loci were analysed on an ABI 3130xl Sequencer (Applied Biosystems) using internal size marker Genescan 400 HD (Applied Biosystems).

Robustness of the parentage analysis

In the present analysis of parentage relationships, 95 adults were used as candidate parents of the 72 young, which might have been only 4–19% of the adults in the school [1]. However, the present analysis was sound, as shown by the following. Assuming an infinite population, we roughly estimated the probability that an adult is identified as a genetic parent of a young by chance even though the adult is not one of the genetic, real parents of the young (p).

$$p = \prod p_i,$$

$$p_i = 1 - (1 - a_i - b_i)^2,$$

where a_i and b_i are the frequencies of the alleles of locus i of the young, when the locus i of the young is heterozygous, or

$$p_i = 1 - (1 - a_i)^2,$$

where a_i is the frequency of the allele of locus i of the young, when the locus i of the young is homozygous. The probability p differed among young, and varied from 1.38×10^{-3} to 2.15×10^{-6} in the 72 young examined. This means that only one of 724 to 464,616 (21,959 on average) adults can be

identified as a genetic parent of a particular young by chance. These probabilities are very low, even though this estimation is conservative because we do not take the sib-ship relationships between young into account. Therefore, the genetic child-parent relationships between young and their mouth-brooding adults detected by the present analysis more likely reflect the real child-parent relationships than result from statistical errors.

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