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Microsatellites reveal high levels of population substructuring in the species-poor Eretmodine cichlid lineage from Lake Tanganyika

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This study investigated fine-scale population substructuring in an apparently monogamous, biparental mouth-brooding cichlid. Microsatellite allele frequencies were determined at four polymorphic loci for nine populations of *Eretmodus cyanostictus*. We provide empirical support for the hypothesis that a species employing this breeding strategy should exhibit high levels of population substructuring. Stretches of sand represent considerable barriers to dispersal and, in contrast to the rock-dwelling cichlids of Lake Malawi, distance alone, along a continuous rocky shoreline, is sufficient to reduce gene flow significantly. There was a significant pattern of isolation by distance both along the whole study area and over the stretch of continuous shoreline, suggesting that this species has poor dispersal capabilities and that juveniles establish territories close to their natal site. Despite limited dispersal, *E. cyanostictus* populations are not significantly more inbred than a more-widely dispersing rock-dwelling cichlid from Lake Malawi. This finding may cast doubt on the hypothesis that polyandry has evolved as a mechanism for maintaining genetic diversity in Lake Malawi cichlids. High levels of substructuring may not always promote high levels of speciation, and other factors, such as the intensity of sexual selection, may be more important in determining the speciation potential of a lineage.

Keywords: microsatellite DNA; cichlid; Lake Tanganyika; *Eretmodus*; population structure; speciation

1. INTRODUCTION

Lake Victoria and Lake Malawi are renowned for their endemic cichlid flocks, both of which are thought to be monophyletic and to have evolved extremely rapidly within the confines of their respective lake basins (Meyer 1993). Lake Tanganyika is inhabited by an older, more diverse and probably polyphyletic cichlid fauna (Poll 1986; Meyer 1993). These three lakes provide a unique opportunity to study both macro- and micro-evolutionary events in a single vertebrate family (Cichlidae), different lineages of which have evolved under different conditions and over different time-spans in lacustrine systems.

Recent research using microsatellite DNA markers has revealed high levels of population substructuring in the rock-dwelling cichlids (mbuna) inhabiting Lake Malawi (Van Oppen *et al*. 1997*a*; Arnegard *et al*. 1999; Markert *et al*. 1999). These studies suggest that these polygamous, maternal mouth-brooding species are highly stenotopic and do not disperse over areas of deep water and/or long stretches of sandy shoreline. This is likely to have been important in the evolution of many species and geographically restricted colour morphs. Lake Tanganyika differs from Lake Malawi and Lake Victoria in that it is inhabited by an assemblage of non-haplochromine cichlids. While all known haplochromines are polygamous maternal mouth-brooders, Lake Tanganyika's non-haplochromine cichlids exhibit a wide range of breeding systems. These include polygamous maternal mouth-brooders, monogamous substrate spawners, harem-based shell spawners and biparental mouth-brooders. Previous, lake-wide studies of rock-dwelling cichlid species from Lake Tanganyika indicate mitochondrial DNA (mtDNA) haplotypes with geographically restricted distributions in some species (Sturmbauer & Meyer 1992; Sturmbauer *et al*. 1997; Rüber *et al*. 1999). Other species show almost no geographical subdivision, suggesting that geological events and physical barriers may differentially affect gene flow in closely related species, even those employing similar breeding systems (Meyer *et al*. 1996; Sturmbauer *et al*. 1997).

We investigated fine-scale population substructuring in a taxon with a markedly different breeding system from that exhibited by the mbuna of Lake Malawi. *Eretmodus cyanostictus* is a member of the tribe Eretmodini, small goby-like cichlids, which inhabit the shallow rocky-pebble shorelines of Lake Tanganyika. *E. cyanostictus* is an apparently monogamous, sexually monochromatic, biparental mouth-brooder that exhibits a female-to-male pattern of mouth-brooding (Yamaoka *et al*. 1986; Kuwamura *et al*. 1989) and is widely distributed over the southern half of the lake. Previous research has revealed considerable differences in mtDNA haplotype and microsatellite frequencies between populations (Rüber *et al*. 1999, 2001). *E. cyanostictus* retains only a vestigial swim bladder (Konings 1998), which is likely to allow it to remain close to the substrate in turbulent conditions.

We predicted that the monogamous biparental mouth-brooding breeding system, coupled with the possibly poor dispersal capabilities of *E. cyanostictus*, is likely to lead to high levels of population substructuring. The absence of high levels of substructuring would suggest either that adult dispersal is much greater than expected or that

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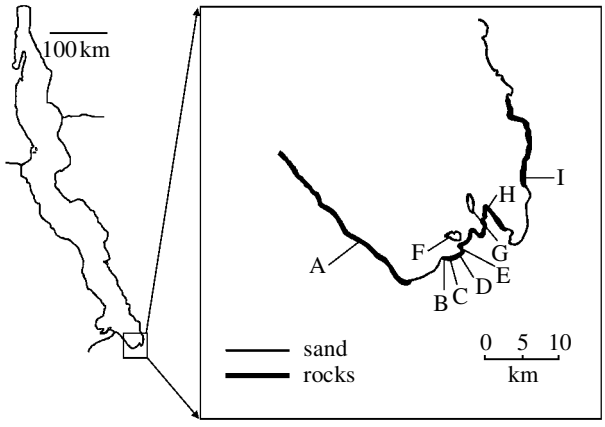

Figure 1. Zambian shoreline of Lake Tanganyika showing the sample collection sites.

significant dispersal occurs before individuals reach adulthood and occupy breeding territories.

We used microsatellite markers that have been previously used to study fine-scale substructuring in African cichlids. We tested the following nested hypotheses: first, that physical barriers, such as sandy beaches and areas of deep water, which act as barriers to gene flow in the polygamous maternal mouth-brooding mbuna, will also act as barriers to gene flow in *E. cyanostictus*, a monogamous, biparental mouth-brooding species; and second, that the predicted poor dispersal capabilities of *E. cyanostictus* lead to a positive correlation between genetic divergence and geographical distance, even in the absence of physical barriers.

2. MATERIAL AND METHODS

(a) *DNA preparation and amplification*

Monofilament gill-nets (mesh size: 0.8–1.2 cm) were used to collect 287 specimens of *E. cyanostictus* from nine localities on the Zambian shoreline of Lake Tanganyika (figure 1), in less than 3 m depth of water, during two collecting trips in April and October 1999. Fin clips were taken from freshly caught specimens and immediately preserved in 70% ethanol.

Total DNA was extracted using proteinase-K digestion and salt precipitation, using a protocol modified from Aljanabi & Martinez (1997). Extracted DNA was re-suspended in 200 µl of autoclaved MilliQ (MQ) H_2O.

All samples were screened for variation at each of four microsatellite loci: *Pzeb1*, *Pzeb3* (Van Oppen *et al.* 1997*b*), *TmoM11* and *TmoM5* (Zardoya *et al.* 1996). Polymerase-chain-reaction amplifications were performed under the following conditions: 94 °C for 120 s, followed by five cycles of 94 °C for 45 s, A°C for 45 s and 72 °C for 45 s, followed by 30 cycles of 91 °C for 30 s, A°C for 30 s, 72 °C for 30 s, followed by 72 °C for 10 min. The annealing temperature (A) was 55 °C for *Pzeb1*, *Pzeb3* and *TmoM5* and 50 °C for *TmoM11*. Reaction mixes (total volume: 10 µl) consisted of 2 µl (*ca.* 20 ng) of template DNA, 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 0.26 units of Taq polymerase (Pharmacia Biotech, Amersham, UK) and 1 µl 10 × reaction buffer (Pharmacia Biotech). The mixture was overlaid with 10 µl of mineral oil. Amplified products were resolved on short 6% denaturing polyacrylamide gels on an ALF Express DNA sequencer (Pharmacia Biotech). Product sizes were determined by comparison with M13mp8 DNA standards, following Van Oppen *et al.* (1997*b*).

Allelelinks (Pharmacia Biotech) was used to size the fragments. Alleles were binned into 1 bp (*Pzeb1*) or 2 bp categories (*Pzeb3*, *TmoM5* and *TmoM11*) and compared with a suite of standard alleles run on each gel.

(b) *Data analysis*

Genotypes at all pairs of loci were tested for linkage disequilibrium and deviations from Hardy–Weinberg equilibrium using the exact test of Genepop (Genepop v. 3.1d; Raymond & Rousset 1995). Significance levels were determined using the Markov chain method (dememorization number = 5000, 100 batches, 2000 iterations).

Population structure was tested using an infinite-allele model (IAM) (Kimura & Crow 1964). IAM-based measures were used in preference to stepwise mutation models for all statistics. In recently isolated populations, and when fewer than 20 loci are used, IAM methods are likely to give more accurate estimates of population structure (Slatkin 1995; Gaggiotti *et al.* 1999).

Pairwise and overall F_{ST} was estimated by θ (Weir & Cockerham 1984) using FSTAT (Goudet 1995). Differences in allele frequencies between samples were calculated using Fisher's exact test (Genepop v. 3.1d; Raymond & Rousset 1995). Fisher's (1954) method was used to combine single-locus probabilities to give a multi-locus estimate. The values from the two statistics were tested for significant departures from zero using permutation tests contained in the respective packages.

Pairwise estimates of the number of migrants per generation were calculated using the corrected private-allele-based model of Barton & Slatkin (1986) in Genepop v. 3.1d (Raymond & Rousset 1995).

Measures of genetic distance are expected to increase with geographical distance under isolation-by-distance models. The Mantel test (Genepop v. 3.1d; Raymond & Rousset 1995) with 5000 permutations was used to test for a significant correlation between $F_{ST}/(1-F_{ST})$ and distance between samples following Rousset (1997). $\mathrm{Log}_{10}M$ was also calculated ($M = 1/4((1/F_{ST})-1)$: equivalent to the number of migrants that would yield the observed levels of population differentiation) and regressed on $\log_{10}$(separation distance). This was used to investigate the geographical pattern of gene flow from the spatial pattern of genetic differentiation (Slatkin & Maddison 1990; Slatkin 1993).

The sequential Bonferroni procedure was used throughout the analyses to adjust significance levels for multiple comparisons (Rice 1989).

3. RESULTS

All loci were polymorphic for all the samples. For the nine samples combined, the mean number of alleles per locus varied from 3.44 for *TmoM11* to 17.7 for *TmoM5*. The mean expected heterozygosity (H_e) per locus ranged from 0.44 for *TmoM11* to 0.89 for *TmoM5*. Within-sample H_e ranged from 0.56 for sample I to 0.73 for sample E. Observed heterozygosity (H_o) and H_e per sample, number of alleles, size ranges and significant deviations from Hardy–Weinberg expectations are shown in table 1. After Bonferroni correction, 3 out of 36 tests revealed significant deviations from Hardy–Weinberg equilibrium. Two of the significant tests were for sample I. This sample has a size of 12, which is extremely small for a microsatellite study. Any

Table 1. *Levels of genetic diversity for nine populations of* Eretmodus cyanostictus *from the Zambian shoreline of Lake Tanganyika using four microsatellite loci*

	population									
locus	A ($n = 32$)	B ($n = 46$)	C ($n = 41$)	D ($n = 23$)	E ($n = 23$)	F ($n = 15$)	G ($n = 47$)	H ($n = 48$)	I ($n = 12$)	mean
Pzeb1										
no. of alleles	9	9	10	10	11	10	10	7	5	9.00
allele min.	129	134	131	132	128	128	128	134	131	128
allele max.	143	146	150	141	148	143	144	142	139	150
H_o	0.688	0.652	0.675	0.478[a]	0.652	0.800	0.783	0.667	0.167[a]	0.618
H_e	0.855	0.763	0.840	0.757	0.822	0.760	0.770	0.654	0.601	0.758
Pzeb3										
no. of alleles	5	6	6	5	3	5	3	6	2	4.56
allele min.	319	317	317	317	319	319	321	317	319	317
allele max.	331	327	327	327	323	337	325	331	323	337
H_o	0.594	0.244	0.447	0.304	0.522	0.286	0.326	0.500	0.167	0.377
H_e	0.607	0.363	0.518	0.274	0.492	0.533	0.377	0.557	0.278	0.444
TmoM11										
no. of alleles	3	5	3	3	4	4	4	3	2	3.44
allele min.	155	159	159	159	159	159	157	159	159	155
allele max.	161	167	163	163	165	165	165	165	161	167
H_o	0.375	0.478	0.405	0.609	0.652	0.333	0.435	0.313	0.000[a]	0.400
H_e	0.330	0.469	0.518	0.507	0.617	0.429	0.540	0.491	0.375	0.475
TmoM5										
no. of alleles	16	21	24	14	16	10	21	22	15	17.67
allele min.	218	216	216	216	216	216	216	216	216	216
allele max.	264	304	282	282	286	268	286	288	296	304
H_o	0.875	0.796	0.854	0.636	0.870	0.733	0.674	0.766	0.917	0.791
H_e	0.799	0.894	0.882	0.835	0.911	0.836	0.779	0.807	0.903	0.849
mean no. of alleles	8.25	10.25	10.75	8.00	8.50	7.25	9.50	9.50	6.00	—
mean H_o	0.633	0.543	0.595	0.507	0.674	0.538	0.553	0.561	0.313	—
mean H_e	0.658	0.629	0.698	0.607	0.727	0.662	0.624	0.634	0.563	—

[a]Denotes significant deviation from Hardy–Weinberg equilibrium after table-wide Bonferroni correction.

Table 2. *Genetic differentiation of Zambian populations of* Eretmodus cyanostictus

(For each pairwise comparison the table indicates distance between populations (km), intervening substrate, multi-locus F_{ST}, number of migrants per generation and multi-locus exact tests and individual-locus exact tests on allele frequencies. Significant p-values at the Bonferroni-corrected level ($p = 0.005$) are indicated by asterisks.)

					exact test p-values				
pairwise comparison	distance (km)	intervening substrate	four-locus F_{ST}	number of migrants	multi-locus p-value	*Pzeb1*	*Pzeb3*	*TmoM11*	*TmoM5*
A–B	23.4	rocky coast; sandy beach	0.134 ($p < 0.001$)*	2.67	$p < 0.0001$*	$p = 0.0006$*	$p = 0.0016$*	$p < 0.0001$*	$p < 0.0001$*
B–C	1.2	rocky coast	0.019 ($p = 0.001$)*	6.07	$p = 0.0003$*	$p = 0.0001$*	$p = 0.396$	$p = 0.052$	$p = 0.202$
C–D	2.0	rocky coast	0.010 ($p = 0.138$)	6.10	$p = 0.091$	$p = 0.018$	$p = 0.082$	$p = 0.946$	$p = 0.753$
D–E	3.1	rocky coast	0.036 ($p < 0.001$)*	3.71	$p < 0.0001$*	$p = 0.331$	$p = 0.0016$*	$p = 0.054$	$p = 0.0018$*
E–F	2.7	sandy bay	0.015 ($p = 0.045$)	3.36	$p = 0.062$	$p = 0.250$	$p = 0.116$	$p = 0.104$	$p = 0.200$
E–H	14.8	rocky coast	0.069 ($p < 0.001$)*	3.39	$p < 0.0001$*	$p < 0.0001$*	$p = 0.0002$*	$p = 0.005$	$p < 0.0001$*
E–G	15.6	rocky coast; sandy bay	0.098 ($p < 0.001$)*	2.25	$p < 0.0001$*	$p < 0.0001$*	$p < 0.0001$*	$p = 0.008$	$p < 0.0001$*
G–H	4.7	rocky coast; sandy bay	0.015 ($p < 0.001$)*	3.55	$p < 0.0001$*	$p < 0.0001$*	$p = 0.393$	$p = 0.003$*	$p = 0.631$
H–I	28.1	rocky coast; sandy beach	0.177 ($p < 0.001$)*	1.07	$p < 0.0001$*	$p < 0.0001$*	$p < 0.0001$*	$p = 0.393$	$p < 0.0001$*
A–I	72.5	(whole study area)	0.175 ($p < 0.001$)*	1.44	$p < 0.0001$*	$p < 0.0001$*	$p = 0.096$	$p < 0.0001$*	$p < 0.0001$*

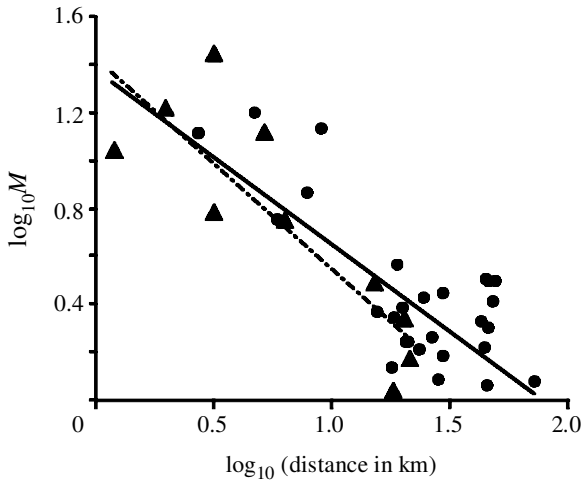

Figure 2. Plot of $\log_{10}M$ (where $M = 1/4((1/F_{ST})-1)$ against $\log_{10}$(separation distance). The triangles correspond to populations not separated by physical barriers; the circles and triangles correspond to the entire data set. The slope of the regression line for the entire data set (solid line) is -0.72 ($r^2 = 0.67$); the slope of the reduced data set (dashed line) is -0.88 ($r^2 = 0.72$).

comparisons involving this sample or sample F ($n = 15$) should be treated with caution.

There was no evidence of linkage disequilibrium in any pair of loci ($p > 0.05$). F_{ST} over all samples was highly significant ($F_{ST} = 0.098$, $p < 0.001$). A highly significant F_{ST} estimate was also obtained for the five samples not divided by physical barriers (B, C, D, E and H) ($F_{ST} = 0.068$, $p < 0.0002$). F_{ST} revealed highly significant differences between all adjacent sample pairs ($p < 0.001$) with the exception of C–D and E–F (table 2). F_{ST}-values ranged from 0.010 for C–D to 0.177 for H–I.

All of the combined multi-locus exact tests for sample pairs were significant (table 2), with the exception of C–D and E–F. Single-locus tests revealed that no single locus or group of loci was solely responsible for the multi-locus differences.

There was a highly significant correlation between $\log_{10}$ (separation distance) (km) and $F_{ST}/(1-F_{ST})$ ($p = 0.0016$) using a one-sided Mantel test. There was a significant correlation between $F_{ST}/(1-F_{ST})$ and $\log_{10}$ (separation distance) (km) for the samples not divided by physical barriers ($p = 0.039$). Plots of $\log_{10}M$ versus $\log_{10}$(separation distance) (figure 2) had regression coefficients of -0.72 ($r^2 = 0.676$) for all samples and a slope of -0.88 ($r^2 = 0.72$) for the samples not separated by physical barriers.

A Mantel test was again used to test for differences in F_{ST}-values between samples separated by physical barriers and those not separated (in the form of a binary matrix). This revealed that populations currently separated by physical barriers had higher F_{ST}-values but this was non-significant ($p = 0.0784$).

4. DISCUSSION

This study investigated fine-scale population substructuring in a monogamous, biparental, mouthbrooding cichlid species. It provides empirical support for the hypothesis that a species employing this breeding strategy should exhibit high levels of substructuring. The results confirm the findings of studies on other rock-dwelling cichlids: that stretches of sand represent considerable barriers to dispersal and, in contrast to the mbuna, that distance alone, along continuous rocky shoreline, is sufficient to reduce gene flow significantly. The study revealed a significant pattern of isolation by distance along both the whole study shoreline ($p = 0.0016$) and over the stretch of continuous shoreline ($p = 0.039$). Microsatellite markers revealed an overall F_{ST} of 0.098 ($p < 0.001$). The presence of such high levels of population substructuring suggests that juveniles do not disperse widely and are likely to acquire territories close to the parental territory.

Low levels of dispersal in *E. cyanostictus* may be expected to lead to high levels of inbreeding and concomitant inbreeding depression. In a direct comparison with a species of mbuna (data from Markert *et al.* 1999) we found no significant difference in average sample inbreeding coefficients ($F = (H_o - H_e)/H_o$, two-tailed *t*-test, $p = 0.11$), which suggests that *E. cyanostictus* are no more inbred than mbuna. This is an unexpected result as the mbuna exhibit both polygamy and polyandry, and this has been suggested to overcome the effects of limited dispersal by increasing effective population size (N_e) and leading to greater allelic diversity than expected from the population size (Parker & Kornfield 1996). This suggests that population sizes are considerably larger in *Eretmodus* than in the mbuna, or that *Eretmodus* are able to differentiate between siblings and non-siblings and thus avoid inbreeding, or that polygamy and polyandry are not, as has been suggested, responsible for the maintenance of genetic diversity in the mbuna.

The correlation between genetic distance and geographic distance allows tentative inferences concerning migration patterns to be drawn (Slatkin & Maddison 1990; Slatkin 1993). A slope of -1.00 is expected for a regression of $\log_{10}M$ on $\log_{10}$(separation distance) if gene flow follows a one-dimensional stepping-stone model, and a slope of -0.5 is expected if a two-dimensional pattern is followed. In this study, a slope of -0.88 was found for all nine samples and a slope of -0.72 was found for the five samples not separated by physical barriers. Both results suggest a migration scenario midway between the two extreme cases. Regular changes in water level coupled with the complex structure of the Zambian shoreline (Kohda *et al.* 1996) are likely to have led to a complicated pattern of dispersal involving the mixing of previously isolated populations, migration between adjacent populations and migration between islands and the main shoreline.

Previous studies of rock-dwelling cichlid species inhabiting Lake Malawi (Van Oppen *et al.* 1997*a*; Arnegard *et al.* 1999; Markert *et al.* 1999) also found high levels of population substructuring. However, in these cases physical barriers, such as sandy areas or stretches of deep water, appear to be necessary to reduce gene flow between adjacent populations significantly. This is not the case in *E. cyanostictus*, in which distance alone appears sufficient to reduce gene flow significantly. Several characteristics of *E. cyanostictus* may result in the higher levels of substructuring than observed in the mbuna. First, they retain only a vestigial swim bladder and swim in short

bursts, which is likely to reduce their dispersal capacity (Konings 1998). Second, breeding pairs appear to defend territories jointly, spawning is synchronized around lunar cycles and the apparently male-biased sex ratio will lead to few opportunities for extra-pair mating and therefore little incentive for males to desert (Neat & Balshine-Earn 1999). Moreover, by remaining with the female and brooding the young, males may increase their reproductive success by allowing the female to recover reproductive condition faster and therefore spawn again sooner (Neat & Balshine-Earn 1999). In contrast, the mbuna are polygamous and polyandrous and as many as seven or eight males are known to sire each brood (Parker & Kornfield 1996). This strategy may not only increase the genetic variation in each brood but, coupled with some male dispersal (Knight *et al*. 1999), also reduce the chances for substructuring to develop along continuous stretches of shoreline.

The high level of substructuring between adjacent populations in the mbuna of Lake Malawi is considered to have been an important factor in their speciation. However, the Eretmodini appear to be even more highly structured, yet have not speciated to the same degree despite being a considerably older lineage (Nishida 1991). One would expect species with poor dispersal capabilities and high levels of substructuring to exhibit high levels of intraspecific variation in some traits. This is not the case in *E. cyanostictus*, and suggests that high levels of genetic substructuring alone are not sufficient to promote high levels of speciation. Other species-specific factors may determine whether groups speciate or not. Sexual selection has been repeatedly suggested as a factor that may be important in cichlid speciation (Dominey 1984; Turner 1994; Seehausen *et al*. 1997). For example, Seehausen *et al*. (1997) suggested that during speciation events in cichlids from Lake Victoria, differences in male colour patterns may evolve first, followed by ecological diversification. This may also be the case in Lake Malawi, where many morphologically similar coexisting species differ principally in the male colour pattern. In contrast, the four nominal species currently described in the tribe Eretmodini (Poll 1986) show considerable ecological diversification (principally tooth shape) but little differentiation in colour pattern. This is unlikely to be purely a result of different conditions in the three lakes, as members of the maternal mouth-brooding tribe Tropheini in Lake Tanganyika also show high levels of intraspecific variation in colour pattern (the colour variants may be independent species) but little morphological variation (Sturmbauer & Meyer 1992). Differences in breeding system may fundamentally affect the rate and mode of speciation in different cichlid lineages.

In summary, high levels of genetic substructuring do not always lead to high levels of speciation, and, as recent data from demersal and pelagic cichlids inhabiting Lake Malawi has revealed, species-rich lineages may be only subtly structured (Taylor & Verheyen 2001) or not structured at all (Shaw *et al*. 2000). Breeding system appears to be a more important factor in the speciation potential of a lineage than the level of substructuring, and this is consistent with claims that strong sexual-selection pressures predispose certain lineages to rapid and frequent speciation events (Dominey 1984; Turner 1994; Seehausen *et al*. 1997).

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