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Rates of Extra-Pair Paternity in a Tree Swallow (*Tachycineta bicolor*) Population from Asheville, NC

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Abstract

Tachycineta bicolor, the tree swallow, is a socially monogamous bird that practices extra-pair paternity, an event where females seek out mates different from their residential partner. Benefits of extra-pair paternity include increased sexual selection and higher genetic diversity among offspring. This behavior is not uncommon among bird species, especially those that are migratory. Tree swallows are typically residents in Canada, the central United States, and parts of Mexico, but the species has recently begun to expand the southern edge of their range. There is interest in whether this shift is associated with differences in other behaviors, specifically extra-pair paternity. It was hypothesized that the rate of extra-pair paternity in this new population of tree swallows would be similar to that in other tree swallow populations in the typical breeding range. To conduct this experiment, DNA was extracted from bird feathers of 82 tree swallows from seven different nest boxes around Beaver Lake (Asheville, North Carolina). PCR was used to amplify two microsatellite loci, *PPi2* and *LTMR6*, from 23 individuals from five nests. Successful PCR products were sent out for fragment analysis. Fragment sizes at each locus were determined via Geneious 2019.0.4™, and GERUD 2.0™ was then used to predict the potential genotype for the maternal and paternal parent of each nest. Results so far indicate that this population has reduced rates of extra-pair paternity. Seeing a major change in this population, like mating differences, could help explain other behavioral changes in the species, including the unusual shift in the tree swallows' breeding range.

1. Introduction

Extra-pair paternity is a phenomenon whereby females in a population seek out other potential males to mate with who differ from their social partner[1]. This is a common mating practice among birds, especially passerines (songbirds). Benefits of extra-pair paternity include higher genetic diversity among offspring and increased sexual selection[2]. Research on blue tits (*Cyanistes caeruleus*) helps to show the genetic benefits to this kind of mating. The researchers manipulated the brood size of various nest boxes of blue tit populations, another species that, like tree swallows, are socially monogamous but use extra-pair mating. For each pair of nest boxes similar in hatching date and brood size, one was randomly selected for enlargement, and three nestlings were added from a donor nest. Adding the extra nestlings was meant to make the nests overcrowded and appear to be a poorer environment. They found that extra-pair offspring had a more efficient immune system, providing evidence for the hypothesis that some genetic benefits are only expressed in certain environments, specifically poor environmental conditions[3].

According to research, environmentally affected traits, like mate availability, can affect the outcome of offspring produced by extra-pair mates[3,4,5]. In research done on the yellow-rumped flycatcher (*Ficedula zanthopygia*), it was seen that females tended to choose distantly located, more heterozygous males rather than their nearest neighbors to engage in extra-pair paternity with. The findings also suggested that some passerine species may have variation in

their instances of extra-pair paternity due to the desire to mate with highly heterozygous males[5]. In another study, researchers examined four years of data and research consisting of various environmental states among a population of tree swallows (*Tachycineta bicolor*). Environmental conditions were determined by looking at egg laying date and the agricultural and population density found around each nest; the later eggs were laid and the higher the densities of each condition were found, the poorer the environment was considered to be. The study found that within-pair offspring were favored in better environmental conditions. Extra-pair offspring were dependent on paternal features and population density, and it was seen that in areas of poor environmental conditions, these offspring might increase in numbers to increase the success of the entire brood[4].

Tachycineta bicolor, tree swallows, are small song birds that are residential in Canada, the central United States, and parts of Mexico; the species undergoes annual migration but then typically returns to previous nesting sites[6]. Unlike many other passerines that are expanding their ranges northward, tree swallows are expanding their range south[6]. Despite being a socially monogamous species, these birds seem to be benefitting from practicing extra-pair paternity[1]. Female tree swallows have been observed rejecting undesired males to actively select their copulations. This example shows that tree swallows do not randomly select extra-pair paternity partners and have more control over their selection. Behavioral evidence has also shown that tree swallow populations tend to be more outbred than inbred, suggesting an increase in population genetic diversity due to selection of mates that may be more distantly related to the females than their social partner[7]. Previous tree swallow research has shown that 67% of the nests analyzed in a population appeared to have offspring fathered by more than one male[2], and the average rate of extra-pair paternity in the center of tree swallow distribution ranges from 50-90% of individuals[1].

This study examined the occurrence of extra-pair paternity in a new population of tree swallows at Beaver Lake in Asheville, North Carolina. The human-made lake is in a residential area, and the tree swallows are banded in labeled nest boxes around the lake monitored by UNC Asheville faculty. As there are only a set number of boxes around the lake, the tree swallows must compete with other local nest boxing species. The analysis here is an update of research previously done on this specific population of tree swallows. The new rate of extra-pair paternity will be compared to the rate of known occurrences in individuals found at the range center. If there are large discrepancies in this population's amount of extra-pair paternity, differences in factors like size of breeding area and population might be the cause. This change in range may make this form of mating even more beneficial due to the novelty of the new environments.

2. Methodology

2.1 Feather Collection

Feathers were collected from 82 individuals found in nest boxes surrounding Beaver Lake in Asheville, North Carolina. The birds were carefully taken from the back of each nest box and bagged. From there, their weight, sex, age, and wing length measurements were taken for a different study. Small non-flight feathers were also taken from the females' brood patches and areas on the males' abdomens. Offspring feathers were collected within a week of hatching and when resident adults were not present. The feathers were stored in labeled vials containing 100% ethanol until DNA extraction.

2.2 DNA Extraction

For each sample, feathers were placed in a mortar, covered in liquid nitrogen, and then ground using a pestle. Once ground, the samples were put into labeled microcentrifuge tubes and combined with 300 µl Buffer ATL and 20 µL Proteinase K from the DNeasy Blood and Tissue Kit®, along with 20 µl of 1 M DTT. The samples were then incubated in a water bath at 56 °C for 48 hours. After that, the samples were vortexed for 15 s, then 300 µl Buffer AL and 300 µl 100% ethanol were added, with vortexing occurring between each addition. Each sample mixture was then put into a DNeasy Mini spin column placed in a 2 ml collection tube and was centrifuged for 1 min at 8000 rpm. The DNeasy Mini spin columns were then placed into new 2 ml collection tubes and 500 µl Buffer AW1 was added before going into the centrifuge for 1 min at 8000 rpm. After, each DNeasy Mini spin column was placed into a new 2 ml collection tube, and 500 µl Buffer AW2 was added before centrifuging for 3 min at 14000 rpm. Last, each DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and 200 µl Buffer AE was directly pipetted onto the DNeasy membrane. Samples were incubated at room temperature for 1 min then centrifuged for 1 min at 8000 rpm. A ND-

1000 Nanodrop Spectrophotometer®, zeroed using AE buffer, was then used to determine each sample's concentration and quality.

2.3 PCR Amplification

Genetic analyses using molecular tools like microsatellite markers do not rely on behavioral observations and have been used to measure extra-pair paternity in this and other passerine species[7,8,9,10,11,12]. Microsatellites, a type of SSRs (simple sequence repeats) or STRs (short tandem repeats), represent repeated one to ten nucleotide long DNA motifs. They are amplified through PCR (polymerase chain reaction), and the success of PCR product amplifications are determined using gel electrophoresis. Microsatellite markers have also been used to estimate animal breeding[13] and estimate paternity in pigeons (*Columba livia domestica*) from feather DNA[14]. Thus, PCR amplification of microsatellite loci was used in this study.

Master mixes and thermocycling conditions were based on published protocols for the two loci: *LTMR6* and *PPi2*[7,8,9,10,11,12]. These protocols had been developed for other species (e.g., *Chiroxiphia linearis*, the long-tailed manakin) and have been found to amplify for a large range of passerines. Studies of other species (including *Chiroxiphia linearis*, the long-tailed manakin, and *Acrocephalus scirpaceus*, the Eurasian reed warbler) have found the allele range for each locus to be 188-190 nucleotides for LTMR6[11] and 242–280 nucleotides for *PPi2*[15] .

The master mix recipe for *LTMR6* called for a 20 µL reaction with 1 µL of 10 µM reverse primer, 0.5 µL of 10 µM M13 tagged forward primer, 0.5 µL of 5 µM M13 tagged 6FAM primer, 6.4 µL of 2.5 mM dNTPs, 2 µL of *New England Biolabs* 10X Standard *Taq* Reaction buffer, 1 µL of 5U/µM *Taq* DNA polymerase, and 8.6 µL genomic DNA. The master mix for *PPi2* was also a 20 µL reaction, and used: 1 µL of 10 µM reverse primer, 0.5 µL of 10 µM M13 tagged forward primer, 0.5 µL of 5 µM M13 tagged 6FAM primer, 2.3 µL of 25 mM $MgCl_2$, 6.4 µL of 2.5 mM dNTPs, 2 µL of *New England Biolabs* 10X Standard *Taq* Reaction buffer, 1 µL of 5U/µM *Taq* polymerase, 0.3 µL of PCR water, and 6 µL genomic DNA.

The samples then underwent thermal cycling using conditions modified from the original literature[7,8,9,10,11,12]. Thermal cycling conditions for *LTMR6* were: 1 cycle at 94°C for 3 min; 9 cycles of touchdown PCR (-0.5°C each cycle), starting with 94°C for 30 s, then 60°C for 40s, then 72°C for 40 s; 24 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 40 s; 1 cycle for 10 min at 72°C[5,9,12,13,14]. The thermal cycling profile for *Ppi-2* was 1 cycle at 94 °C for 10 min; 1 cycle at 50 °C for 1 min; 1 cycle at 72 °C for 1 min; 34 cycles of 94 °C for 30 s, 50°C for 40 s, and 72°C for 40 s[5,9,12,13,14].

2.4 Fragment Analysis and Familial Determination

Successful amplification of the samples was tested using 1% agarose TBE gel electrophoresis. Amplicons were sent to North Carolina State University's Genomic Sciences Laboratory for fragment analysis. Geneious 2019.0.4™ software was used to analyze the microsatellite loci *PPi2* and *LTMR6*. For each sample, peaks were looked for in the predicted allele range, and allele sizes (haplotypes) then were saved to use in subsequent GERUD 2.0™ analyses. GERUD 2.0™ was used to reconstruct parental genotypes using offspring data from *PPi2* and *LTMR6*. The data came from the allele sizes downloaded from Geneious 2019.0.4™. For boxes with a resident male and female, GERUD 2.0™ predicted parental genotypes for the offspring found in the nest. This then allowed the program to estimate the probability that the resident adults were the actual parents of that box's offspring. For boxes without a resident male and/or female, GERUD 2.0™ predicted parental genotypes and estimated hatchling parentage.

3. Results

GERUD 2.0™ reconstructed and predicted parental genotypes for five different nests from this population using haplotype results from Geneious 2019.0.4™ (Table 1). For Nest 1, there were potentially 17 different parent combinations for the nest, each having two fathers; groups 1 and 2 equally had the highest likelihood of being the true combination (Table 2). To help explain differences between these predicted groups, groups 1 and 3 will be used as an example. In parent group 1, father 1 sired 1 offspring and father 2 sired other offspring, whereas in group 3, both fathers were equally likely to sire all offspring (Table 2). For Nest 2, there were 50 potential families in the nest with 2 fathers each. Also, each group had an equal likelihood of being the true combination (Table 3). For Nest 3, there were 6 potential families in the nest with 1 father each, and each group had an equal likelihood of being the true

combination (Table 4). For Nest 4, there were 16 potential families in the nest with 1 father each, and each group had an equal likelihood of being the true combination (Table 5). Finally, for Nest 5, there were 6 potential families in the nest with 1 father each, and Groups 1 and 2 equally had the highest likelihood of being the true combination (Table 6). Overall, looking at the number of predicted maternal and paternal parents for each nest, it was found that the rate of extra-pair paternity in Asheville tree swallows was 40% (Table 7).

Table 1. Haplotypes for hatchlings and resident parents from five nest boxes for loci *PPI2* and *LTMR6*.

	Nest 1: *PPi₂*	Nest 1: *LTMR6*	Nest 2: *PPi₂*	Nest 2: *LTMR6*	Nest 3: *PPi₂*	Nest 3: *LTMR6*	Nest 4: *PPi₂*	Nest 4: *LTMR6*	Nest 5: *PPi₂*	Nest 5: *LTMR6*
Resident Maternal Parent	244/250	163/165	none	none	none	none	233/237	358/360	none	none
Resident Paternal Parent	254/254	163/165	none	none	none	none	none	none	none	none
Offspring	250/250	165/165	248/250 250/260 246/250	163/165 163/165 163/165	246/250 250/262	163/163 163/163	235/245	250/254	246/250 246/250 246/250	163/163 163/163 163/163

Table 2. Top 5 out of 17 predicted parent combinations for Nest 1 with 2 paternal parents predicted. Groups 1 and 2 equally have the highest probability of being the true combination.

Parental Group	Parent	*PPi2*	*LTMR6*	# Progeny	Probability
1	Mother	250/254	163/165		
	Father 1	244/244	163/163	1	0.0017
	Father 2	254/250	165/165	2	
2	Mother	250/254	163/165		
	Father 1	244/244	165/165	1	0.0017
	Father 2	254/250	165/165	2	
3	Mother	250/254	163/165		
	Father 1	244/254	163/163	2	0.0013
	Father 2	244/250	165/165	2	
4	Mother	250/254	163/165		
	Father 1	244/254	163/163	2	0.0013
	Father 2	254/250	165/165	2	
5	Mother	250/254	163/165		
	Father 1	244/254	163/163	2	0.0011
	Father 2	250/250	165/165	1	

Table 3. Top 5 out of 50 predicted parent combinations for Nest 2 with 2 paternal parents predicted. All predicted groups have equal probability of being the true combination.

Parental Group	Parent	*PPi2*	*LTMR6*	# Progeny	Probability
1	Mother	250/250	165/165		
	Father 1	248/246	163/163	2	0.5
	Father 2	260/260	163/163	1	
2	Mother	250/250	165/165		
	Father 1	248/260	163/163	2	0.5
	Father 2	246/246	163/163	1	
3	Mother	250/250	165/165		
	Father 1	248/248	163/163	1	0.5
	Father 2	260/246	163/163	2	
4	Mother	250/250	163/163/		
	Father 1	248/246	165/165	2	0.5
	Father 2	260/260	165/165	1	
5	Mother	250/250	163/163		
	Father 1	248/260	165/165	2	0.5
	Father 2	246/246	165/165	1	

Table 4. Top 5 out of 6 predicted parent combinations for Nest 3 with 1 paternal parent predicted. All predicted groups have equal probability of being the true combination.

Parental Group	Parent	*PPi2*	*LTMR6*	# Progeny	Probability
1	Mother	246/250	163/163		
	Father 1	246/262	163/163	2	0.0625
2	Mother	246/250	163/163		
	Father 1	250/262	163/163	2	0.0625
3	Mother	246/262	163/163	2	
	Father 1	250/250	163/163	2	0.0625
4	Mother	250/250	163/163	2	
	Father 1	246/262	163/163	2	0.0625
5	Mother	250/262	163/163		
	Father 1	246/250	163/163	2	0.0625

Table 5. Top 5 out of 16 predicted parent combinations Nest 4 with 1 paternal parent predicted. All predicted groups have equal probability of being the true combination.

Parental Group	Parent	*PPi2*	*LTMR6*	# Progeny	Probability
1	Mother	233/235	358/250		
	Father 1	237/245	360/254	2	0.0039
2	Mother	233/245	358/250		
	Father 1	237/235	360/254	2	0.0039
3	Mother	237/235	358/250	2	
	Father 1	233/245	360/254	2	0.0039
4	Mother	237/245	358/250	2	
	Father 1	233/235	360/254	2	0.0039
5	Mother	233/235	358/254		
	Father 1	237/245	360/250	2	0.0039

Table 6. Top 5 out of 6 predicted parent combinations Nest 5 with 1 paternal parent predicted. Groups 1 and 2 equally have the highest probability of being the true combination.

Parental Group	Parent	*PPi2*	*LTMR6*	# Progeny	Probability
1	Mother	246/246	163/163		
	Father 1	250/254	163/163	3	0.3750
2	Mother	246/250	163/163		
	Father 1	246/254	163/163	3	0.3750
3	Mother	246/250	163/163	2	
	Father 1	250/254	163/163	3	0.1406
4	Mother	246/254	163/163	2	
	Father 1	250/246	163/163	3	0.1406
5	Mother	246/254	163/163		
	Father 1	250/254	163/163	3	0.0469

Table 7. Summary data for 5 nests and 2 loci.

Nest	# of Predicted Maternal Parents	# of Predicted Paternal Parents
1	1	2
2	1	2
3	1	1
4	1	1
5	1	1

4. Discussion

I expected that there would be one maternal parent predicted per nest. However, it was interesting to see that in Nests 1 and 4, the predicted maternal haplotypes for the two loci did not match the actual resident mother's haplotype. This could be due to errors made during PCR amplification or in the feather collecting stage. Bird feathers that were collected from each nest may not have been from the true resident, as the birds could freely move around. Also, tree swallows, like many other birds, use found feathers to build their nests, so some of those may have gotten combined with the collected samples. One other possible explanation could be females egg dumping into nests of others, a phenomenon seen with many brood parasites. Research has shown this to happen less often in tree swallow nests than with other types of swallows, but it is still a possibility as they have been seen to accept parasitic eggs if added to their nest within three days of their first laid egg[16].

Out of the five nests examined, only two of them (40%) had offspring fathered by two different males, which is lower than 67% found in previous research[3]. Also, in Nest 1, while only one male appeared to be the father of the offspring, he was not the resident male of the nest. This nest in particular provides evidence of extra-pair paternity as the female clearly has both a resident mate and one other to help her produce offspring. Unfortunately with the four other nests, it is unclear whether extra-pair paternity is truly occurring or not. It is clear that the nests' offspring are coming from one male, but due to difficulties during feather collection, the haplotypes of the resident males are unknown.

For further research, the collection of the resident male's feathers needs to be prioritized. Since it appears that female tree swallows in this population are mainly producing offspring with only one male, the resident male's DNA is needed to see if he is the one male being mated with, or if it is another. Also, not all of the hatchlings in each nest were able to be analyzed, so a more complete data set should be created. To make the data set more complete, loci Tabi-1, Tabi-3A, and Tabi-4[7,8,9,10,11,12] as well as loci PPi2 and LTMR6 should be analyzed for all 82 birds. PCR recipes and thermocycling protocols for these loci are already available.

Forty percent extra-pair paternity is much lower than the known range of 50-90% of individuals in a population engaging in this mating behavior[8]. Since this range is so much lower, factors like the population being smaller than typical populations and being found in a more limited area are most likely the cause for this new populations' difference in extra-pair paternity. Due to these different factors, unlike the yellow-rumped flycatcher (*Ficedula zanthopygia*)[5], they may be forced to choose neighboring males, most likely the resident male, as mates. Once further genetic analysis has been done to confirm this rate, more in-depth research surrounding this new populations' habitat would be beneficial. Examples of this could include amount and type of nesting competition, residential human disturbances, and comparisons of average weather to areas located in the species' typical breeding range.

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