The Wilson Journal of Ornithology 134(1):000–000, 2022 DOI: 10.1676/20-00095

No genetic evidence for parent–offspring relatedness in post-breeding social groups of Black-crested Titmouse (*Baeolophus atricristatus*)

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ABSTRACT—After the breeding season, territorial adult Black-crested Titmouse (*Baeolophus atricristatus*) and residing juveniles form social groups that may persist until the following spring. Under the prolonged brood care hypothesis, one would expect these juveniles to be retained offspring with delayed dispersal of the breeding pair. To test if Black-crested Titmouse juveniles that reside in post-breeding territories are offspring of the territorial adult male, we performed microsatellite-based paternity analyses of 6 juvenile–adult male social dyads on 6 different territories. None of the juveniles were offspring of the adult male with which it shared a territory. We discuss several possible evolutionary explanations for this result. *Received 13 August 2021. Accepted 14 December 2021.*

Key words: delayed dispersal, genotyping, microsatellites, non-kin, parentage, paternity.

No hay evidencia genética de parentesco en grupos sociales posreproductivos del carbonero *Baeolophus atricristatus*

RESUMEN (Spanish)—Después de la estación reproductiva, los adultos territoriales del carbonero *Baeolophus atricristatus* y juveniles residentes forman grupos sociales que pueden persistir hasta la siguiente primavera. Según la hipótesis del cuidado prolongado de la nidada, podría esperarse que esos juveniles fueran descendencia retenida con dispersión retrasada de la pareja reproductiva. Para someter a prueba si estos carboneros juveniles que residen en territorios posreproductivos son descendientes del macho adulto territorial, llevamos a cabo un análisis de paternidad basado en microsatélites de 6 díadas de juveniles y machos adultos en 6 diferentes territorios. Ninguno de los juveniles fue descendiente del macho adulto con el cual compartían territorio. Discutimos varias posibles explicaciones evolutivas para este resultado.

Palabras clave: dispersión retrasada, genotipos, microsatélites, no-parentesco, parental, paternidad.

In multiple bird species, sexually mature individuals stay in their natal territory and form social family groups (e.g., Brown 1987, Emlen 1995, Koenig and Dickinson 2016). This behavior is widespread across bird families, occurring for example, in Paridae, Picidae, and Corvidae (Ekman 1989, Stacey and Ligon 1987, Ekman et al. 1994, respectively). After fledging, individuals may disperse and establish a territory of their own. However, constraints on obtaining an independent territory and the benefits of philopatry may explain why some individuals may remain as philopatric subordinates rather than disperse (i.e., delayed dispersal; Emlen 1982, Stacey and Ligon 1987, Koenig et al. 1992, Hatchwell and Komdeur 2000, Cockburn 2006). Kin selection is often important in determining the benefits to this strategy (Hamilton 1964, Ekman et al. 1994, Griffin and West 2003, Dickinson and Hatchwell 2004, Green et al. 2016).

For juveniles unable to obtain an independent territory, an alternative to staying at home exists. Individuals can disperse and join non-related birds on a foreign territory (Koenig et al. 1992, Ekman and Griesser 2002). The formation of non-related social groups within a territory is not common, even though unrelated residents have been documented in almost half of cooperative breeding birds (Riehl 2013). The reasons why a territorial resident would allow non-relatives to reside on its territory poses an evolutionary conundrum—because the costs of increased group size (e.g., food sharing) are not counterbalanced by kin-selected benefits (Kingma et al. 2014).

A territorial resident is therefore expected to allow its own offspring on its territory instead of non-related juveniles (the prolonged brood care hypothesis; Ekman et al. 1994). One possible benefit gained by allowing non-kin juveniles to reside on one's territory is that group members may increase effectiveness of antipredator detection and territory defense, as well as group foraging efficiency (Davies and Houston 1981, Elgar 1989, Beauchamp 1998, Brouwer et al. 2005, Beauchamp 2008, Mares et al. 2012,

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Kingma et al. 2014). To better understand the formation of social groups, the relative importance of delayed dispersal and settlement on a foreign territory should be studied in species with both kin and non-kin within social territorial groups (e.g., Siberian Jay [*Perisoreus infaustus*], Ekman et al. 1994; Tufted Titmouse [*Baeolophus bicolor*], Pravosudova and Grubb 2000).

Titmice are small birds from the Paridae family. Some titmouse species (Tufted Titmouse, Bridled Titmouse [*B. wollweberi*]) form prolonged post-breeding social groups (Brawn and Samson 1983, Ekman 1989 and references therein, Pravosudova and Grubb 2000) and occasionally exhibit cooperative breeding (Brackbill 1970, Davis 1978, Tarbell 1983, Christman and Gaulin 1998, Nocedal and Ficken 1998). Titmouse social groups can be composed of territorial adults with both kin and non-kin juveniles (Pravosudova et al. 1999, Pravosudova and Grubb 2000). For the Tufted Titmouse, Pravosudova et al. (1999) showed that among 12 territories that were each occupied by a group of 3 birds (an adult pair and 1 juvenile), the residential juvenile was not related to either adult in 7 of the 12 groups. For the same species, Pravosudova and Grubb (2000) showed that among 17 territorial groups (each with 2 adults and 1–3 juveniles), all juveniles were offspring of the adults in 3 groups, non-offspring in 8 groups, and a combination of both in 6 groups. This versatility in social group composition makes titmice a very interesting system to study the selective pressures favoring social groups.

Here, we studied the post-breeding social group composition in territories of the Black-crested Titmouse (*B. atricristatus*), a sister species of the Tufted Titmouse. Although, based on their close relatedness, we might expect similar genetic group compositions in these species, knowledge on genetic relationships in Black-crested Titmouse groups is currently lacking. Our goal was therefore to establish if the juveniles that reside in post-breeding social groups of this species were offspring of the territorial male.

Methods

Study species

The Black-crested Titmouse is a socially monogamous passerine. Its distribution ranges from southern Oklahoma and Texas (USA) to northeast Mexico (Patten and Smith-Patten 2008). The species breeds in cavities and also uses nest boxes (Grubb 1998). Males are highly territorial, defending confined breeding territories in spring and summer, and have larger home ranges in the winter (Brawn and Samson 1983, Rylander 2015). After the breeding season, 2 paired adults often form social groups with up to 6 juveniles, and such groups may persist until the following breeding season (Rylander 2015, Queller and Murphy 2017, Borger et al. 2020). Cooperative breeding does not regularly occur in this species, although its occurrence has been reported (Rylander 2015).

Study site and sampling

This study was conducted on a site near San Antonio, Texas, USA (29°41′15 N, 98°19′49 W) during the post-breeding season, between 18 May and 12 July 2018. As part of a larger study of territorial defense (see Borger et al. 2020), territorial intrusion experiments STIs (Simulated Territorial Intrusions; with taxidermic models and conspecific vocalization) were carried out on 25 territories. During these experiments, 12 of these territories were defended by adults and accompanying juveniles (the remaining territories were defended by adults alone). We describe the methods of that experiment here because, for the current study, we captured birds that responded to the STIs from that experiment.

Immediately after the intrusion experiment, we captured and banded as many group members as possible using mist nets placed in the immediate vicinity to where the taxidermic models were placed. To attract birds to the nets, we again used a taxidermic model and the same conspecific vocalizations. Although individuals were not banded prior to our study on group territoriality, we observed no aggression between group members, yet observed strong aggression toward the simulated intruder (taxidermic model). This suggests that all the birds that approached were members of the same group. Additionally, we never observed more than one adult pair responding to our STI, suggesting that only the defending territorial group responded. Distance between the STIs was greater than 200 m to ensure that we monitored different territories. If a focal male from a previous trial was observed, the trial was ended. This occurred on one occasion.

Table 1. Fluorescent label, allele range, number of alleles (A), and observed and expected heterozygosity (H_o, H_e) for 11 microsatellite loci amplified in 2 multiplex PCRs (PCR MP) for 24 adult Black-crested Titmouse. Asterisk denotes large heterozygote deficiency.

Msat locus	Fluor-label	PCR MP	Allele range	A	H_o	H_e	Reference
CtA8	Fam	2	439–459	8	0.91	0.85	Tarvin (2006)
Cuu4	Fam	2	156–175	7	0.92	0.81	Gibbs et al. (1999)
Escu6	Fam	1	110–135	11	0.83	0.90	Hanotte et al. (1994)
Mjg1	Ned	2	121–141	7	0.62	0.70	Li et al. (1997)
Pca4	Ned	1	163–184	6	0.79	0.80	Dawson et al. (2000)
Pdo5	Fam	2	234–279	12	0.75	0.81	Griffith et al. (1999)
PmaD22	Fam	1	410–485	19	0.88	0.94	Saladin et al. (2003)
PmaTGAn42	Hex	2	277–318	9	0.42	0.86*	Saladin et al. (2003)
Pocc6	Hex	2	178–210	8	0.75	0.75	Bensch et al. (1997)
Titgata02	Fam	1	223–273	14	0.83	0.93	Wang et al. (2005)
Titgata79	Hex	1	179–332	26	0.96	0.97	Wang et al. (2005)

On 6 territories, we captured 6 dyads composed of the territorial male and a resident juvenile. On the other territories only the adult male or only juveniles were caught; adult females were never caught. From each bird we collected a small blood sample (20 μL) by puncture of the brachial vein, which was stored in Queen's lysis buffer (Seutin et al. 1991) at room temperature and later in the refrigerator. DNA extraction from blood samples followed Richardson et al. (2001). In summary, 2 μL red blood cells were digested using a proteinase K solution. Proteins were removed from the solution by ammonium acetate precipitation. Supernatant was transferred to a new clean tube, and DNA was precipitated using 100% ethanol. Finally, DNA was washed with 70% ethanol to remove excess salt, and dissolved in TE (10 mM Tris, 0.1 mM EDTA).

Molecular sexing

Molecular sex of the birds was assessed using the method of Griffiths et al. (1998) and/or Van der Velde et al. (2017). PCR reactions were carried out in 10 μL volume containing 0.2 mM of each dNTP, 0.5 μM of each primer P2 and P8 (Griffiths et al. 1998) or 2602F and 2669R (Van der Velde et al. 2017), 10 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.25 U Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), and 2 μL DNA template. The PCR-program consisted of 1 min: 94 °C, 35 cycles of 94 °C for 30 s, 50 °C for 60 s (primers P2 and P8), or 60 °C for 60 s (primers 2602F and 2669R), and 72 °C for 45 s, followed by 72 °C for 2 min. PCR products were separated on a 2% agarose gel by electrophoresis and visualized by ethidium bromide staining.

Microsatellite markers and genotyping

To obtain genetic markers for paternity analyses in Black-crested Titmouse, 59 published microsatellite loci from (related) passerine species were tested in initially 8 unrelated Black-crested Titmouse individuals for amplification and polymorphism. These loci were selected because they were reported to be useful in a different titmouse species (Tufted Titmouse; Tarvin 2006), in other Paridae species (Saladin et al. 2003, Wang et al. 2005, Olano-Marin et al. 2010), or reported to have cross species utility (Dawson 2005; Dawson et al. 2010, 2013). Over 60% ($n = 36$) of the tested loci amplified and showed polymorphism. From these 36 loci, we selected 11 loci (Table 1) that showed relative high levels of polymorphism (>4 alleles in 8 individuals; see Table 1), and clear peak patterns that could be scored reliably. These 11 highly polymorphic loci were amplified in the 6 dyads (6 adult males and 6 juveniles) in 2 multiplex PCRs (see Table 1) using Qiagen MP PCR kit and manufacturer's protocol (Qiagen GmbH, Hilden, Germany). The loci were also amplified in 18 additional adults to characterize the level of polymorphism of these markers in the study population.

We separated fluorescent-labeled PCR products on an AB3730 DNA analyzer and allele sizes were automatically scored using Genemapper 4.0 software (Applied Biosystems 2005; for fluorescent-labels used see Table 1). Subsequently, genotypes

Table 2. Multi-locus microsatellite genotypes and molecular sex (M = male, F = female) for 6 adult (Age = A) and 6 juvenile (Age = J) Black-crested Titmouse from 6 different territories (Ter = Territory number). ID = band number of the bird. Numbers in the table indicate allele-sizes for the 11 microsatellite loci (? = missing data). Bold alleles indicate mismatches between the adult male and the juvenile within the same territory.

				Microsatellite loci									
ID	Ter	Age	Sex	Escu6	Pca4	PmaD22	Titgata02	Titgata79	CtA8	Cuu4	Mjg1	Pdo5	PmaTGAn42	Pocc6
244102019	26	A	M	127 131	163 184	426 466	**235 259**	**203 273**	**449 455**	163 169	139 141	238 242	302 302	198 200
244102020	26	J	F	125 127	163 175	? ?	**251 263**	**269 293**	**441 451**	163 175	139 139	238 265	302 302	198 204
244102003	28	A	M	123 127	**184 184**	418 470	**223 259**	**261 281**	**445 459**	163 169	**139 141**	**238 248**	**282 318**	**200 204**
244102002	28	J	F	123 123	**163 182**	450 470	**252 263**	**233 293**	**449 449**	163 163	**134 134**	**240 240**	**294 294**	**184 206**
244102004	30	A	M	131 135	163 175	**418 478**	259 271	**179 233**	**443 443**	**175 175**	? ?	240 271	**285 297**	200 202
244102005	30	J	M	131 137	163 163	**470 474**	231 271	**221 229**	**445 445**	**135 165**	139 139	240 269	**290 290**	202 206
244102008	33	A	M	125 133	163 175	450 485	235 247	213 233	445 449	**158 163**	134 134	240 242	**290 290**	202 204
244102009	33	J	M	133 135	163 175	450 485	235 235	233 281	445 449	**173 175**	134 134	240 240	**282 302**	198 202
258115483	34	A	M	131 133	169 175	**474 478**	**255 263**	**269 293**	455 459	158 165	**134 134**	240 248	302 302	202 202
258115482	34	J	F	127 133	175 175	**418 470**	**227 243**	**221 265**	451 455	163 165	**141 141**	240 273	297 302	198 202
244102017	47	A	M	**127 127**	**163 182**	**426 470**	**227 231**	277 301	449 449	**156 169**	134 139	240 240	285 285	200 202
244102016	47	J	F	**125 133**	**175 198**	**418 430**	**239 255**	221 277	443 449	**160 165**	132 134	240 240	302 302	198 202

of within territorial adult males and juveniles were compared. For each microsatellite locus we scored whether the adult male and offspring shared at least 1 allele. If the male and offspring did not share an allele at a specific locus then this was scored as a mismatch (microsatellite loci with bold alleles in Table 2). To be conservative, we excluded parentage between the male and juvenile if their genotypes mismatched at 2 or more loci. Although relatively rare, mismatches between genotypes for a single locus might be due to genotyping error (Magrath et al. 2009) and are therefore not reliable indicators for excluding paternity. In 2 individuals the PCR failed for a single locus (see missing data in Table 2), but this did not affect the assessment of their genetic relationship.

Results

All 6 dyads showed mismatches for 2–8 of the examined microsatellite loci (Table 2), indicating that none of these juveniles were offspring of the adult male within the same territory. Among the 6 dyads tested, 4 juveniles were female and 2 were male (Table 2).

Testing 59 microsatellites resulted in 11 highly polymorphic markers for genotyping Black-crested Titmouse (Table 1). The combined exclusion power of these 11 markers was high, 0.00015 for first and 0.0000014 for second parent (Cervus 3.0; Kalinowski et al. 2007). This statistic indicates that, if an adult–juvenile dyad is not parent and offspring, this marker-set has a >99.98% chance of detecting that, making it very suitable for paternity analyses. Locus PmaTGAn42 showed a high level of heterozygote deficiency (Table 1). This deviation from Hardy-Weinberg equilibrium might be explained by the presence of a 0-allele (non-amplifying allele) at this locus. Individuals that are heterozygote for a 0-allele and a "normal" allele at locus PmaTGAn42 are scored as homozygotes for the "normal" allele because the 0-allele is not amplified with PCR and therefore not visible, creating an excess of homozygotes for this locus (Dakin and Avise 2004; Table 1).

Discussion

We found no evidence of paternal relationship between the adult territorial male and the resident

juveniles. This was the case among 6 social groups of titmice where both the territorial male and one of the resident juveniles were sampled. This finding is in line with two genetic studies on the closely related Tufted Titmouse, both of which found that multiple juvenile group members were not the offspring of the dominant adults in the group (Pravosudova et al. 1999, Pravosudova and Grubb 2000). Taken together, these results indicate that it is not uncommon among titmice for non-kin members to join social groups, and this pattern may be more widespread across parids than previously understood.

High levels of extrapair paternity (EPP) may be a factor contributing to the lack of parent–offspring relatedness between territorial adult males and the residential juveniles. At the moment, we do not have estimates of EPP in the Black-crested Titmouse, but with the genetic markers presented here, we will be able to determine EPP in the future. A low EPP rate of 8.8% was reported in the closely related Tufted Titmouse (Pravosudova et al. 2002). If the EPP rate is similar for these closely related species, such a low rate of EPP would not account for 100% unrelatedness between the 6 randomly sampled adult–juvenile dyads in the current study. Furthermore, high divorce rates of breeding pairs could also explain the lack of relatedness between territorial adult males and juveniles. Too little is known about pair-bond longevity in titmice to assess the importance of divorce for our results. But again, a very high divorce rate in a limited time period (breeding season) would be required to explain the high level of unrelatedness in our study. As such, we argue that EPP and divorce alone are unlikely to explain the results of our study, and we suggest that juveniles immigrate to foreign territories and form non-family (or mixed kin and non-kin) social groups.

Why then would adult territorial residents allow non-kin juveniles in their territory? Under the prolonged brood care hypothesis, adults are expected to prefer offspring above non-kin juveniles in their territory (Ekman et al. 1994). In the Siberian Jay, adults are more aggressive toward non-kin juveniles than toward their own offspring in their territory, especially when resources are limited (Ekman et al. 1994). Consequently, one might expect social groups of titmice to mainly consist of kin rather than non-kin.

As the opposite pattern is observed in the Black-crested Titmouse, there must be circumstances in which the direct benefits of adopting non-kin juveniles overcome the costs (e.g., food sharing; Riehl 2013, Taborsky et al. 2016). Increased predator vigilance, reduced risk during predator mobbing, and increased foraging success have been suggested as important direct benefits of a larger group size (Brown and Hoogland 1986; Elgar 1989; Poiani 1991; Beauchamp 1998, 2008). A larger group size might also be beneficial in territory defense (Davies and Houston 1981, Brouwer et al. 2005, Mares et al. 2012). In the Black-crested Titmouse, there is evidence that the subordinates are heavily involved in territory conflicts (Borger et al. 2020) and that juveniles participate in predator mobbing (TGM, pers. obs.). Taken together these factors may be important in titmouse ecology and may therefore explain the presence of non-kin in social groups.

The presence of non-kin in social groups could also depend on the reproductive success of the dominant pair. If a pair's reproduction failed or if their reproductive success is low, they might benefit from an increased group size—and thus be more likely to allow non-kin to join their group. On the other hand, parents that managed to successfully produce multiple offspring might benefit less from allowing non-kin individuals in their group. Future work will need to take into account reproductive success of the resident pair in order to understand the complex patterns of non-kin, or mixed, social groups.

Why would juveniles join a group of non-relatives? The answer may be as simple as increased access to resources, and a safe haven in which to develop into adulthood. Additionally, juveniles may increase their chance of establishing a (higher quality) breeding territory (Riehl 2013), potentially through the process of "budding" off part of the adult's territory (Kingma et al. 2016). It has been shown in the Black-crested Titmouse that offspring sometimes obtain a breeding territory near their putative parent's territory (Rylander et al. 2020), suggesting a benefit of delayed dispersal and group formation may somehow relate to the establishment of independent territories. We might speculate that these benefits may accrue for both kin and non-kin group members. It is also possible that larger territories may afford more opportunity for group members to settle, especially if through

the mechanism of budding. These hypotheses require further study.

Our results provide evidence that unrelated juveniles do sometimes join post-breeding territorial groups in the Black-crested Titmouse. However, because we present evidence from only 6 juvenile–adult male dyads, we suggest that our results be interpreted with care. Our data indicate that 100% of juveniles we sampled were unrelated to the adult male, which suggest that a substantial fraction of juveniles in these social groups are unrelated to the adult male territory owner. However, we are not able draw firm conclusions about the prevalence of this phenomenon. Additional research is needed to understand how often unrelated juveniles are present in these territorial groups, and how many juveniles within a group are related to the adult territorial pair. We recommend that all studies on territorial groups or cooperative breeding groups consider the possibility that group membership may have mixed relatedness.

Acknowledgments

We thank Bat Conservation International and the Nature Conservancy for access to their property, and we extend our gratitude to F. Hutchins for generously supporting our research. We also thank C. Dreghorn and N. Salazar for their help with the fieldwork. We thank three anonymous reviewers for their helpful comments and suggestions on an earlier version of this paper. This research was supported by a grant from the Nikolaas Mulerius Foundation to JK, MJB, and TGM, a grant from The Groningen University Fund and the Marco Polo fund to MJB, a grant from the Bricker Ecology to TGM, and a Trinity University Murchison Summer Undergraduate Research Fellowship to LEJ. Animal use followed USDA and PHS guidelines for the ethical use of vertebrate animals in research, reviewed and approved by Trinity University (011818_TM5).

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