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EXTRA-PAIR PATERNITY RATE OF WILLOW WARBLERS ON KOLA PENINSULA

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The study of extra-pair paternity (EPP) provides valuable insights into the evolutionary and ecological factors that shape reproductive strategies in birds. The Willow Warbler *Phylloscopus trochilus* (Linnaeus 1758), a predominantly socially monogamous species, has been a focal point for EPP research; however, its geographic and interannual variability in EPP frequencies remains underexplored. In this study, we re-assessed EPP frequencies in Willow Warblers at the Luvenga outpost of the Kandalaksha Nature Reserve (southern Kola Peninsula, northwestern Russia), replicating methodologies from prior surveys conducted in 2015. In 2023, DNA samples from 104 nestlings and 54 adult individuals from 17 broods were analyzed using microsatellite markers for parentage verification. The 2023 findings revealed an unexpectedly low frequency of EPP, with only 2.9 % of chicks being extra-pair young (EPY) and 17.6 % of broods experiencing EPP. This contrasts significantly with 2015 data from the same site, where higher EPP levels were reported in Willow Warblers (37.5 % EPP frequency and 83.3% of nests containing EPY). Our results underscore pronounced interannual intraspecies fluctuation in EPP frequency within a single geographic location. These findings emphasize the importance of long-term, multi-site studies to elucidate the temporal and spatial variability of EPP in avian populations, cautioning against overgeneralization from short-term data and reinforcing the need for continued examination of both genetic and ecological drivers.

Keywords: birds; breeding biology; extra-pair paternity; molecular-genetic techniques; *Phylloscopus trochilus*

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М. В. Матанцева*, С. А. Симонов, А. Ю. Кретова. УРОВЕНЬ ЭКСТРАПАРНОГО ОТЦОВСТВА ПЕНОЧКИ-ВЕСНИЧКИ НА КОЛЬСКОМ ПОЛУОСТРОВЕ

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Изучение экстрапарного отцовства (ЭПО) предоставляет важные данные для понимания эволюционных и экологических факторов, влияющих на репродуктивные

стратегии у птиц. Пеночка-весничка *Phylloscopus trochilus* (Linnaeus, 1758), преимущественно социально моногамный вид, является важным объектом исследований ЭПО, однако географическая и межгодовая изменчивость частоты ЭПО у этого вида остается недостаточно изученной. В настоящем исследовании проведена повторная оценка частоты ЭПО у пеночек-весничек в окрестностях кордона «Лувеньга» Кандалакшского заповедника (юг Кольского полуострова, северо-запад России) с применением методик, аналогичных использованным в исследованиях 2015 года. В 2023 г. для проверки отцовства были проанализированы образцы ДНК 104 птенцов и 54 взрослых особей из 17 семей с использованием микросателлитных маркеров. Результаты 2023 года показали неожиданно низкую частоту ЭПО: экстрапарное потомство составило лишь 2,9 % от общего числа птенцов и было отмечено только в 17,6 % выводков. Эти данные существенно отличаются от результатов 2015 года для того же района, где был зафиксирован более высокий уровень ЭПО пеночки-веснички (37,5 % ЭПО и 83,3 % гнезд, содержащих экстрапарное потомство). Наши результаты подчеркивают выраженную межгодовую внутривидовую изменчивость частоты ЭПО в пределах одной географической зоны. Это свидетельствует об актуальности проведения долгосрочных исследований в разных регионах для выявления временной и пространственной изменчивости ЭПО в популяциях птиц, предостерегая от обобщений на основе краткосрочных данных и подтверждая необходимость дальнейшего изучения как генетических, так и экологических факторов, влияющих на ЭПО.

Ключевые слова: птицы; гнездовая биология; экстрапарное отцовство; молекулярно-генетические методы; *Phylloscopus trochilus*

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Introduction

The study of extra-pair paternity (EPP) is critical for understanding the evolutionary and ecological factors that shape reproductive strategies in living organisms [Bennett, Owens, 2002; Brouwer, Griffith, 2019]. Research on EPP in birds has made substantial contributions to broader evolutionary theories, such as sperm competition, sexual selection, and the maintenance of genetic diversity within populations [Petrie, Kempenaers, 1998; Neodorf, 2004]. Investigating this phenomenon reveals complex behavioral, genetic, and ecological interactions that influence reproductive success and adaptation, underscoring its significance in evolutionary biology [Arnold, Owens, 2002; Westneat, Stewart, 2003].

Despite substantial interest in this topic, interspecific variation in EPP remains understudied, highlighting the importance of assessing EPP levels across different populations within the same species [Brouwer, Griffith, 2019]. Among birds, the Willow Warbler *Phylloscopus trochilus* (Linnaeus 1758) stands out as one of the first predominantly socially monogamous species in which EPP was documented [Bjørnstad, Lifjeld, 1997; Fridolfsson et al., 1997]. However, data on EPP in Willow Warblers from various geographic regions remain sparse, with relatively few investigations conducted [Bjørnstad and Lifjeld, 1997; Fridolfsson et al., 1997; Prøven, 2005; Gil et al., 2007; Lapshin et al., 2017].

In European Russia, EPP frequency estimates have been derived from surveys carried out between 2006–2008 and 2015–2017 in the Murmansk and Pskov Regions, Karelia, and Mordovia [Lapshin et al., 2017]. These studies reported notably high EPP frequencies in the northernmost surveyed area – the Luvenga outpost within the Kandalaksha Nature Reserve on the southern Kola Peninsula (Murmansk Region) – while lower rates were observed in more southerly regions [Lapshin et al., 2017]. This geographic variation prompted the hypothesis that EPP frequency may increase toward the northern edge of the species' breeding range [Lapshin et al., 2018]. The current study aims to re-evaluate EPP frequencies in Willow Warblers at the Luvenga site using methodologies consistent with prior research [Lapshin et al., 2017], with the objective of generating supplementary data on EPP variation and facilitating interannual comparisons within the same study region.

Material and methods

The Willow Warbler *Phylloscopus trochilus* (Linnaeus, 1758) is a small passerine bird, which is considered predominantly monogamous with rare cases of polygyny and extra-pair paternity [reviewed by Lapshin et al., 2017]. The species name is given here according to the Bird Checklists of the World [Avibase..., 2019].

We carried out surveys of EPP in Willow Warblers in May – July 2023 at the Luvenga outpost of the Kandalaksha Nature Reserve (67°10′N, 32°69′E) situated on the shore of the Gulf of Kandalaksha, White Sea (southern Kola Peninsula, Northwest Russia) (Fig.). We found 19 nests in the study area. All the nests were regularly checked. We captured parent females with an nest box trap while they were feeding 7–8-day-old nestlings and putative parent males with mist nets using playback of conspecific songs. With the same method, we also trapped all other males appearing at the study plot. We took blood samples (~3μl) from all the captured adults by clipping off 2 mm tips of their claws. This technique was chosen as a safer method instead of puncturing the brachial vein – *Phylloscopus* Warbler claws fully regrow within several weeks, and such operations are not known to have ever caused negative consequences [Lapshin et al., 2017; our data]. Nestlings from 17 nests (which were not preyed) were sampled at 7–8 days of age. We collected 1–2 growing greater coverts as samples from nestlings. The samples were stored in centrifuge tubes with 0.5 ml of 75 % Ethanol in a refrigerator at 4 °C for 1–2 weeks. After field work, the samples were stored in the laboratory at – 20 °C. A total of 158 individuals were used in parentage analyses: 104 chicks and 54 adults (17 females and 37 males).

DNA was isolated using Proteinase K digestion and extracted using DNA-Extran-2 reagent kit (NPK Syntol). Three Willow Warbler microsatellites (Table) were used for paternity exclusion and assignment: Phtr1, Phtr2, and Phtr3 [Fridolfsson et al., 1997]. PCR amplifications were performed on a Maxygene thermal cycler (Axygene, USA). Screen Mix-HS reaction mixture (Eurogen, Russia) of 25μl volume contained 5 μl of PCR mix, 1 pmol of forward and reverse primers (Table), and ~50 ng of DNA. The primer annealing temperature was +55 °C.

The PCR products were separated in 8 % polyacrylamide gel with Tris-acetate buffer. Putative parents were run with their nestlings on the same gel. Phtr3 is sex-linked in the Z chromosome, so females have only one allele. However, we could distinguish between male homozygotes and females by sexing the chicks using a sex-linked gene CHD [Griffiths et al., 1998]. The amount of extra-pair paternity was estimated in PROBMAX program [Danzmann, 1997]. We also tried to reveal the paternity of other than the social male by searching the genotype database for males that could be

Kola Peninsula: asterisk – study area

consistent with the paternally inherited genotypes of the given extra-pair nestling.

Results and discussion

We sampled parents and nestlings of17 broods to identify extra-pair young (EPY). Of these broods, 17.6 % contained EPY (3 out of 17, in all cases one EPY in the nest), and only 2.9 % of all chicks were EPY (3 out of 104). No instances of intra-specific brood parasitism, where offspring were unrelated to both parents, were observed. Additionally, we found no evidence of kinship between EPY and other tested males.

In contrast, surveys conducted in the same area using identical methods in 2015 showed a notably higher EPP level among Willow Warblers, with a 37.5 % EPP frequency and 83.3 % of nests containing EPY [Lapshin et al., 2017]. Comparatively lower proportions of families with EPY were documented in Karelia and the Pskov Region (Northwest Russia) – 67–68 %, and in Mordovia (central European Russia) – 75 %. The EPP frequencies were reported as 15.3 % in Karelia, 11.8 % in the Pskov Region, and 18.4 % in Mordovia [Lapshin et al., 2017]. Furthermore, one instance of intra-specific brood parasitism was detected in both Karelia and Mordovia [Matantseva et al., 2021]. Similar to our 2023 results, no fathers of the EPY were identified among the males tested [Lapshin et al., 2017; Matantseva et al., 2021].

In Europe, the first attempt to detect EPP in a Swedish Willow Warbler population found no EPY at all [Gyllensten et al., 1990]. The absence of EPY could have resulted from either a genuinely low EPP level or from the limited sensitivity of earlier methodologies. Since the advent of species-specific genetic markers [Fridolfsson et al., 1997], also employed in this study, EPP detection rates in European populations have generally been higher. Recorded EPP frequencies range from 24 % to 28 % in Sweden and 33 % to 40 % in Norway, with 47–58 % of nests containing EPY [Bjørnstad, Lifjeld, 1997; Fridolfsson et al., 1997; Prøven, 2005; Gil et al., 2007].

Variability in EPP levels within species across different regions and years has also been documented in other bird populations, although the underlying causes remain insufficiently understood [Brouwer and Griffith, 2019]. The factors contributing to the low EPP levels observed in the studied population in 2023 are not yet clear. EPP rates are primarily associated with population density [Westneat, Sherman, 1997], breeding synchrony [Stutchbury, Morton, 1995; Brouwer, Griffith, 2019], and sex ratio within the population [Lapshin et al., 2017]. However, data on these relationships can be contradictory [Griffith et al., 2002; Brouwer, Griffith, 2019]. In our study, these parameters were comparable between 2015 and 2023, yet the EPP estimates differed significantly. This discrepancy indicates that additional factors, such as environmental changes, individual variation in mate choice, mate-guarding behaviors, parental investment strategies, or changes in genetic compatibility, may also play a role in influencing EPP rates [e.g., Hamilton, 1990; Tregenza, Wedell, 2000; Arnold, Owens, 2002; Arct et al., 2015]. While our 2023 data are limited in assessing the specific factors that drive EPP variation, they offer additional evidence of the significant variability in EPP within this species.

Conclusions

In 2023, we reassessed the frequency of extra-pair paternity (EPP) in Willow Warblers (*Phylloscopus trochilus*) at the Luvenga outpost on the southern Kola Peninsula and compared these results to data collected in 2015 at the same site. Our analysis revealed an unexpectedly low EPP frequency in 2023, markedly lower than the levels observed in 2015 for this population and below those reported from other regions of Euro-

Primer sequences (F – forward, R – reverse) and other characteristics of the microsatellite lociused in the study [Fridolfsson et al., 1997]

Locus	Primer sequences 5'…3'	Repeat motif	Access number in NCBI*
Phtr1	F: CTGGGAGAAGACTCTAAGCCTT R: CTACTTTTTAATGTGAGATCCAAACT	(CA)9	AM056069.1
Phtr2	F: CGCAGGCTCAGAAATACTTGA R: GCCCACAGCTCAATAGTCTT	(CA)12	EF621527.1
Phtr3	F: ATTTGCATCCAGTCTTCAGTAATT R: CTCAAAGA AGTGCATAG AGATTTCAT	(CA)23	AM056070.1

Note. * NCBI – National Center for Biotechnology Information.

pean Russia and Northern Europe. These findings highlight that EPP rates within a species can demonstrate substantial interannual variability within the same region, as well as across different geographic locations. Consequently, researchers should interpret EPP data with caution when based on short-term studies. This study underscores the critical need for longitudinal research across multiple sites to gain a comprehensive understanding of the temporal and spatial dynamics of EPP in avian populations.

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