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Genetic Isolation of a *Luscinia luscinia* population (Aves: Muscicapidae) in Eastern Hungary

***Dávid Kováts*[1], *Zoltán Ács*[2]**

[1]Department of Evolutionary Zoology and Human Biology, University of Debrecen, 4010 Debrecen, Egyetem tér 1., Hungary; E-mail: david.kovats@gmail.com
[2]Vénic Nature Conservation Foundation, 8400 Ajka, Tölgyfa u. 3. Hungary

Abstract: The Carpathian basin is located on the south-western edge of the geographical range of *Luscinia luscinia*. Until recently, this species has only been documented in the north-eastern part of Hungary (specifically in the Upper-Tisza region), where a small breeding population formerly occurred. In this study, we analysed mitochondrial DNA sequences of 30 individuals sampled at five sites along river Bódva and river Tisza via Szatmár-Bereg Landscape Protection Area (LPA) to Bátorliget. The mitochondrial cytochrome *c* oxidase subunit I (COI) fragments were amplified and sequenced directly, and isolated haplotype of *L. luscinia* was distinguished from the easternmost portion of Hungary (Vámosatya, Bockerek forest, 48°11'N, 22°23'E). The new paraphyletic clade suggests a genetically well isolated population of *L. luscinia* in eastern Hungary.

Key words: mt COI gene, phylogeny, *Luscinia luscinia,* Vámosatya

Introduction

In the region of the Carpathian basin, two closely related nightingale species occur. The common nightingale (*Luscinia megarhynchos*) is widely distributed in the western Palaearctic (Cramp 1992). The thrush nightingale (*L. luscinia*) is frequently found in north-eastern Europe (e.g. Poland), and the Carpathian basin is situated in the south-western edge of the geographical range of the species (Moreau 1972, Cramp 1992).

In Hungary, *L. megarhynchos* is very frequent in ecotones of woodlands of bushy areas and usually occurs in high numbers in wetlands and dry habitats. *L. luscinia* has special habitat requirements and prefers wet low-laying areas with rather patchy vegetation, especially riverine forests close to large rivers or oxbow lakes (Sorjonen 1986). This species is a regular migrant but rarely nesting in north-eastern Hungary (Schmidt 1986, Kováts 2012). In this region, the two species occur in a narrow overlapping zone (Reifová *et al*. 2011). Until recently, only a few pairs of *L. luscinia* were found in the Upper-Tisza region (between Tiszabercel and Tiszatelek); however, its population decreased strongly in the last decades, probably due to habitat fragmentation or elimination of coppices and undergrowths (Schmidt 1986, Kováts *et al*. in press). However, we have no information about the quantitative aspects of co-occurrence of these two congeneric nightingale species.

Phylogenetic relationships of *L. luscinia* and *L. megarhynchos* have not been investigated in Hungary. The aim of our study is to provide molecular characterisation of their breeding populations and to determine their phylogenetic relationships.

Materials and Methods

Study sites and data collecting

Our field work was carried out in study sites at river Bódva (48°27'N, 20°43'E) [circle No. 1 on

Fig. 1], the floodplain areas of the Upper-Tisza Region [(Kesznyéten Landscape Protected Area (LPA) 48°01'N, 21°06'E [circle No. 2a on Fig. 1] and between Tiszabercel and Tiszatelek (48°10'N, 21°42'E) [circle No. 2b on Fig. 1], the Szatmár-Beregi LPA (Bockerek-forest, near Vámosatya, 48°11'N, 22°23'E) [circle No. 3 in Fig. 1] and Bátorliget (47°45'N, 22°26'E) [circle No. 4 in Fig. 1]. In general, the vegetation is quite diverse, formed by soft-wood riparian forests (*Salicetum albae-fragilis*).

Data were collected from early May to mid-June between 2006 and 2010. In each site, only Ecotone® mist-nets (dimensions of 2.5 × 7 and 12 m) with tape luring were used. Birds were caught within the peak of the breeding season, except individuals with the GenBank accession numbers of JQ740231, JQ740233 and JQ740246, which were migrants. New sequences were deposited in the GenBank. All scientific names, sample codes, GenBank accession numbers and locations are given in Table 1. After handlings, birds were released back to their territories.

DNA extraction, amplification and COI sequencing

In total, 30-40 µl blood was collected from the brachial vein of 30 individuals of *L. luscinia* and *L. megarhynchos*. All blood samples were deposited in 1.5-2 cm^3 microtest tubes in ethanol during field surveys and were deep-frozen at -20°C. For purification, Qiagen Blood & Tissue DNA kit (Cat. No. 69504) was used. To use the kit, the blood samples were alcohol discharged. The samples were centrifuged at 6000 rpm and the supernatant alcohol was drawn off using a pipette. The centrifuge tubes were left open and the samples were allowed to dry for 24 hours at room temperature to ensure that the remaining ethanol was completely evaporated from the blood samples. The dry blood samples were suspended in 1× PBS to provide a sample suitable for the Qiagen DNA purification kit. Clumped blood pieces were triturated using a single-use polypropylene homogenizer which fits onto the end of the Eppendorf tubes. The final elution step was performed using 150 µl purified H_2O at pH 7. The purified DNA samples were then stored at -20°C. A PCR procedure was used to amplify the DNA strands and the phylogenetic origin of the individuals was determined based on the analysis of the cytochrome-*c* oxidase subunit I (COI) sequences. Gene frequencies of each subgroup were determined, and differences between the marker gene sequences and the mtDNA sequences of the sample groups were identified. The mtDNA bar coding based on the COI gene is considered a successful approach to a broad range of taxa (Hebert *et al.* 2004, Hogg, Hebert 2004, Johnson, Cicero 2004, Tavares, Baker 2008). For the PCR amplification of the 5' region of COI, primers described by Kerr *et al*. (2009) were used. Forward primer: BirdF1 (5'-TTCTCCAACCACAAAGACATTGGCAC-3'), reverse primer: COIbirdR2 (5'-ACGTGGGAGA-TAATTCCAAATCCTGG-3'); 1 µl of each 150-µl elution mixture was used for the COI PCR reaction.

Each reaction was matched in 25 µl blood sample as follows: 10*PCR puffer (Fermentas) 2.5 µl, Mg_2Cl (25mM) 1.2 µl, dNTP (2.5 mM) 2 µl, BirdF1 (10 pmol/µl) 0.6 µl, COIbirdR2 (10 pmol/µl) 0.6 µl, Taq DNS polymerase (Fermentas) 0.25 µl (1 U), mtDNS templat 1 µl. With each PCR reaction, Peqlab-gradient PCR appliance was used based on the temperature program of the following process: initial denaturation at 94°C for 2 min and 40 cycles: at 94°C for 1 min, at 54-62°C for 50 sec and at 72°C for 2 min. The final elongation was at 72°C for 5 min. The annealing temperature was often modified depending on the mtDNA samples (54°C to 62°C). In each case, negative controls were prepared without the addition of the template mtDNA. The success of the PCR reactions was confirmed on 1.5% agarose gels (GIBCO); 2 µl of each PCR mixture was loaded to the agarose gels, which were stained with ethidium bromide and photographed under UV light using an UVP gel documenting system. From the PCR products giving a single band when subjected to gel electro-

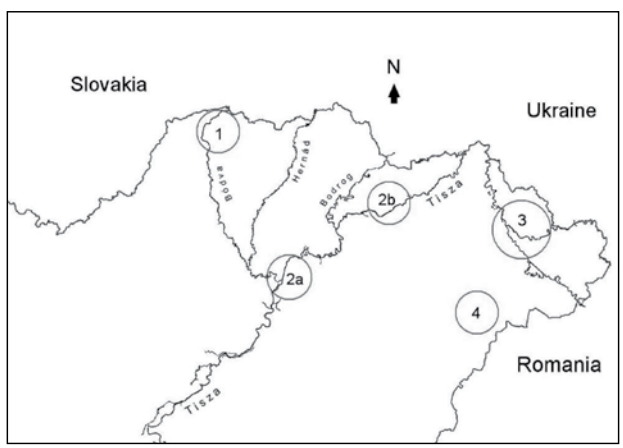

Fig. 1. Location of the study areas (1: river Bódva, 2: Upper-Tisza region (2a: Kesznyéten LPA, 2b: Tiszabercel-Tiszatelek, 3: Szatmár-Beregi LPA, 4: Bátorliget)

Table 1. Complete list of sequenced individuals and museum specimens examined in this study. The scientific names, museum labels or ring numbers, GenBank accession numbers and locality of the collection are referred.

Species	Specimen label	GenBank accession No. (COI)	Locality
Luscinia luscinia	UWBM49179	GQ482129	Moscow, RU
L. luscinia	UWBM49577	GQ482128	Moscow, RU
L. luscinia	UWBM49411	GQ482130	Sverdlovska, RU
L. luscinia	UWBM49514	GQ482131	Sverdlovska, RU
L. luscinia	UWBM74235	GQ482132	Kirov, RU
L. luscinia	UWBM59669	GQ482133	Smolensk, RU
L. luscinia	ZMMU 59a	GQ482134	Kaliningrad, RU
L. luscinia	NRM20026317	DQ683476	Malmon, SW
L. luscinia	BISE-Aves392	GU571964	Orebro, SW
L. luscinia	NHMO-BC40	GU571473	Telemark, NO
L. luscinia	NHMO-BC39	GU571474	Telemark, NO
L. luscinia	AE80017	JQ740221	Vámosatya, E-HU
L. luscinia	AE36813	JQ740231	Tiszadob, NE-HU
L. luscinia	AE49320	JQ740233	Tiszadob, NE-HU
L. luscinia	AE26575	JQ740246	Szalonna, N-HU
L. megarhynchos	UWBM64638	GQ482135	Krasnodar, RU
L. megarhynchos	UWBM46491	GQ482136	Alma-Ata, KA
L. megarhynchos	UWBM61111	GQ482137	Krasnodar, RU
L. megarhynchos	MIUT200359	DQ683477	Bazangan, IR
L. megarhynchos	USNM: Drov. 3745	JQ175292	MC
L. megarhynchos	USNM: Drov. 3733	JQ175293	MC
L. megarhynchos	AE36868	JQ740216	Perkupa, N-HU
L. megarhynchos	AE36874	JQ740217	Tornanádaska, N-HU
L. megarhynchos	AE36876	JQ740218	Tornanádaska, N-HU
L. megarhynchos	AE36879	JQ740219	Szögliget, N-HU
L. megarhynchos	AE36881	JQ740220	Szögliget, N-HU
L. megarhynchos	AE80019	JQ740222	Tarpa, E-HU
L. megarhynchos	AE80023	JQ740223	Tivadar, E-HU
L. megarhynchos	AE80027	JQ740224	Fehérgyarmat, E-HU
L. megarhynchos	AE80035	JQ740225	Bátorliget, E-HU
L. megarhynchos	AE80128	JQ740226	Tiszabercel, NE-HU
L. megarhynchos	AE80014	JQ740227	Tiszalúc, NE-HU
L. megarhynchos	AE36900	JQ740228	Tiszalúc, NE-HU
L. megarhynchos	AE36899	JQ740229	Tiszalúc, NE-HU
L. megarhynchos	AE36898	JQ740230	Tiszalúc, NE-HU
L. megarhynchos	AE80127	JQ740234	Tiszabercel, NE-HU
L. megarhynchos	N115813	JQ740235	Tiszalúc, NE-HU
L. megarhynchos	AE36889	JQ740237	Tiszalúc, NE-HU
L. megarhynchos	AE36892	JQ740238	Tiszalúc, NE-HU
L. megarhynchos	AE36896	JQ740239	Tiszalúc, NE-HU
L. megarhynchos	AE44802	JQ740240	Tiszabercel, NE-HU
L. megarhynchos	AE80018	JQ740242	Vámosatya, E-HU
L. megarhynchos	AE36781	JQ740243	Perkupa, N-HU
L. megarhynchos	AE36814	JQ740244	Tiszadob, NE-HU
L. megarhynchos	AE80022	JQ740245	Tarpa, E-HU
L. megarhynchos	AE80024	JQ740247	Tivadar, E-HU
L. megarhynchos	AE36870	JQ740248	Perkupa, N-HU
L. brunnea	USNM 620607	JQ175290	Chin, MM
L. brunnea	USNM 620595	JQ175291	Chin, MM
L. calliope	UWBM 44150	GQ482112	Kamchatka, RU
L. calliope	UWBM 51743	GQ482113	Krasnoyarsk, RU

Table 1. Continued.

Species	Specimen label	GenBank accession No. (COI)	Locality
L. calliope	UWBM 47214	GQ482114	Khabarovsk, RU
L. calliope	UWBM 52532	GQ482115	Magadansk, RU
L. calliope	UWBM 73298	GQ482116	Irkutsk, RU
L. calliope	UWBM 59869	GQ482117	Dornod, MO
L. calliope	ZMMU RYA 1681	GQ482118	Sopochnoe lake, RU
L. calliope	MMU RYA 1682	GQ482119	Sopochnoe lake, RU
L. calliope	ZMMU RYA 1680	GQ482120	Sopochnoe lake, RU
L. calliope	ZMMU RYA 1658	GQ482121	Sopochnoe lake, RU
L. cyane	UWBM 47130	GQ482122	Khabarovsk, RU
L. cyane	UWBM 74757	GQ482123	Primorskiy Kray, RU
L. cyane	UWBM 52522	GQ482124	Magadansk, RU
L. cyane	UWBM 51739	GQ482125	Krasnoyarsk, RU
L. cyane	UWBM 46940	GQ482126	Sakhalinsk, RU
L. cyane	UWBM 59709	GQ482127	Dornod, MO
L. sibilans	UWBM 47493	GQ482138	Sakhalin, RU
L. sibilans	UWBM 44562	GQ482139	Kamchatka, RU
L. sibilans	UWBM 78240	GQ482140	Irkuts, RU
L. sibilans	UWBM 47106	GQ482141	Khabarovsk, RU
L. sibilans	KRIBB338	EF515794	KR
L. svecica	UWBM 74242	GQ482142	Kirov, RU
L. svecica	UWBM 59422	GQ482143	Labytnangi, RU
L. svecica	UWBM 49697	GQ482144	Murmansk, RU
L. svecica	UWBM 75800	GQ482145	Tyva, RU
L. svecica	UWBM 44132	GQ482146	Chukotskiy Avtonomnaya, RU
L. svecica	ZMMU RYA 1926	GQ482147	Tormanskoe swamp, RU
L. svecica	ZMMU RYA 1927	GQ482148	Tormanskoe swamp, RU
L. svecica	UWBM 67624	DQ433776	Tyva, RU
L. svecica	UWBM 44078	DQ433777	Kamchatka, RU
L. svecica	NHMO-BC477	GU571475	Oppland, NO
L. svecica	NHMO-BC478	GU571476	Oppland, NO
L. svecica	USNM 608996	DQ433005	Lappland, SW
L. svecica	BISE-Aves310	GU571965	Norrbotten, SW
L. svecica	BISE-Aves157	GU571966	Norrbotten, SW
Ficedula albicollis	UWBM 49299	GQ481892	Kursk, RU
F. albicollis	UWBM 49388	GQ481893	Kursk, RU
F. albicollis	UWBM 49425	GQ481894	Kursk, RU
F. hypoleuca	UWBM 49352	GQ481896	Kursk, RU
F. hypoleuca	ZMMU 10a	GQ481897	Kaliningrad, RU
F. hypoleuca	UWBM 49395	GQ481898	Kursk, RU
F. hypoleuca	UWBM 49648	GQ481899	RU
F. hypoleuca	UWBM 61029	GQ481901	RU
F. hypoleuca	NHMO-BC494	GU571395	Oslo, NO
F. hypoleuca	NHMO-BC493	GU571396	Oslo, NO
F. semitorquata	UWBM 61130	GQ481913	Krasnodar, RU
F. semitorquata	UWBM 61175	GQ481914	Akhmetovska, RU
F. semitorquata	UWBM 64706	GQ481915	Krasnaya Polyana, RU
Monticola gularis	UWBM59864	GQ482168	New-Barag, MO

Abbreviations: KA: Kazakhstan, KR: Korea, MC: Macedonia, MM: Myanmar, MO: Mongolia, E-HU: eastern Hungary, NE-HU: north-eastern Hungary, N-HU: northern Hungary, IR: Iran, RU: Russia, SW: Sweden, NO: Norway; specimen labeled of „AE" or „N" are numbered aluminum rings (after ringing and measurement, birds were released back into the wild).

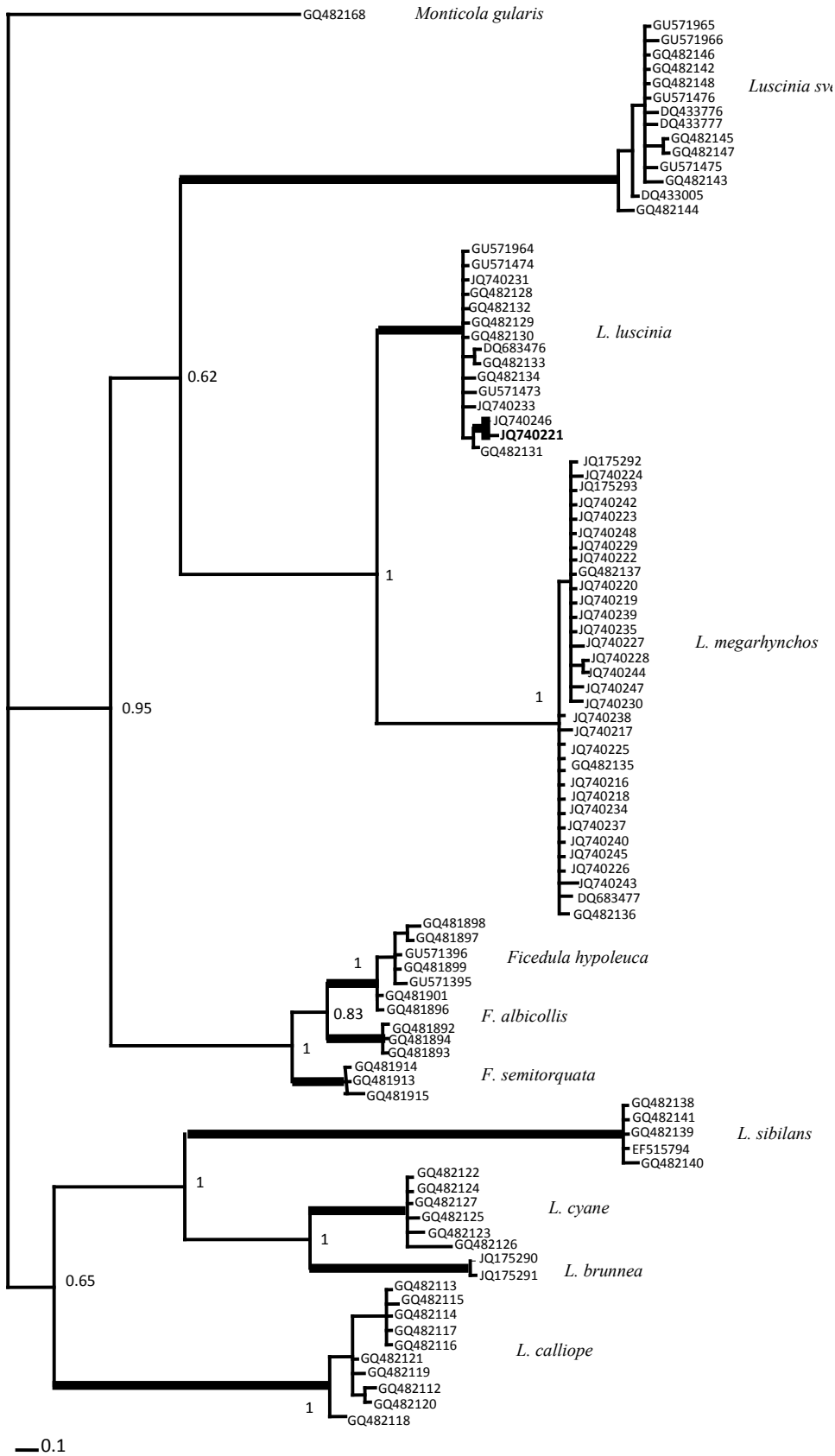

Fig. 2. Patterns of sequence divergences of different nightingale populations using *Ficedula* species and *Monticola gularis* as outgroups based on cytochrome-*c* oxidase subunit I gene (COI) sequences set up by MrBayes algorithm. Only the posterior probabilities of 50% are shown in the dendrogram. GenBank accession numbers and species identification are given to the right of each clade. The new paraphyletic clade of *L. luscinia* (JQ740221) is bolded.

phoresis, COI gene fragments were purified using the SAP-ExoI method. To 23 µl of the PCR mixture, 1 µl Shrimp Alkaline Phosphatase (USB) and 1.5 µl 10× diluted Exonuclease I (USB) was added, incubated for 45 minutes at 37°C, and then for 15 minutes at 94°C to inactivate the enzymes. COI fragments giving multiple bands when subjected to gel electrophoresis were excised from the gels and purified using the Qiagen Gel Extraction kit. The final elution step was performed using 20 µl H_2O. The purified COI fragments were sequenced by the sequencing laboratory of Macrogen in Amsterdam using the BigDye cyclic sequencing (Applied Biosystems). In each case, mtDNA was sequenced from both sides using the above-mentioned BirdF1 and COIbirdR2 primers. The electropherograms from the DNA sequencer were analyzed using the Bioedit software program. For each forward and reverse base sequence from the COI fragment sequencing, consensus sequences were generated using the ClustalX v1.83 software program. Sequences from previous studies (Yoo *et al.* 2006, Aliabadian *et al.* 2007, Kerr *et al*. 2007, Kevin *et al*. 2009, Schindel *et al*. 2011,) were downloaded from GenBank and compared with our sequences. All scientific names, sample codes, GenBank accession numbers and locations are given in Table 1. The closely related *Luscinia* species such as: *L. brunnea*, *L. calliope*, *L. cyanea*, *L. sibilans* and *L. svecica*, three *Ficedula* species (*F. semitorquata, F. albicollis* and *F. hypoleuca*) as well as *Monticola gularis* were used as outgroups. To set up their phylogenetic relationships, MEGA5 software program (Tamura *et al.* 2011) and MrBayes 3.2.1 program (Ronquist, Huelsenbeck 2003) were used.

Results and Discussion

In this study, we investigated a 663 bp long part of the COI gene of 98 individuals belonging to 11 species. Our molecular results can be divided into two parts. Firstly, the phylogenetic analysis revealed that interspecific genetic distance between the haplotypes of *L. luscinia* and *L. megarhynchos* was 5.5%. The intraspecific variability was 0.0020 in *L. luscinia,* while it was slightly lower, 0.0016, in the case of *L. megarhynchos*. Based on the phylogenetic patterns, haplotypes of *L. megarhynchos* indicate a genetically and geographically consistent population, even if some marker sequences of Eastern Europe are interspersed in the current subdivision. Further, the general pattern of haplotype differentiation suggests that *L. megarhynchos* is currently more common in the Upper-Tisza region (Fig. 2), while no individuals of *L. luscinia* were found in this area. Its population probably decreased due to general habitat loss as it was assumed previously (Schmidt 1986), but more ecological investigations are needed to verify this hypothesis.

Secondly, isolated haplotype of *L. luscinia* (GenBank accession number JQ740221, see Fig. 2) was distinguished from the easternmost portion of Hungary (Vámosatya, Bockerek forest, 48°11'N, 22°23'E). The current paraphyletic clade suggests a genetically well isolated population of *L. luscinia* in eastern Hungary. It represents a new population of *L. luscinia* in eastern Hungary (Szatmár-Bereg Landscape Protection Area, Vámosatya, Bockerek-forest 48°11'N, 22°23'E), where the species has not previously been documented. This haplotype was unambiguously different from other clades and revealed as a paraphyletic lineage in this respect. However, future field work is needed which directly compares the morphometrical characteristics of other populations and the newly found population reported in this study. We leave the question open, what types of impacts caused the *L. luscinia* population to expand in the eastern portion of Hungary?

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