

The first record of a female hybrid between the Common Nightingale (*Luscinia megarhynchos*) and the Thrush Nightingale (*Luscinia luscinia*) in nature

Radka Reifová · Pavel Kverek · Jiří Reif

Received: 12 September 2010/Revised: 11 March 2011/Accepted: 27 April 2011/Published online: 17 May 2011
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Abstract Understanding the mechanisms causing reproductive isolation between incipient species can give important insights into the process of species origin. Here, we describe a record of a hybrid female between two closely related bird species, the Common Nightingale (*Luscinia megarhynchos*) and the Thrush Nightingale (*L. luscinia*). These species are separated by incomplete prezygotic isolation, and the occurrence of fertile hybrid males in nature has been documented before. Our record represents the first genetically confirmed evidence of the occurrence of hybrid females between these two species in nature. Although the hybrid female was captured in the peak of the breeding season in suitable habitat, it did not show any sign of reproductive activity, suggesting that it was sterile. This observation together with previous

experimental work in captivity suggests that hybrid female sterility plays an important role in reproductive isolation in nightingales. Unexpectedly, the hybrid female had highly developed fat reserves and was in the process of moulting, which in nightingales normally occurs approximately 1 month later. This unusual moult pattern could contribute to lower fitness of hybrid females and thus to speciation in nightingales.

Keywords *Luscinia megarhynchos* · *Luscinia luscinia* · Hybridization · Hybrid sterility · Bird moult

Zusammenfassung Die Mechanismen zu verstehen, die zu reproduktiver Isolation werdender Arten führen, kann wichtige Einblicke in den Prozess der Artentstehung gewähren. Hier beschreiben wir den Nachweis einer weiblichen Hybriden von zwei eng verwandten Vogelarten, der Nachtigall (*Luscinia megarhynchos*) und dem Sprosser (*L. luscinia*). Diese Arten sind durch unvollständige präzygotische Isolation getrennt, und fruchtbare Hybridmännchen sind bereits zuvor in der Natur dokumentiert worden. Unser Nachweis stellt den ersten genetisch abgesicherten Beleg für das Auftreten weiblicher Hybriden dieser beiden Arten in der Natur dar. Obwohl das Hybridweibchen zum Höhepunkt der Brutsaison in geeignetem Habitat gefangen wurde, zeigte es keinerlei Anzeichen reproduktiver Aktivität, was darauf hindeutet, dass es steril war. Diese Beobachtung deutet nebst früheren experimentellen Arbeiten in Gefangenschaft darauf hin, dass weibliche Hybridsterilität eine wichtige Rolle für die reproduktive Isolation von Nachtigallen spielt. Unerwarteterweise hatte das Hybridweibchen sehr gut entwickelte Fettreserven und befand sich in der Mauser, die bei Nachtigallen normalerweise etwa einen Monat später beginnt. Dieses ungewöhnliche Mausermuster könnte zur

Communicated by T. Friedl.

Sequence data from this article have been deposited with the GenBank Data Libraries under accession nos. HQ191443-HQ191473.

R. Reifová (✉)
Department of Zoology, Faculty of Science,
Charles University in Prague, Prague, Czech Republic
e-mail: radkas@natur.cuni.cz

P. Kverek
Vilová 246, 294 02 Kněžmost, Czech Republic

J. Reif
Faculty of Science, Institute for Environmental Studies,
Charles University in Prague, Prague, Czech Republic

J. Reif
Department of Zoology and Laboratory of Ornithology,
Faculty of Science, Palacky University Olomouc,
Olomouc, Czech Republic

niedrigeren Fitness von Hybridweibchen und daher zur Artbildung bei Nachtigallen beitragen.

Introduction

The nature of reproductive isolation between closely related species provides important information about the selective forces driving the species origin. In birds, speciation research has focused mainly on prezygotic barriers to gene flow including mating behavior and species recognition systems (reviewed in Price 2008). Mechanisms of postzygotic isolation are much less well understood. According to Haldane's rule (Haldane 1922), postzygotic barriers such as hybrid sterility and reduced viability preferentially affect the heterogametic sex, i.e. the females in birds. The proximate causes that are responsible for this female-specific fitness reduction are, however, largely unknown.

Naturally occurring hybrid zones between recently diverged species provide a unique opportunity to study the mechanisms of postzygotic isolation in birds (Barton and Hewitt 1985; Randler 2006). The reduced fertility or inviability of hybrid females has been observed, for example, in the flycatchers *Ficedula hypoleuca* and *F. albicollis* (Gelter et al. 1992; Sætre et al. 2001) and crows *Corvus corone* and *C. cornix* (Saino and Bolzern 1992; Saino and Villa 1992). In addition, molecular data suggest that female-specific hybrid sterility or inviability might also occur in other bird species such as the buntings *Passerina amoena* and *P. cyanea* (Carling and Brumfield 2008) or the chiffchaffs *Phylloscopus collybita* and *P. ibericus* (Helbig et al. 2001). Besides hybrid sterility and inviability, some other forms of postzygotic reproductive barriers have been observed in avian hybrid zones. In the crow hybrid zone, for example, hybrid females lay eggs with thin shells, making the eggs susceptible to breakage (Saino and Villa 1992). In addition, crow hybrids show lower flight initiation distances than both parental species, suggesting that they might be at higher predation risk (Randler 2008). Detailed descriptions of hybrid phenotypes could shed more light into the proximate causes that are responsible for the reduced fitness of avian hybrids.

Two closely related species of nightingales, the Common Nightingale (*Luscinia megarhynchos*) and the Thrush Nightingale (*L. luscinia*), are a suitable model system for studying the mechanisms of postzygotic reproductive isolation in birds. These species diverged approximately 1.8 Mya and today their breeding distributions overlap in central and eastern Europe (Storchová et al. 2010). Despite their overall morphological and ecological similarity (Cramp 1988), they can be clearly distinguished by slightly

different body size, wing feather characteristics, plumage coloration and song (Svensson 1992). The two nightingale species also differ in the timing of migration and annual reproductive cycle (Hudec 1983; Stadie 1991), which is likely caused by different wintering places in Africa for both species, as well as the fact that the overall breeding distribution is more northerly in *L. luscinia* (Cramp 1988; Hagemeyer and Blair 1997). In central Europe, *L. megarhynchos* arrives from the wintering grounds and starts to breed approximately 1 week earlier than *L. luscinia* (Stadie 1991). The incubation period is equally long in both species; however, *L. luscinia* fledglings leave the nest 1 or 2 days younger compared to *L. megarhynchos* (Hilprecht 1965; Stadie 1991). The complete moult takes around 45 days in *L. megarhynchos*, but only 30 days in *L. luscinia* (Stresemann and Stresemann 1966; Berger 1967; Ginn and Melville 1983).

In sympatry, nightingales occasionally hybridize and produce viable hybrid progeny, which can be recognized according to their intermediate morphology (Stadie 1983; 1991; Becker 1995). Morphological as well as genetic data suggest that approximately 3–5% of males in sympatry are hybrids (Becker 2007; Reifová et al., unpublished). Hybrid males are fertile and can reproduce with either of the parental species (Becker 2007). In a few cases, morphologically intermediate individuals with unknown sex showing no reproductive activity have been observed in nature (Stadie 1983, 1991). It has been suggested that these individuals could be sterile hybrid females. However, since nightingales are not sexually dimorphic and the sex can be determined only in reproductively active individuals (Cramp 1988), genetic evidence is needed to confirm this assumption.

In this study, we describe the record of a nightingale hybrid lacking any signs of reproductive activity. Genetic analyses confirmed the hybrid status of the individual and showed that it was a female. We describe its phenotype and discuss what it can tell us about the mechanisms of postzygotic reproductive barriers in nightingales.

Methods and results

Description of the record

During regular field work studying breeding nightingales, on 3 June 2009 we captured, using a collapsible trap, a nightingale that phenotypically resembled *L. megarhynchos* in some traits and *L. luscinia* in others. The bird was found near the town Mladá Boleslav in central Bohemia, Czech Republic (geographic coordinates: 50°24'39"N, 14°56'14"E), at a locality with dense shrubby vegetation consisting mainly of *Cornus mas*, *Acer* sp. div., *Ulmus laevis*,

Syringa vulgaris, *Symphoricarpos albus* and *Rosa canina*. The area falls within the breeding range of *L. megarhynchos* (Hagemeijer and Blair 1997; Štastný et al. 2006), although very rarely single *L. luscinia* individuals have been observed in this region (Kverek 1998; Štastný et al. 2006). During the last few years, several nightingales with intermediate phenotype have been reported in this region (Kverek 2002, 2007; Kverek et al. 2008). These cases were all males, as could be recognized by the raised cloaca, and most of them expressing singing activity. Genetic analysis performed in one of these individuals confirmed its hybrid origin (Kverek et al. 2008).

The individual captured on 3 June 2009 had morphological characteristics very similar to those of previously captured hybrids in this area. It showed a reduced tenth primary (3 mm shorter than the longest primary covert) and the absence of emargination on the outer vane of the seventh primary, which are typical traits for *L. luscinia* (Svensson 1992). However, an unspotted breast and lower tail coverts, as well as a shorter ninth primary compared to the seventh primary, were consistent with diagnostic traits for *L. megarhynchos* (Svensson 1992). According to the absence of pale patches on the tips of the greater coverts, the relatively large width of these coverts as well as the large width and rounded tips of the tail feathers, we classified its age as being older than the second calendar year.

Surprisingly with respect to the date of the capture, when the peak of the breeding season normally occurs in nightingales, the hybrid individual was in the process of moulting (Fig. 1). On both wings, the first three primaries, which normally initiate the regular moult, were new feathers growing from follicles, while the fourth to tenth primaries had not yet been replaced. On the right wing, the fifth secondary had also been replaced by a new feather. No secondaries were moulting on the left wing. The bird also showed a few new feathers on its belly. From the length of the growing primaries, we estimated that the moult had started approximately 6 days before we captured the bird (on 28 May). Interestingly, the bird also had highly developed fat reserves (the furcula was completely covered by fat, and the belly was almost completely covered). Nightingales normally gather fat reserves immediately before the onset of autumn migration (Stadie 1991), and fat gathering sometimes overlaps with the last stages of the moult process (Pavel Kverek, own unpublished data).

Unlike the hybrids previously captured within the Mladá Boleslav area, this individual did not show any reproductive activity—the brood patch was completely absent and the cloaca was not raised. Identification of its sex was thus impossible in the hand. The individual was caught in the territory of a male singing a typical *L. megarhynchos* song.

This male approached within close proximity and called with a contact voice when we were taking measurements of the hybrid. After releasing the hybrid, the male actively followed it and sang. However, the hybrid did not show any response to this male; it was observed foraging and later disappeared in the scrub. This observation raised suspicion that the hybrid bird could be a female. The singing male in this territory was captured later, on 18 June 2009, using an Ecotone mis-net with tape luring. The male was strictly territorial as it responded aggressively to a playback. According to all morphological traits, the male was *L. megarhynchos*. The male showed a raised cloaca suggesting that it was prepared for copulation.

Genetic analysis

A blood sample from a brachial vein was taken from the hybrid individual to determine sex and confirm the hybrid origin. Sex determination was performed using PCR amplification of the CHD-Z and CHD-W genes located on the avian sex chromosomes (Griffiths et al. 1998). We performed the assay in several nightingales of known sex and in the hybrid. PCR amplification resulted in a single approximately 340-bp-long band in all males and two distinct bands, approximately 340 and 370 bp, in all females. The hybrid individual showed the same pattern as the females, supporting our suspicion that the individual was genetically a female.

To confirm the hybrid status of the captured female and to identify the species origin of its parents, we sequenced a part of two Z-linked genes, *ADAMTS6* and *SPINZ-2*, and a part of the *ND2* gene located on the mtDNA. Previous analysis of 25 allopatric individuals of *L. megarhynchos* and 25 allopatric individuals of *L. luscinia* revealed two species-specific single nucleotide polymorphisms (SNPs) in a 650-bp fragment of *ADAMTS6* and seven species-specific SNPs in a 950-bp fragment of *SPINZ-2* (Storchová et al. 2010). We PCR amplified the same fragment of both Z-linked genes from DNA of the hybrid female. PCR conditions are described in Storchová et al. (2010). These fragments were sequenced in both directions and base composition was determined at positions carrying previously identified species-specific SNPs. The hybrid female showed the same base composition in all species-specific SNPs as *L. megarhynchos*, suggesting that its Z chromosome is of *L. megarhynchos* origin. This can also be seen from the neighbor-joining trees for the two Z-linked genes (Fig. 2a). The trees represent genealogical relationships among unique haploid sequences obtained from 25 individuals of *L. megarhynchos*, 25 individuals of *L. luscinia* and the hybrid female. The haploid sequences were reconstructed using the program PHASE (Stephens et al. 2001; Stephens and Donnelly 2003) as described in

Fig. 1 Female hybrid between *L. megarhynchos* and *L. luscinia* captured on 3 June 2009 near Mladá Boleslav, Czech Republic (*top left*). Unexpectedly, considering the date of capture, the hybrid female was in the process of moulting as can be seen on the belly (*top right*) as well as both wings (*bottom*). Photos: Pavel Kverek



Storchová et al. (2010). The tree was reconstructed in MEGA 4.0 software (Tamura et al. 2007) using maximum composite likelihood method for estimating evolutionary distances. The sequence of the hybrid female clearly clusters with sequences of *L. megarhynchos* in both genes, indicating that the Z chromosome of the hybrid female comes from this species.

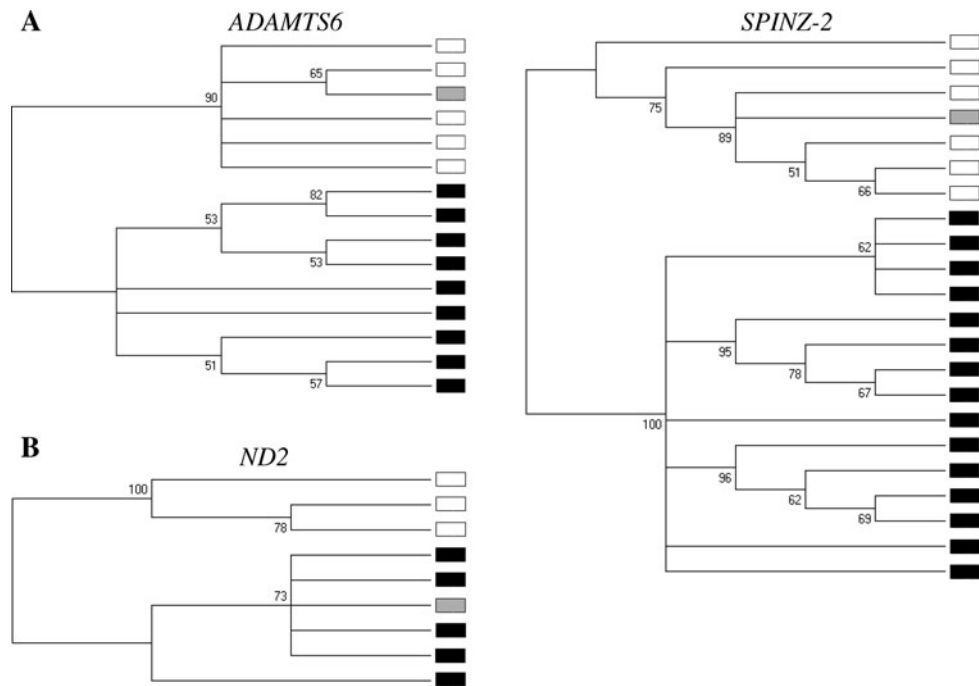
To amplify and sequence the part of the *ND2* gene, we used the following primers: ND2-F: CCCACATCATGCC TAATCCT, ND2-R: AATTAGTCATTTGGGGAGGAA. PCR conditions were as follows: 95°C for 2 min, (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) 39 times, 72°C for 10 min. PCR amplification resulted in a 525-bp PCR product, which was sequenced in both direction using the PCR primers. Since between-species nucleotide differences are not known for the *ND2* gene, we sequenced 15 individuals of both species in addition to the hybrid. The high quality 373-bp sequence obtained, which was determined from all sequenced individuals, was highly divergent between the species and contained 16 species-specific SNPs. The sequence from the hybrid female was clearly of *L. luscinia* origin according to all 16 SNPs, as can also be seen from the neighbor-joining tree (Fig. 2b). As females always inherit mtDNA from their mothers, while the Z chromosome comes from their father, our results suggest that our female really is a hybrid and originated from the mating of a female *L. luscinia* with a male *L. megarhynchos*. The sequences obtained have been deposited in GenBank under accession numbers HQ191443–HQ191473.

Discussion

Investigations of interspecific hybridization can provide important insight into the mechanisms responsible for the origin of bird species. In this study, we describe the first genetically confirmed record of a hybrid female between *L. megarhynchos* and *L. luscinia* in nature. The hybrid female arose from the crossing of a *L. luscinia* female with a *L. megarhynchos* male. *L. luscinia* is a very rare species in the Czech Republic, while *L. megarhynchos* is quite common (Štátný et al. 2006). The direction of interspecific crossing from which the hybrid female arose is thus consistent with the idea that a scarcity of conspecific males leads to hybridization (Randler 2002, 2006). Although the female was found within the breeding territory of a male *L. megarhynchos* and the breeding season was in its peak phase, it did not show any sign of reproductive activity, suggesting that it was sterile. In contrast, the male *L. megarhynchos* was prepared for copulation and sang frequently, which at that time indicates that it was unmated and searching for a mating partner (Amrhein et al. 2002). The absence of reproductive activity in the hybrid female is thus unlikely to be caused by the unavailability of a potential mate or its infertility.

Our observation is in agreement with experiments on interspecific breeding of nightingales in captivity. Stadie (1991) reported that females raised by mixed pairs of *L. megarhynchos* and *L. luscinia* were viable but not able to reproduce. Although often followed by males in the cage,

Fig. 2 Neighbor-joining trees for two loci on the Z chromosome (**a**) and one locus on mitochondrial DNA (**b**). Sequences of *L. megarhynchos* are represented by white rectangles, sequences of *L. luscinia* by black rectangles and the sequence of the hybrid female by a gray rectangle. Only unique haploid sequences of the two species are shown. The nodes that do not have majority bootstrap support have been compressed. The trees show that the hybrid female inherited the Z chromosome from *L. megarhynchos* and mitochondrial DNA from *L. luscinia*



they were never observed to copulate. Females also did not pay any attention to breeding material for the nest and the brood patch was always completely absent. Reproductive failure of nightingale hybrid females thus seems to arise very early in the reproductive cycle both in captivity as well as in nature. This is in contrast with other well-described examples of the sterility or reduced fertility of hybrid females in closely related bird species. In the flycatchers *Ficedula hypoleuca* and *F. albicollis*, hybrid females are able to mate and lay eggs, but their eggs never hatch (Svedin et al. 2008). In the crows *Corvus corone* and *C. cornix*, hybrid females are inferior in egg laying performance and their chicks less frequently survive (Saino and Bolzern 1992; Saino and Villa 1992). This shows that female-limited hybrid sterility in birds can affect different stages of the reproductive cycle.

Interestingly, our hybrid female was in the process of moulting at the time of capture. Nightingales normally moult after the end of the breeding season before the onset of migration to wintering grounds. A complete moult usually takes around 45 days in *L. megarhynchos* and 30 days in *L. luscinia*, and occurs between the second half of June and late August (Stresemann and Stresemann 1966; Berger 1967; Ginn and Melville 1983). In the Mladá Boleslav region, where we found the hybrid, most *L. megarhynchos* moult between 17 June and 21 August (Kverek 2008). We estimated that the hybrid female had already started to moult on 28 May. Such an unusually precocious moult could have been initiated by the accidental loss of the right fifth secondary, which was in the most

advanced moulting stage (Fig. 1). Such an accidental feather loss could slightly quicken the start of the moult. It is, however, unlikely that it could initiate the moult as early as May. We thus suggest that the early onset of the moulting process may be a natural part of the bird's annual cycle. This idea is also supported by the fact that the hybrid female had highly developed fat reserves, which nightingales normally gather much later, just before the onset of autumn migration (Stadie 1991). This precocious moult is very unusual and was never observed in any of the three hybrid males captured in the same region during the first half of June in previous years (Kverek et al. 2008). This suggests that precocious moult may be specific to sterile hybrid females.

The precocious moult of the nightingale hybrid female could theoretically have two different causes. First, it has been observed that birds that do not breed in a particular year sometimes start the process of moulting a few weeks earlier than breeding birds (Stadie 1991). The early moult of the hybrid female could thus be a consequence of its sterility. Second, an inappropriately timed moult in the hybrid female could result from diverging timing of the annual reproductive cycle in parental species (Hudec 1983; Stadie 1991). This has been observed for example in hybrids between African and European Stonechats (*Saxicola torquata rubicola* and *S. t. axillaris*) (Helm and Gwinner 1999). Since a properly timed moult is an important part of the nightingale annual reproductive cycle, an early start could have a dramatic effect on the fitness of hybrid individuals. Data on a larger number of nightingale female hybrids are, however, needed to draw any

conclusions about the possible role of the unusual moult pattern in nightingale speciation.

Acknowledgments We are grateful to Lukas Jenni for discussions on the progress of moulting in the captured individual, David Hardkopf for English revision and three anonymous referees for valuable comments to earlier drafts of this manuscript. This research was supported by post-doctoral grant of the Czech Science Foundation (GACR 206/08/P160) to R.R., and by the Ministry of Education, Youth and Sport of the Czech Republic (MSM0021620828, MSM6198959212). Experiments comply with the current laws of the Czech Republic.

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