

# **Vocalization of two nightingale species in their hybrid zone**

Hlasové projevy dvou druhů slavíků v jejich hybridní zóně

MSc. thesis – Diplomová práce



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**Declaration:**

I declare this thesis is a result of my own work, except for the contribution of people mentioned below. My own work consisted of recording in the field, song analyses, writing the thesis and manuscript draft. Samples for genetic analyses were collected by Jiří Reif, Radka Reifová and myself. The method of song analyses was developed by Silke Kipper and Michael Weiss and the data from DNA analyses were provided by Radka Reifová and her team. Conclusions and interpretations in presented manuscript draft represent only my own opinions which do not necessarily cohere with those of coauthors. I properly cite all information sources used. The work presented in the thesis has not been used to obtain any other academic degree.

In Prague, August 28, 2011

Signature

## **ACKNOWLEDGEMENT / PODĚKOVÁNÍ**

General acknowledgement is a very personal matter, which I would like to write in my mother tongue. That is why the following part is written in Czech.

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## ABSTRAKT

Ptačí zpěv je jedním ze znaků podléhajících pohlavnímu výběru, které mají zásadní význam pro výběr partnera a udržování prezygotických reprodukčně-izolačních mechanismů. U blízce příbuzných a částečně reprodukčně izolovaných druhů pěvců může při jejich druhotném kontaktu docházet ke změnám v jejich zpěvech, jež mohou buď divergovat a posílit tak izolační mechanismy mezi těmito druhy, nebo se mohou naopak sblížovat, což může přispět k mezidruhovému toku genů. Mým modelovým druhem je slavík tmavý (*Luscinia luscinia*), u něhož bylo v oblasti společného výskytu se slavíkem obecným (*Luscinia megarhynchos*) dokumentováno mezidruhové křížení a zároveň i konvergence zpěvů, způsobená kopírováním heterospecifických zpěvů. Toto kopírování může být způsobeno buď geneticky – introgresí, nebo chybným učením druhově specifických zpěvů. Tyto dvě hypotézy jsme posuzovali jak genetickými analýzami DNA tak analýzami nahrávek zpěvů obou druhů slavíků z alopatrie (Česká republika a severovýchodní Polsko) a sympatrie (jihozápadní Polsko – oblast kolem řeky Prosny). Srovnáním svých nahrávek s katalogem zpěvů slavíka obecného z alopatrické populace (Německo) se podařilo zjistit, že naprostá většina samců slavíka tmavého z kontaktní zóny má ve svém repertoáru fráze okopírované od slavíka obecného, tzv. „smíšené zpěváci“, přičemž relativní zastoupení heterospecifických zpěvů bylo u jednotlivých smíšených zpěváků různé. Ptáci z oblastí, kde se slavík obecný nevyskytuje, takové fráze nezpívají. Dodatečně bylo do analýz zahrnuto pět samců s morfologicky přechodnými znaky, což naznačovalo hybridizaci či introgresi. Získané výsledky analýz DNA ukázaly, že těchto pět jedinců bylo mezidruhovými hybridy. Ve svém repertoáru kombinovali zpěvy obou druhů stejně jako smíšené zpěváci slavíků tmavých. Podíl frází slavíka obecného v jejich zpěvech byl signifikantně vyšší než u slavíků tmavých. Z výsledků lze usuzovat, že přejímání cizích prvků zpěvu může být v oblasti společného výskytu druhů způsobeno spíše chybným učením jedince během jeho senzitivní periody v mládí a nebo kopírováním zpěvů od svých teritoriálních sousedů v dospělosti. Vzhledem k tomu, že přibližně 5 % samců v sympatrii jsou hybridy, vliv mezidruhového křížení na tento jev je pravděpodobně nevýznamný přestože hybridní původ jedinců může zvyšovat tendenci k učení zpěvů obou rodičovských druhů.

klíčová slova: *Luscinia luscinia*, *Luscinia megarhynchos*, struktura zpěvu, hybridní zóna, introgrese, bioakustika

## ABSTRACT

Bird song is a sexually selected trait that is crucial for mate choice and for maintenance of pre-mating reproductive barriers. Secondary contact of closely related and partially reproductively isolated song bird species may result in changes in their songs; these can either diverge and strengthen the reproductive barrier between the two species, or converge and contribute to mixing of their gene pools. The Thrush Nightingale (*Luscinia luscinia*) and its congener Common Nightingale (*L. megarhynchos*) may serve as model species suitable for studying these phenomena. In their secondary contact zone, an interspecific hybridization has been documented, as well as convergence of songs of Thrush Nightingales caused by copying of heterospecific songs. Such copying may be a result of erroneous learning of species-specific songs or by genetic introgression. We tested these hypotheses by simultaneous analyses of DNA and song recordings of both species from allopatry (Czech Republic and northeastern Poland) and sympatry (central Poland). Comparisons between our recordings and a catalogue of songs recorded in a Common Nightingale population from allopatry (Germany) confirmed that most of Thrush Nightingale males from the sympatric region were ‘mixed singers’ that use Common Nightingale phrases in their repertoires; the proportion of heterospecific songs varied among individuals. In contrast, songs of allopatric Thrush Nightingales do not include these heterospecific phrases. Additionally, we included in the analyses five individuals with a phenotype indicating potential hybridization or introgression; DNA analyses confirmed that these five individuals were interspecific hybrids. Their repertoires also combined song types of both species, as in Thrush Nightingale ‘mixed singers’, and the proportion of Common Nightingale songs sung by hybrids was significantly higher than in Thrush Nightingales. Heterospecific copying of songs by Thrush Nightingales may be caused either by copying from territorial neighbours in adulthood or by erroneous learning of juveniles. Given the fact that only ca 5% of sympatric males are hybrids, the direct contribution of hybridization to this phenomenon is probably unimportant, although the hybrid origin might increase the tendency for learning of songs from both parental species.

Key words: *Luscinia luscinia*, *Luscinia megarhynchos*, song structure, hybrid zone, introgression, bioacoustics

## GENERAL INTRODUCTION

For more than 150 years, since the time of Charles Darwin, the study of crossing between species is one of the main topics of evolutionary biology. Although the study of hybridization and understanding the mechanisms causing reproductive isolation between incipient species is often difficult, it can give important insights into the process of species origin. In contrast to previous studies, interspecific hybridization seems to be distributed uniformly across taxonomic groups within animal kingdom (Schwenk et al. 2008) and may result in disruption of reproductive barriers by mixing of gene pools. One of the approaches to study genetic basis of reproductive isolation involves naturally occurring hybrid zones between closely related species (Grant and Grant 1992). One of the greatest advantages of this approach is a possibility to study non-model organisms that cannot be crossed in the laboratory. The most famous model systems for study of speciation process in birds include closely related sympatric species of Darwin's finches (Geospizinae) on the Galapagos Islands or *Ficedula* Flycatchers in their hybrid zone in Sweden (Grant and Grant 1992, Saetre et al. 1997). Secondary contact of closely related and partially reproductively isolated songbird species may result in changes in traits that are crucial for mate choice, hence for the maintenance of pre-mating reproductive barriers. Their songs are among such sexually selected traits, which can either diverge and thus strengthen the reproductive barriers, or converge and contribute to mixing of gene pools instead (Grant 1972).

Our model system involves two sympatric, closely related species of nightingales, which are partially reproductively isolated. They are suitable for studying mechanisms of reproductive isolation, assessing the role of sex chromosomes and adaptive evolution in this phenomenon. The project also focuses on adaptive changes in bird ecology and morphology associated with the coexistence of the two very similar species in sympatry. Integration of the genetic and ecological data provides much deeper insight into the mechanisms than each of these approaches alone, which are responsible for the origin of new species. My MSc. thesis, which is a part of this project, focuses on changes in songs of these species in their contact zone, with an aim to assess whether heterospecific copying of songs by one of the species is influenced genetically.

In the first part of this thesis I (i) review contemporary knowledge about the role of vocal communication on interspecific hybridization in birds and vice versa and (ii) describe study system of the two Nightingale species. The second part of the thesis deals with the question of causes of heterospecific copying in congeneric species and has the form of a

manuscript draft. In addition, analyses and information, which are not included in the manuscript section, are provided in Appendices I – IV. These appendices include for example data about all analyzed individuals, analyses of their song frequencies, examples of sonograms etc.

## **1. Current state of knowledge**

### **1.1. Interspecific hybridization**

Interspecific hybridization is a relatively widespread phenomenon in many plant and animal taxa, which attracted the attention of scientists for over 150 years (Schwenk et al. 2008). As such, it is one of the most intensively studied processes in ecology and evolution. There are several hypotheses that test when and under what circumstances hybridization takes place in birds, many of which relate to bird songs.

There are various definitions of the term hybridization that have been used in scientific literature. Each of these relates to levels of divergence between the individuals that undergo reproduction. The two extreme definitions define hybridization as: (i) crosses between genetically distinct individuals and (ii) crosses between individuals from different species (Harrison 1993). As a result, it is hard to choose one general definition of this phenomenon. According to Harrison (1990), hybridization is a successful crossing between individuals from two populations or groups of population that are distinguishable in one or more heritable characters.

### **1.2. Causes of hybridization in birds**

Hybridization in birds seems to be relatively common, especially in song birds (Passeriformes) and ducks, geese, and swans (Anseriformes; Grant and Grant 1992). There are numerous hypotheses that explain hybridization in birds. According to one of the most widely accepted hypothesis, hypothesis of rarity, hybridization occurs mostly in contact area of closely related species, one of which is significantly rarer than another. In such situation, mates of the common species are more easily available to widely dispersed individuals of the rare species than conspecific mates. In congruence with this hypothesis, there are many cases where females of rare species are paired with males of the common species and vice versa (reviewed in Wirtz 1999, Randler 2002). Similar situation eventually resulting in hybridization appears also in populations with biased sex ratios. In such

populations, heterospecific pairing is likely to occur when all conspecific mates in the contact zone of two species are already paired (Baker 1996).

Interspecific hybridization can also be a result of error in female choice. This may be caused by lack of time during short breeding period or by competition between females (Gowaty 1997). Female choice errors appear relatively commonly also in young and inexperienced individuals. There can also be errors in acoustic, visual or behavioural recognition of conspecifics, especially in closely related species with only small differences in key character(s) facilitating recognition of conspecific mates (Helb et al. 1985).

In addition, mate choice errors can result from supranormal stimuli, which make heterospecific mates more attractive than conspecifics. Typical examples of traits with a potential to serve as supranormal stimuli include feather colouration patterns, more complex songs and social dominance (or their combination). For example, more complex songs with heterospecific elements acting as supranormal stimuli are known in several bird taxa, such as *Sylviidae* and *Certhia* spp. (e.g., Helb et al. 1985).

Finally, wrong mate preference as a culturally influenced experience can be passed horizontally in one generation, followed by vertical transfer to next generations. This phenomenon has been showed by Freeberg et al. (1999) as a cause of interspecific hybridization in the Brown-headed Cowbird (*Molothrus ater*).

### **1.3. The role of song in hybridization**

Sexually selected traits, socially transmitted in particular, are considered to have the most important role in mate recognition in birds and thereby act as important prezygotic reproductive isolation mechanism. Bird song is one of these traits and its role can be crucial in speciation (Grant and Grant 1996, Bensch et al. 2002).

Sympatrically occurring species (and also closely related and partially reproductively isolated species coming into secondary contact) can, depending on the type of interspecific interaction, show convergent or divergent character displacement in song patterns, as well as in other key characteristics of species recognition (Grant 1972).

Divergent character displacement may be the result of selection pressure on reproductive isolation (selection against hybrids with lower fitness and selection for individuals with easily distinguishable conspecific songs) or ecological segregation (e.g., in population from different habitats). This may lead to reproductive isolation between populations (Saetre 1997, Boughman 2002). Songs of closely related species may also diverge

due to interspecific competition for common resource (Schluter 2000). In such case, divergence in songs can be considered as a result of coevolution of songs with other divergent morphological or ecological traits (Podos 2001).

In some cases, convergence of song patterns or wide plasticity of song may increase the rate of interspecific gene flow. This may be a result of heterospecific copying of songs (Alatalo et al. 1990, Grant and Grant 1997) or adjusting song features to the acoustic environment (de Kort 2002). There are many studies describing this phenomenon in closely related species in sympatry (e.g., den Hartog et al. 2007, Haavie et al. 2004). Songs enriched by elements from another song (i.e., mixed songs), which were established due to copying of songs between species, can be followed by broad heterospecific pairing or changes in preferences resulting from such a song convergence. This was documented in recently established sympatry of two *Ficedula* flycatchers in Sweden. In this area, the majority (65%) of male Pied Flycatchers (*Ficedula hypoleuca*) include various parts of Collared Flycatcher (*F. albicollis*) song in their song repertoire (but not vice versa). Collared Flycatchers males respond similarly to these mixed songs as to conspecific songs, and long-term data on pairing patterns showed that males singing a converged song attract females of the other species. Female Collared Flycatchers only pair with male Pied Flycatchers if the males sing the mixed song types. This is the reason why hybridization rate in this population reaches high levels of ~ 20%, in contrast to very low proportion of hybrids in populations that have been sympatric for longer time (Qvarnström et al. 2006). Convergent shifts in songs (e.g., as an adaptation to acoustic environment) may be also due to lack of interaction between species caused by interspecific territoriality (Secondi et al. 2003). Moreover, convergence in songs can also play only an additional role in mate choice, e.g., in association with plumage traits (Patten et al. 2004).

#### **1.4. Effect of hybridization on songs in birds**

In contrast to the well-studied role of song in hybridization, the effect of hybridization on songs in birds is assessed in a relatively small number of studies. Introgression through hybridization may cause song convergence, especially in birds with genetically determined song structure (de Kort et al. 2002). Hybridization may induce changes of various extent in functional parameters (temporal or some spectral) or in structure (songs, elements) of vocalizations. In some cases, hybrids can learn songs only from one parental species. This may happen in areas where one species is significantly more common than the other one (Gelter 1987). However, hybrids often show mixed songs which contain song elements of

both parental species (Beier et al. 1997). Mixed songs occur both in hybrids of species with genetically determined calls (e.g., de Kort et al. 2002, Ceugniet et al 1999), and in species with learned songs (e.g., Bensch et al. 2002, Helb et al. 1985).

## **2. Nightingales – my study system**

My MSc. study focuses on changes in songs of two closely related nightingale species hybridizing in their secondary contact zone – the Common Nightingale (*Luscinia megarhynchos* Brehm, 1831) and its congener Thrush Nightingale (*Luscinia luscinia* L., 1758). Because of their similarity in many traits and characters, I decided to describe them together.

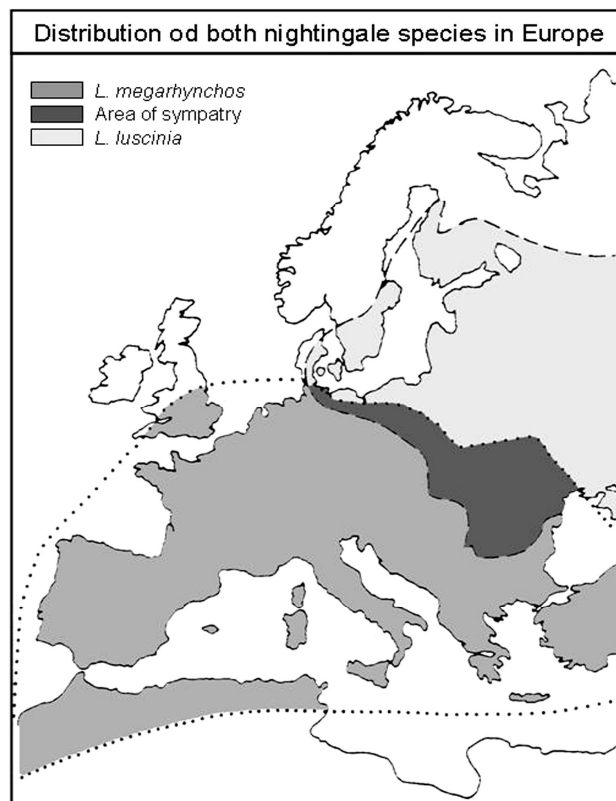
### **2.1. Distribution of the species and their contact zone**

The Common Nightingale, of which three subspecies are recognized, occurs from Southwestern Europe and North Africa to Central Asia (Turkmenia and Mongolia). Only the nominate subspecies *L. m. megarhynchos* inhabiting Europe and North Africa to central Turkey is relevant for my study. The second one is the Thrush Nightingale, which is monotypic and inhabits Northeastern Europe from western Germany to the east to Black Sea and further northeast to Ukraine and Russia (Cramp 1997). The area of the Thrush Nightingale has been expanding in recent decades slightly to west and south (Orr 1976 cited in Cramp 1997). Both species diverged approximately 1.8 MY ago - during the Pleistocene (Storchová et al. 2010) and subsequently came into secondary contact. The area of their co-occurrence (and hybridization) is a relatively narrow zone crossing Europe from southern Denmark, across central Poland, eastern Slovakia to southern Romania (Fig. 1, adapted from Sorjonen 1986a).

### **2.2. Morphology and ecology of the species**

These two nightingale species are very similar in morphological traits. They have similar size and colour of plumage (brown upperparts and greyish underparts; Fig. 2). However, they can be distinguished by subtle differences in wing feather measurements (see, e.g., Hromádka et al. 1992 for details) and plumage coloration of adults (slight spots on chest in the Thrush Nightingale and more reddish tail in Common Nightingale). The Thrush Nightingale is heavier and has also a bit longer tail and larger wing span (Cramp 1997).

Both species live mainly in lowlands and also occupy similar breeding habitats (e.g., Sorjonen 1986a). They establish territories mostly in shrubby habitats. At study sites in central Europe, typical habitats are characterized by the presence of vegetation such as willows (*Salix* spp.), blackthorn (*Prunus spinosa*) and European bird cherry (*Prunus spinosa*) with dense understorey. This is usually dominated by nettles in which they often built their nests. Nightingales inhabit also field thickets and edges of broadleaved groves, often near the rivers or wetlands (pers. obs., for localities see Appendix I).



**Figure 1.** Distribution of the Common Nightingale (*Luscinia megarhynchos*, dark grey) and the Thrush Nightingale (*L. luscinia*, light/pale grey) in Europe and their contact zone (black).

In sympatry, the Common Nightingale seems to be weaker competitor and the two nightingale species have a tendency to differ slightly in breeding habitats. The Common Nightingale shifts more often to hilly habitats, whereas the Thrush Nightingale remains mostly in lowlands. This pattern may be a result of interspecific territoriality (Sorjonen 1986a, Ranozsek 2001). Recent detailed investigations showed that interspecific competition in sympatry results also in diverged relative bill size in these species when compared to body size (increased bill size in the Common Nightingale), which can reflect segregation of their feeding niches (Reifová et al. 2011a). This process of phenotypic segregation of sympatric

populations—an asymmetric ecological character displacement in this case—seems to be a result of in situ evolution of a novel phenotype which could drive species divergence. It has been speculated that such divergence may enhance reproductive isolation between the species even in the face of ongoing hybridization (Reifová et al. 2011a).



**Figure 2.** Slight differences in phenotype of the Thrush Nightingale (*Luscinia luscinia*, left) and the Common Nightingale (*L. megarhynchos*, right).

### 2.3. Songs and song learning

Both species of nightingales commonly sing from dense growths, usually close to the edge (Sorjonen 1983). The nightingale song is typically delivered during the night, usually at higher perch, but they sing also during the day, often near the ground (Sorjonen 1977). The peak of their singing activity on breeding grounds is approximately from mid-April to mid-June (depending on various factors such as arrival date of females from wintering grounds, latitude of the breeding grounds, and on mating status of males, e.g., Kipper et al. 2004, Armhein et al. 2002, Sorjonen 1986b). Interestingly, a few reports showed that the nightingale males sing also on their wintering grounds in Africa (e.g., Pearson 1984).

In spite of general similarities in song production of these congeneric species, the Common Nightingale and the Thrush Nightingale differ in their songs (Sorjonen 1983, 1986a). These differences play the most important role in analyses used in my study.

The Common Nightingale is famous for its large song type repertoire; the adult males may sing about two hundred different types of songs (on average 190, Hultsch and Todt 1981; Kipper et al. 2004). In comparison with Common Nightingales, Thrush Nightingale

repertoires are medium-sized (on average 11, Sorjonen 1987). They sing songs with a longer duration and a lower song rate (Lille 1988, Cramp 1997, see Fig. 3c). Differences in song organisation between species (at the level of elements) are shown on spectrograms in Fig. 3.

Songs in nightingales are learned the same way as in other oscine birds, through social learning from models (father or neighbours). This combination of cultural and genetic evolution causes the song of oscines to be generally a very plastic trait (reviewed in Marler 1997). Song learning has been thoroughly studied in Common Nightingales (reviewed in Hultsch 1993). The use of such large repertoires seems to be related to the ability of individuals not only to learn during their natal summer, but also to memorise and imitate new songs later in life. During early stages of ontogeny, juvenile males sing firstly non-structured sequences of elements (subsong), subsequently add species-typical song structure and timing (plastic song), and their song crystallizes at the age of about 11 to 12 months (adult song). Birds can still incorporate new songs in their repertoire after sexual maturity in their first year of life (Todt and Geberzahn 2003), and thus they are considered ‘open ended learners’ (reviewed in Hultsch and Todt 2008).

In areas where these two nightingales co-occur, cases of males singing songs with elements of both congeneric species are known. Among first studies describing this phenomenon, there are reports by Kux and Weisz (1977) from Slovakia, and by Stadie (1983) from Schleswig Holstein region in Germany. First song comparisons from areas of sympatry documented an asymmetric convergence in songs (Schmidt 1973, Sorjonen 1986a). A relatively large proportion of Thrush Nightingales living in sympatry used phrases copied from the Common Nightingale in their songs. These birds have been called ‘mixed singers’ (Lille 1988, Sorjonen 1986a). On the other hand, imitations of heterospecific songs seem to be very rare in the latter species (Becker 2007). Apparently, both species substantially differ in their tendency to imitate each others songs. The above mentioned reports on song convergence (i.e., on the presence of mixed singers among Thrush Nightingales) were so far limited either to a subjective evaluation (Schmidt 1973, Kux and Weisz 1977, Stadie 1983, Becker 1995, 2007) or to an approach based on the quantity of syllable types used in songs in combination with spectral measures (Sorjonen 1987, Lille 1988). Although such observations are very interesting, the actual extent of the song type copying between species has not been directly quantified so far.

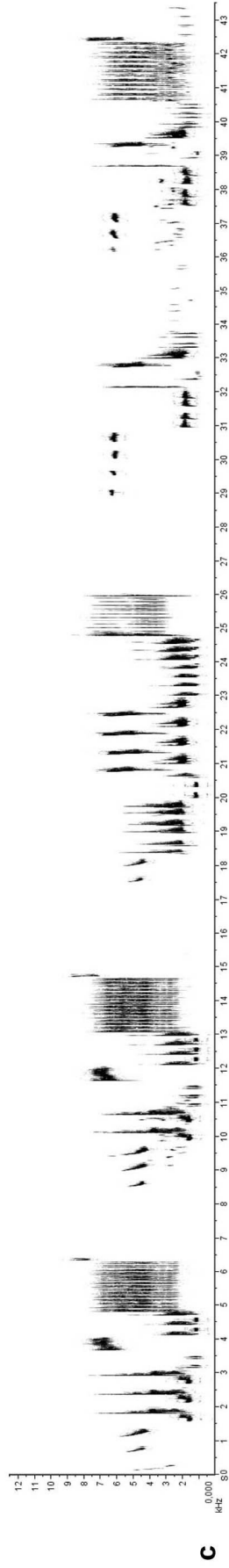
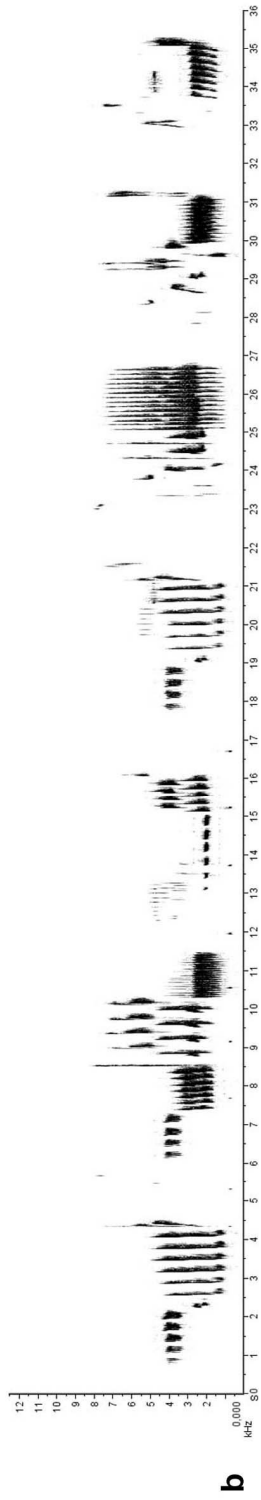
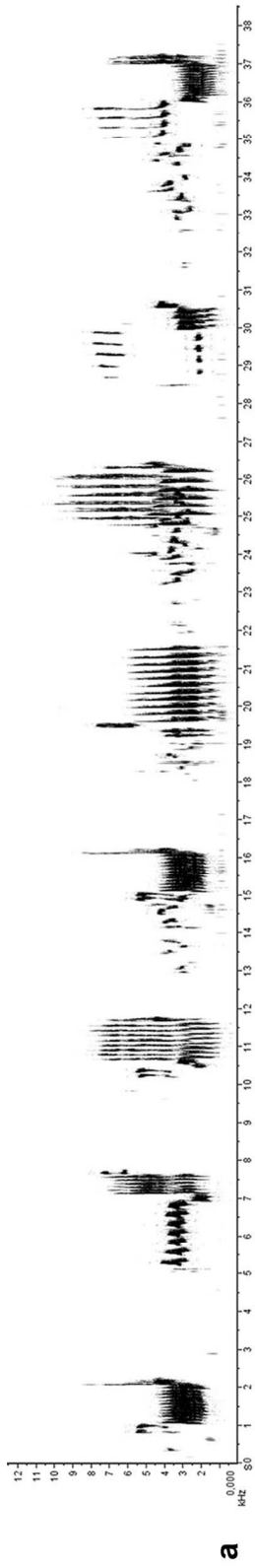
## 2.4. Hybridization of nightingales in sympatry

Observations of mixed pairs and the occurrence of individuals with intermediate morphological traits confirm that the two species occasionally hybridize in the narrow zone of sympatry (e.g., Stadie 1983, Becker 1995, Kverek 1998, 2002). Interspecific hybridization of these two nightingale species in captivity then showed that the F1 hybrid females are sterile, while the F1 hybrid males are fertile (Stadie 1991) which is in accordance with Haldane's rule (Haldane 1922).

Until recently, the estimations of hybridization rates in sympatry (3-5%) have been based solely on morphological studies (Becker 2007). The first genetically analysed interspecific hybrid was caught in 2007 in the Czech Republic. Interestingly, this was outside of the main distribution area of the Thrush Nightingale. The hybrid status of this male was confirmed by gene sequence analysis on the sex chromosome Z compared with corresponding sequences from males of both species from allopatric areas (Kverek et al. 2008). A more recent genetic study from sympatry of both species (Reifová et al. 2011a) confirmed similar rate of interspecific hybridization in a much larger sample of males (3 hybrids of 102 analysed males, i. e., 3%). Genetically determined hybrid female was described only recently by Reifová et al. (2011b).

**Figure 3.** Spectrograms of songs (following page).

- a) Common Nightingale – pure species specific song from allopatry (38s)
- b) Thrush Nightingale - mixed song from sympatry (36s)
- c) Thrush Nightingale – pure species specific song from allopatry (43s)



### 3. Aims of the thesis

Based on the facts that the hybridization between these two nightingale species occurs occasionally (Becker 2007, Reifová et al. 2011a), and a substantial convergence in songs has been reported from the area of sympatry (e.g., Sorjonen 1986a), there are two alternative scenarios that may explain the phenomenon of mixed singers. According to the first scenario, hybridisation and/or introgression affects the song display of individuals in sympatry and mixed singers could be in fact interspecific hybrids. The second one, on the other hand, explains the song convergence as a result of learning of songs from heterospecifics, independent of hybridization. Therefore my major aim is to confirm or reject these two alternative scenarios.

In this thesis, I used a new method (developed by Silke Kipper and Michael Weiss) for assignment of analysed songs to species-specific songs of the Common Nightingale. This approach is based on comparison with a catalogue of distinct song types recorded from *Luscinia megarhynchos*. The catalogue consists of more than four hundred song types of a German Common Nightingale population. Correlations were calculated in the software Avisoft and the final classification was performed by visual comparison of the songs of interest with best matched catalogue songs previously selected by software (see Methods section in the next chapter for details). In contrast to relatively subjective or repeatable but rough methods used in previous song comparisons (see section 2.3.), the approach used herein is well repeatable, applicable to and comparable with other studies using analogical methods.

The above mentioned approach makes possible to determine the proportion of song types of Common Nightingale in songs recorded from the studied individual. This allows me to fulfil the first aim, (i) to confirm whether the use of Common Nightingale songs by the sympatric Thrush Nightingale is common or not. If so, the obtained information can be used to (ii) suggest causes of such song convergence. The third aim of the thesis is (iii) an evaluation of songs of interspecific hybrids for which both song recordings and genetic data are available.

The data from DNA analyses of sympatric individuals used in this thesis were provided by Radka Reifová. They are based on sequences of two genes located on the sex chromosome Z. This method reliably determines F1 hybrids but does not allow more detailed identification of later-generation hybrids or backcrosses. However, more sensitive markers could prove the effect of introgression in finer scale in the future.

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# MANUSCRIPT DRAFT

## **‘Mixed singers’ among closely related songbird species: a consequence of erroneous learning rather than interspecific hybridization**

(UNPUBLISHED)

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### **Introduction**

Song plays an important role in sexual selection and the establishment and maintenance of pre-reproductive barriers in birds (Grant and Grant 1997a). When two closely related and partially reproductively isolated species come into the contact, their characteristics may diverge as a result of natural selection in order to avoid maladaptive hybridization (Haavie et al. 2004). On the other hand, species may also converge in some features, including morphology, ecology or behaviour (e.g., Price et al. 2007). Among behavioural traits, sexually selected ones such as singing behaviour may be particularly interesting. Since females often base their mate choice decisions on song characteristics (reviewed in Searcy and Yasukawa 1996), song may considerably contribute to maintenance or breakdown of reproductive isolation.

The song convergence between closely related species in sympatry was studied in several model species, in which it apparently arose by various mechanisms (e.g., Secondi et al. 2003, Haavie et al. 2004, den Hartog et al. 2007). Convergence as a result of heterospecific copying of songs (Alatalo et al., 1990, Grant and Grant 1997b) or adjusting song features to the acoustic environment (Badyaev and Leaf 1997) may lead to increased hybridization rates. At vice versa, introgression through hybridization may cause song convergence, especially in birds with genetically determined song structure (de Kort et al. 2002).

Our study focuses on changes in songs of two closely related nightingale species, which hybridize in their secondary contact zone. The Thrush Nightingale (*Luscinia luscinia* L., 1758) inhabiting Northeastern Europe and the Common Nightingale (*Luscinia megarhynchos* Brehm, 1831) from Central to Southwestern Europe diverged approximately 1.8 MY ago (Storchová et al. 2010). They were geographically separated in the late

Pleistocene and subsequently got into secondary contact in Central and Eastern Europe (Sorjonen 1986a, Storchová et al. 2010). Their sympatric area spreads diagonally across Europe - from northern Germany to southern Romania (Fig. 1.). Despite their considerable similarities in phenotype and ecology, both species can be distinguished by subtle differences in morphological traits especially on wing feathers (Cramp 1997). In sympatry, some ecological and morphological differences have been documented between the two species, including slightly different breeding habitats (Sorjonen 1986a, Ranoszek 2001) and divergence in relative bill size (Reifová et al. 2011). Such differences may be caused by interspecific territoriality and subsequent adaptation to different food sources (Reifová et al. 2011).

The songs of the two nightingale species belong to the most complex among songbirds; the repertoire of an average Common Nightingale male consists of some 190 song types (Hultsch and Todt 1981; Kipper et al. 2004). In contrast, the Thrush Nightingale repertoires are medium-sized (on average 11 song types; Sorjonen 1987). The latter species sings songs with a longer duration and a lower song rate (Lille 1988; Cramp 1997; see also Fig. 3 in General Introduction). Song learning has been particularly thoroughly studied in Common Nightingales (reviewed in Hultsch 1993). As in other oscine passerines, songs in nightingales are learned through social learning from a model (father or neighbour) both during juvenile and adult periods of their lives. Nightingales are therefore considered 'open ended learners' (reviewed in Hultsch and Todt 2008). This combination of cultural and genetic evolution causes the song of oscines to be generally a very plastic trait (reviewed in Marler 1997).

First song comparisons of both species described an asymmetric convergence in songs in areas where the two nightingale species co-occur (Sorjonen 1986a, Schmidt 1973). A relatively large proportion of Thrush Nightingales living in sympatry used phrases copied from the Common Nightingale in their songs. These birds have been called 'mixed singers' (Lille 1988, Sorjonen 1986a). On the other hand, imitations of heterospecific songs appear to be very rare in the latter species (Becker 2007). Apparently, both species substantially differ in their tendency to imitate each others songs. Reports on song convergence (i.e., on the presence of mixed singers among Thrush Nightingales) were so far limited either to a subjective evaluation (Becker 1995, 2007) or to a method based on the quantity of syllable types used in songs in combination with spectral measures (Sorjonen 1987).

In the narrow zone of sympatry of these species, several cases of mixed breeding pairs and interspecific hybrids were reported. Evidence of hybridization, suggesting that about 5%

of birds in sympatry were hybrids, was until recently based solely on morphological studies (Becker 2007). A more recent study on much larger sample of males confirmed similar proportions of hybrids also genetically (Reifová et al. 2011).

Based on the facts that the hybridization between these two nightingale species occurs occasionally and substantial convergence in songs is known in the area of sympatry, there are two alternative scenarios that may explain the phenomenon of mixed singers: a) hybridization and/or introgression substantially affects the song display or b) song convergence is caused by learning of songs from heterospecifics, independent of hybridization.

We used a new objective approach for a quantitative assignment of our recorded songs of nightingales with a species repertoire catalogue for Common Nightingale. A comparison of song repertoires in combination with genetic analyses allowed to test the hypothesis that heterospecific copying of songs in Thrush Nightingales is not affected genetically, and that the mixed singers are males of Thrush Nightingales rather than interspecific hybrids. These methods also allowed us to compare song composition of hybrids and Thrush Nightingale males in sympatry.

## **Methods**

### **Field sampling and song recording**

The fieldwork was carried out during spring 2008 and 2009 in allopatric and sympatric breeding sites of both species. Allopatric areas were represented by localities in the Czech Republic and Germany for the Common Nightingale and in northeastern Poland for the Thrush Nightingale. Study sites in sympatry were located in central Poland (Fig. 1, Appendix I Table 1).

All samples were collected during April and May at the beginning of the breeding season of both species. Most of the birds were captured by mist netting, lured into nets by playback of commercial recording of either Common Nightingale or Thrush Nightingale (Roché 1995), depending on the presumed species identity of the respective male. The males were then individually marked by metal and colour rings for identification. Preliminary species identification of captured birds was based on measurement of species-specific morphological characteristics (Hromádka et al. 1992). Samples of blood for subsequent genetic analyses were collected from each individual (except Common Nightingale males from Germany) by brachial vein puncture.

The songs were recorded on a digital recorder Marantz PMD660 using a directional Sennheiser ME67 microphone. Most of recordings of individual males were collected during days and evenings (from 6 am to 9 pm), except for the six Common Nightingale males from the German population that were recorded at night.

For birds that did not sing spontaneously, a very short song bout was played to stimulate singing. Each bird heard its species-specific song (Roché 1995); potential hybrids heard randomly either Common Nightingale or Thrush Nightingale song. Playbacks did not seem to have an effect on the bird's singing.

### **Genetic analysis**

Blood samples were taken from sympatric individuals for determination of their species identity using partial sequences of ADAMTS6 (650 bp fragment) and SPINZ-2 (950 bp fragment) genes on sex chromosome Z. These markers have been selected due to their suitability for species identification (Storchová et al. 2010). Conditions of PCR and selection of primers followed Storchová et al. (2010). The two fragments from each sympatric individual were sequenced in both directions and base composition has been determined at positions carrying previously identified species-specific single nucleotide polymorphisms (Reifová et al. 2011).

### **Analysis of songs**

Recordings of 34 individuals were analysed using the software Avisoft SASLab Pro versions 4.5 to 5 (Raimund Specht, Berlin). Song analyses and the classification of song types were conducted without a priori knowledge of the results of genetic analyses. We used recordings of 11 Common Nightingale males from allopatric populations in the Czech Republic (five individuals) and Germany (six individuals), and of five Thrush Nightingale males from allopatry in northeastern Poland. From sympatry, we analyzed recordings of nine Thrush Nightingale and four Common Nightingale males. In addition, five males of intermediate phenotype (as assessed by morphological measurements) from sympatry were analyzed. In most cases, we analyzed ca 20 minute long recordings of each individual (ranging between 19:45 and 23:00 min, see Appendix IV); only a recording of one male with an intermediate phenotype was shorter (8 minutes). The recordings consisted on average of 190 songs per individual in Common Nightingale (range 132-261), 148 songs per individual in Thrush Nightingale (range 88-206) and 131 songs per individual in males of intermediate phenotype

(range 64-176). Numbers of songs analysed per each individual and other details are given in Appendix I Table 1.).

We compared our songs to a catalogue of songs from German Nightingale populations. This catalogue consisted of 425 song types and was derived from analyses of nocturnal singing of 50 Common Nightingales (6 years, 3 populations; Kipper and Kiefer, unpublished). To investigate the species repertoire, long song sequences of 533 successive songs per bird (equalling about 1hr singing) were analysed. Each song was either assigned to a type that was already included in the catalogue or founded a new song type. Nightingales are known for their precise song copying and singing, so that despite their large repertoires, song types can be reliably assigned and compared across individuals, populations, and years (see Kipper et al. 2004 or Sprau and Mundry 2010 for examples).

For a semi-automated assignment of analysed recordings, we compared separately each single analysed song with templates characterising each song type included in the catalogue (547 templates, each of 0.5 s duration; some song types consisting of two repetitive phrases were represented by two different templates). Templates were sections of the 'loudest' part of the respective song type (i.e., the part with the maximum amplitude). Correlations between analysed songs and templates were calculated by using a spectrogram image cross correlation technique provided in Avisoft. The final classification was performed by visual comparison of a spectrogram of the song of interest with spectrograms of those song types from the catalogue with the highest correlation coefficients.

For the semi-automated assignment of our recorded songs, all recordings were first split into files containing single songs. These files were subsequently converted into a format suitable for crosscorrelation analysis with the following settings: sampling frequency conversion 22.05 kHz (Accuracy 256), normalize volume (85%), noise reduction filter (FFT 512, precision 4, threshold -40 db reduce by 90 db).

From each file, a spectrogram file was created (FFT 256, Frame size 100, Window Hamming, Overlap 50%). We conducted quantitative comparisons (using the cross correlation analysis in Avisoft) and calculated correlation coefficients of the songs in our recordings with all 547 templates. Our recorded songs were assigned to catalogue song templates using the function 'Classify .wav or .son files' in Avisoft-SASLab Pro, and final visual comparison was facilitated by an Excel macro (programmed by M. Weiss) that allowed quick inspection of spectrograms of the best-matching song types from the catalogue.

Songs that were not identified by cross correlation were re-checked by a visual comparison with the catalogue and categorized. The categories to which individual analyzed songs were

assigned reflected the degree of their similarity to the template song types in the catalogue. The categories were defined as follows. **‘Very good match’** – songs matching a catalogue song type exactly or resembled it by at least 95 % of the element sequence of the catalogue. **‘Partial match’** – songs similar to a catalogue song type, but differing from it by missing some elements, with resemblance of at least 75 % of the catalogue song element sequence. Songs that could not be assigned to a catalogue type following this definition were further categorized into three subcategories: **‘LM’**: typical Common Nightingale song organization (alpha-beta-gamma-omega, see Appendix II) and recognizable catalogue gamma parts. **‘LL’**: typical Thrush Nightingale song organization (beginning with a repeated part, no beta-part, no omega) and no recognizable Common Nightingale catalogue gamma parts. All disputable cases were included into a category **‘Unclear’**: no Common Nightingale song organization, but recognisable gamma parts, and none of the characteristics above to apply. By these subcategories, we evaluated the overall similarity of the analyzed songs to species-specific song display. Fragmented songs exceptionally found in our recordings, resembling less than approx. 15 % of the catalogue song element sequence were treated as **‘Artifacts’**, and these were not included into song proportion data analysis (for examples, see Appendix II).

After the assignment of individual songs to the categories reflecting their similarity to the catalogue templates, the songs of each individual were pooled into two overall categories referring to the match to Common Nightingale songs, **‘Identified’** and **‘Unidentified’**. These were then used in final evaluation of song composition. We performed the analyses with two versions of these overall categories, differing in stringency of the conditions. In the first, stricter version (‘strict’), only categories with resemblance over 75 % (‘Very good match’ and ‘Partial’) were considered ‘Identified’, while the ‘Unidentified’ group included ‘LM’, ‘Unclear’ and ‘LL’ categories. In the second, more relaxed version of assignment (‘relaxed’), the ‘Identified’ group included the categories ‘Very good match’, ‘Partial’ and ‘LM’, whereas ‘Unidentified’ group included only categories ‘Unclear’ and ‘LL’. This relaxed version of ‘Identified’ group included also songs which did not match strictly to the catalogue but were likely of a Common Nightingale origin, and the inclusion of which was thus useful when evaluating the proportion of heterospecific songs used by mixed singers among Thrush Nightingales.

## Statistical analysis

To test if there is any difference between songs of conspecifics living in allopatry and sympatry, we used generalized linear models (GLM, binomial or quasibinomial family, logit link function). Proportions of identified/unidentified songs were used as a response variable, and the area of occurrence (sympatry or allopatry) was used as a categorical explanatory variable. We first fitted the models using binomial family and checked for overdispersion. When residual deviance was higher than residual degrees of freedom, we used quasibinomial family instead of the binomial one. The same statistical approach was used for evaluation of differences between songs of studied taxa. These two tests were computed by using both versions of the response variable ('strict' and 'relaxed', as defined above). For comparison of song rate between species we used a non-parametric test (Mann-Whitney U test). All statistical analyses were performed in R (R Development Core Team 2009).

## Results

The results of sequence analyses of the two genes on sex chromosome Z showed that only five individuals (all with intermediate phenotype) out of the 34 individuals analyzed in this study were interspecific hybrids, i.e., heterozygous in the ADAMTS6 and SPINZ-2 genes. Genetic analyses also confirmed the species status of males which were morphologically identified as pure Common and Thrush Nightingale.

Altogether, we analyzed 5576 songs from all 34 males. Of these, 2845 songs were from 15 Common Nightingale males, 2076 songs from 14 Thrush Nightingale males, and 655 from 5 interspecific hybrids. The Thrush Nightingale males sung significantly less frequently than the Common Nightingale males (on average 7.3 songs per minute vs. 9 songs per minute); Mann–Whitney U = 47.0, N1 = 14, N2 = 15, P = 0.011<sup>1</sup>.

Common Nightingale males from Germany belonged to one of the populations used for deriving the catalogue. For this reason, their songs corresponded by 100% with the catalogue. On average 82 % (range 72–97 %) of songs of allopatric Common Nightingale males from the Czech Republic were identified in the catalogue. Of the remaining songs, 88 % were also categorized as species-typical display of Common Nightingale song ('LM'), and the rest (12 %, i.e., 2.2 % of all songs) as 'Unclear'. Common Nightingale males in sympatry sung on

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<sup>1</sup> For more detailed analyses see Appendix III.

average 71 % (range 53–95 %) of songs identifiable in the catalogue. Remaining songs were categorized in 94 % as ‘LM’, and in 6 % (i.e., 1.7% of all songs) as ‘Unclear’.

All nine analyzed Thrush Nightingale males from the sympatric region were indeed ‘mixed singers’, which used at least some Common Nightingale songs or songs with its species-typical display in their repertoires. However, the proportion of these heterospecific phrases varied substantially among individuals (on average 45%, range 2 – 76 %), and song categories were represented differently in different individuals. Average proportions of identified and unidentified songs assigned to different categories for each group of males is shown in Fig.1; more details for individual males are given in Fig. 2.

There were no ‘mixed singers’ among the analyzed Thrush Nightingale males from allopatry, all their songs were categorized as song types typical for that species. Thus, the differences in the proportion of identified heterospecific songs between sympatric and allopatric Thrush Nightingales was highly significant in both versions of the assignment rules: stricter version (DF=13, F=19.971, p=0.0008), relaxed version (DF=13, F=16.89, p=0.0014).

All five interespecific hybrids detected by genetic analyses were also evaluated as ‘mixed singers’, songs of which were dominated by Common Nightingale phrases. Proportion of these phrases was on average 90 % (range 84 – 98 %; see also Fig. 2.), i.e., higher than in average Thrush Nightingale mixed singers. The difference in proportion of identified songs between Thrush Nightingale males from sympatry and interspecific hybrids was significant (‘strict’: DF=13, F=10.598, p=0.0068; ‘relaxed’: DF=13, F=6.1667, p=0.02878).

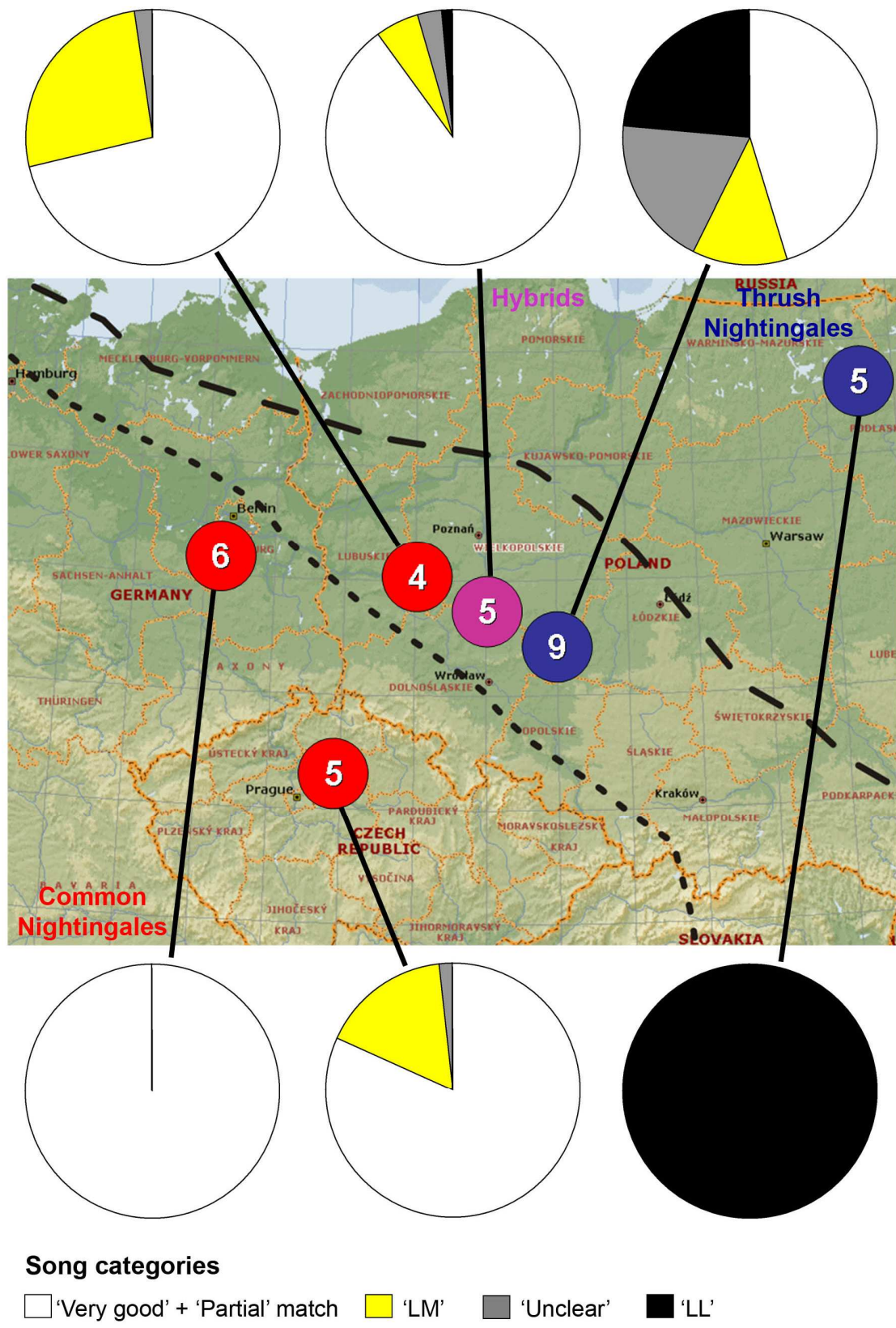
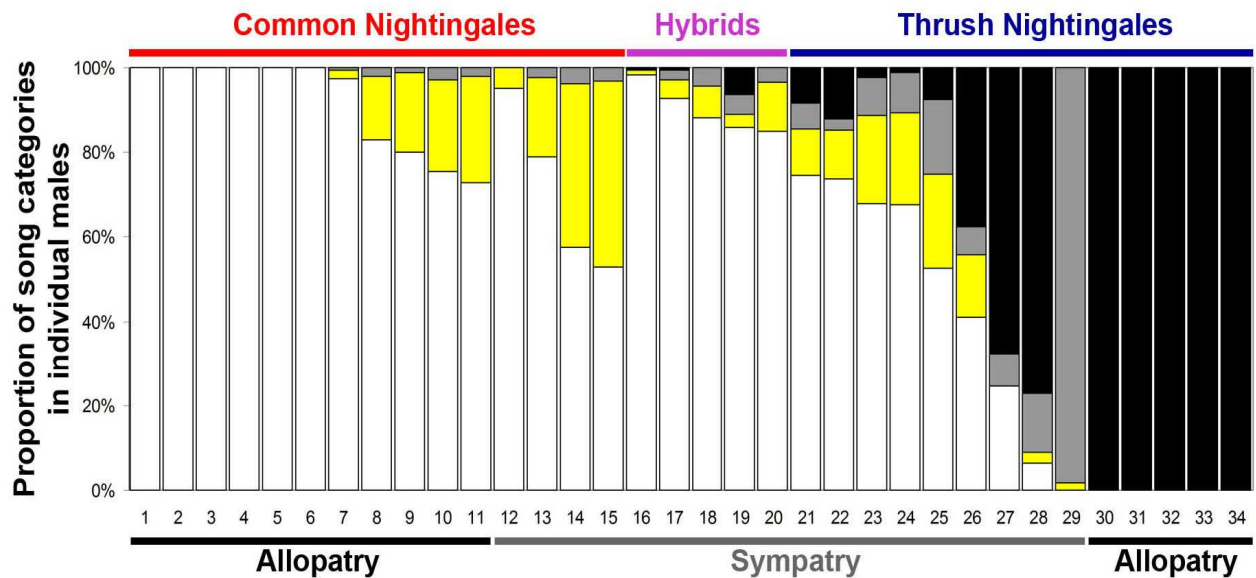


Figure 1. The area of sympatry, geographic position of localities, numbers of analysed males, and average proportion of songs in all categories per each group of males.



### Song categories

□ 'Very good' + 'Partial' match    ■ 'LM'    ■ 'Unclear'    ■ 'LL'

Figure 2. Proportion of songs in each category for each analysed individual. Males are ranked according to their areas of occurrence and to the proportion of identified song types in their songs.

## Discussion

Phenomenon of the two nightingale species in sympatry is a long-studied evolutionary model, which is currently being used in studies on bird speciation. Former studies described an asymmetric convergence in their songs in sympatric areas and suggested relatively large proportion of Thrush Nightingales that, in contrast to Common Nightingales, use heterospecific phrases in their songs (Sorjonen 1986a, Lille 1988, Schmidt 1973, Becker 1995). Nohring (1943, cited in Sorjonen 1986a) suggested that Thrush Nightingale is more flexible in imitating heterospecific songs than the Common Nightingale.

An asymmetric ecological character displacement reflected by a slight shift in breeding habitats (Ranoszek 2001) and increased relative bill size (Reifová et al. 2011) have been documented in Common Nightingales in sympatry. Interspecific territoriality has been suggested as an explanation of these observations (Sorjonen 1986a, Reifová et al. 2011). Although mixed breeding pairs and later interspecific hybridization have been confirmed, the proportion of detected hybrids was consistently low in different studies (Becker 2007, Reifová et al. 2011). Despite their overall similarity, the two nightingales seem to differ substantially

in competitive ability in areas of their co-occurrence, where the Thrush Nightingale appears to be a stronger competitor than the Common Nightingale. As suggested by Reifová et al. (2011), the ongoing movement of the area of sympatry into the regions formerly occupied only by Common Nightingale might be facilitated by the dominance of the Thrush Nightingale.

Our data for the first time quantitatively confirmed the occurrence of mixed singers in Thrush Nightingales in the area of sympatry. Although the proportion of Common Nightingale phrases in sympatric Thrush Nightingale songs varied between individuals, all males analysed in this study were mixed singers. Importantly, combination of song comparisons and genetic analyses showed that heterospecific copying of songs, detected in these males, is not a direct consequence of interspecific hybridization. In congruence with former studies (Sorjonen 1986a, Becker 1995), our results imply that mixed singers probably occur in sympatry in large numbers. Similar observations were previously reported by Sorjonen (1986a) and Lille (1988), but these studies could not directly quantify the actual extent of the song type copying between species. Sorjonen (1987) defined song types based only on the repetitive section in a given song. In contrast, our definition of song types in Common Nightingales catalogue (criteria for song types suggested in Kipper et al. 2004) may better reflect the versatility in song structure and probably may be more appropriate to evaluate eventual mixed nature of a particular song. In addition, the approach used herein is well reproducible, applicable to and comparable with other studies using analogical methods.

The high proportion of mixed singers among Thrush Nightingales opens the question whether including heterospecific songs in their repertoires is adaptive or not. It has been suggested that secondary contact of closely related bird species which results in aggressive interspecific interactions can cause selection on traits that affect encounter rates (e.g., habitat preferences) or competitor recognition and fighting ability, and that competitor recognition can be influenced by coloration or song (Grether et al. 2009). Convergence in songs in Thrush Nightingale might facilitate better recognition of heterospecific neighbours in areas of co-occurrence, and might increase heterospecific aggression. Sorjonen (1986a) already suggested that character convergence gives rise to interspecific territoriality which results in the exclusion of food competitors from a territory. For a stronger competitor, able to defend the territory without eliciting physical encounters, such territoriality might be beneficial.

The weaker competitor in interspecific interactions seems to be the Common Nightingale, as suggested by its morphological and habitat shifts, which most likely reflect niche segregation between the two nightingale species in areas of sympatry (Ranozsek 2001,

Reifová et al. 2011). The Thrush Nightingale is probably the stronger competitor than the Common Nightingale because of its better ability of song matching to its neighbours and ability to respond to interspecific songs (Sorjonen 1986a). According to Cody (1969), increased similarity in songs could evolve concurrently with a decreasing territorial overlap between two species, and actually promote a behavioural response which separates territories and decreases competition over food resources. In other words, song convergence (mixed singing) might be adaptive for the Thrush Nightingale by means of increasing its competitive ability to defend territory in interactions with its congener. This might be of special significance in areas where Thrush Nightingales are rarer than Common Nightingales, as was suggested by Becker (1995).

As in other oscine birds, songs in nightingales are learned through social learning from a model - father or neighbour (Todt and Geberzahn 2003). Because both juveniles and adults are able to learn songs, they are considered 'open ended learners' (reviewed in Hultsch and Todt 2008). Individuals of both species are morphologically similar and in sympatry, they breed approximately at the same time and often near each other. For young birds, it might be difficult to discriminate between males of the two species, thus they might learn erroneously from a heterospecific tutor as suggested by Lille (1988). This was experimentally confirmed in captivity by Stadie (1983), who also showed that young birds retain species-specific song display even when the tutor is heterospecific. Erroneous learning thus might be an alternative explanation suggesting non-adaptive nature of heterospecific song copying.

The combination of cultural and genetic evolution causes the song of oscines to be generally a very plastic trait (reviewed in Marler 1997). Song plasticity and ability to learn new song also in adulthood might further facilitate song convergence. The Thrush Nightingale is commonly mixing its species-specific songs with those of the Common Nightingale in sympatry, and thus seems to be more plastic in heterospecific song learning than its congener. This conforms to the fact that in allopatry, the Thrush Nightingale is also known to have repertoires more similar to neighbouring males than to more distant males at particular locality (Sorjonen 1986a, Griesmann and Naguib 2002). It is in contrast with Common Nightingale, in which repertoires are rather similar to more distant males within the locality than to close neighbours (Hultsch and Todt 1981, Kipper et al. 2004). In addition, the Thrush Nightingale song has substantial geographic variation in contrast to Common Nightingale song, which is relatively similar between distant populations (Sorjonen 1986b, Hultsch 1980 cited in Lille 1988).

This crucial difference between the two nightingale species might indicate some species-specific predispositions for song copying. Possibly, nightingales might learn heterospecific songs also on common wintering grounds. Observations of singing, directly interacting and apparently competing males of both species in Sub-Saharan Africa (Pearson 1984) prove that they may have an opportunity to do so. This hypothesis might be supported by the observation that songs of Thrush Nightingale seemingly include Common Nightingales songs or syllables which are not present in neighbouring Common Nightingales on breeding grounds in sympatry, suggesting that these songs originate from elsewhere (Sorjonen 1986a).

As in the song acquisition in males, female preferences for conspecific songs are also the result of combination of genetic and cultural evolution (ten Cate and Vos 1999). Therefore, juvenile females in areas of sympatry may acquire future preferences for song by imprinting from both conspecific and heterospecific males. More variable song of mixed singers may function for such females as an additive attractant enriching otherwise species-specific song display. If mixed songs in Thrush Nightingales are more attractive for conspecific females, the proportion of mixed singers among males may increase in time and preferences for mixed song can eventually become established in a particular population. The high proportion of mixed singers among Thrush Nightingale males in sympatry might be a result of such scenario. In case of interspecific crossing, such preferences should not be transferred genetically into the next generation by females because of their sterility in F1 generation according to Haldane's rule and thus the rate of interspecific hybridization may remain at low levels which were observed by Becker (2007) or Reifová et al. (2011).

The patterns observed in nightingale sympatry can be compared with sympatry of two flycatcher species in their old European hybrid zone, where heterospecific copying of songs as well as hybridization rates are low (Haavie et al. 2004). This has been explained by reinforcement on plumage traits, increasing their role as a premating reproductive barrier, which decreased the role of songs for mate recognition and reduced the frequency of mixed singing in the pied flycatchers and caused divergence in songs of the collared flycatchers. Such scenario is not applicable in nightingales, where both species are morphologically very similar and females probably choose mates mainly based on their song. Lille (1988) actually considered species-specific songs in nightingales to be the most significant reproductive barrier between congeneric species.

Interestingly, high rates of both heterospecific copying and interspecific hybridisation have been documented in a recently established flycatcher hybrid zone in Sweden (Qvarnström et al. 2006). The situation in nightingale sympatry resembles the latter situation

by high rate of heterospecific copying of songs, but differs in the low hybridization rate. This suggests that some features of species-specific song display keep the proportion of mixed pairs and rate of hybridization low. While the song convergence in flycatchers seems to be rather increasing the rate of maladaptive hybridization (Haavie et al. 2004) the song convergence in nightingales obviously does not increase the rate of hybridization. The song convergence might be adaptive – although the mixed songs are attractive for females of Thrush Nightingales, species-specific song characteristics (which are retained in mixed songs) still remain crucial in mate recognition and keep the low rate of hybridization. In nightingales, the song convergence seemingly does not necessarily affect the role of song as a prezygotic reproductive barrier.

Song analyses of five genetically confirmed hybrid males revealed that all these hybrids were mixed singers. Interestingly, their songs included significantly higher proportion of Common Nightingale phrases than songs of mixed singers of sympatric Thrush Nightingale males. This might be caused by more opportunities to learn songs of both parental species during sensitive period as juveniles, by genetically enhanced ability to learn Common Nightingale songs in adulthood, or both.

Eventual relationship between increased ability of hybrids to compose more variable songs and consequently, their increased reproductive success, might facilitate introgression and existence of backcrosses in sympatry. If frequent, introgression could affect both morphology and behaviour in the sympatric zone. Then, it would be interesting to test whether and to what degree is the relative ability to copy heterospecific songs in Thrush Nightingales affected by introgression. Such scenario is difficult to assess using the markers used herein for genetic identification of hybrids. More detailed genetic analyses in the future, using more variable autosomal markers, may allow testing this challenging hypothesis.

## **Conclusions**

As only five interspecific hybrids were detected by genetic analyses, our data do not support the hypothesis that interspecific hybridization is the most important cause of the song convergence of Thrush Nightingale males in sympatry. We cannot rule out, however, that introgression has subtle effects on the tendency for heterospecific copying, and may influence the variation in proportion Common Nightingale songs among mixed singers. Heterospecific singing in the Thrush Nightingale is thus probably caused either by learning of juveniles or by copying from territorial neighbours in adulthood. Given the findings mentioned above, this model system is suitable for study of convergence of acoustic signals in birds and the effects

of hybridization and introgression on ecology and phenotype of these species in the contact zone. It justifies future experiments focused for example on study of male territoriality and effect of female preferences. Results of such studies might address the question whether song convergence in nightingales is adaptive or not.

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## CONCLUSIONS OF THE THESIS

- Data from this thesis for the first time quantitatively confirmed the occurrence of mixed singers in Thrush Nightingales in the area of sympatry.
- Heterospecific singing in the Thrush Nightingale is probably caused either by learning of juveniles or by copying from territorial neighbours in adulthood. The direct contribution of hybridization to this phenomenon is probably unimportant.
- Song analyses of five genetically confirmed hybrid males revealed that all these hybrids were mixed singers. Their songs included significantly higher proportion of Common Nightingale phrases than songs of mixed singers of sympatric Thrush Nightingale males.
- Additional analyses and further studies are needed for evaluation of role of introgression and to answer the question of adaptivity of song convergence.