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Subspecies-specific song preferences and diverged heterospecific discrimination in females of the bush-cricket *Isophya kraussii* (Orthoptera: Phaneropterinae)

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Divergence of the acoustic signals used in mate calling may be an important driver of speciation. Male song may vary accordingly to, in mismatch with or independently of female preferences. Therefore, to estimate the importance of male signal variation between subspecies, female preferences against subspecies-specific signal variants must be tested. We examined the female response probability in the two subspecies of the bush-cricket *Isophya kraussii* for consubspecific and heterosubspecific male signals, and also for the song of a closely related species (*I. camptoxypha*), which is sympatric with one, but allopatric to the other subspecies. Performing no-choice playback experiments, we found that females of both subspecies responded to the male song of their own subspecies with significantly higher probability than to heterosubspecific and heterospecific songs. Response specificity for consubspecific vs. heterosubspecific signals was not significantly asymmetric comparing the two subspecies. A significant difference was found, however, in the discrimination of heterospecific calls: females showed stronger discrimination against the song of *I. camptoxypha* in the subspecies sympatric with that species. Our results are best explained by a tightly covarying, stepwise coevolution of male signals and female preferences.

ADDITIONAL KEYWORDS: acoustic communication – mate recognition – signal evolution – speciation.

INTRODUCTION

Interpopulation divergence in sexual acoustic communication can be a crucial element of speciation (Wilkins, Seddon & Safran, 2013). It may be either an initial step when sexual acoustic communication evolves along different trajectories in allopatric populations due to sexual selection (e.g. Grace & Shaw, 2011; de Oliveira Gordinho *et al*., 2015), ecological constraints (Zuk, Simmons & Cupp, 1993; Ballentine, 2006; Cocroft, Rodriguez & Hunt, 2010), genetic drift or mutation (Campbell *et al*., 2010; Goodman *et al*., 2015) and cultural evolution (Lachlan & Servedio, 2004; Linossier *et al*., 2016) or it may arise during the secondary contact of previously isolated populations diverged in traits unrelated to sexual communication during allopatry. In those cases, divergence in sexual acoustic communication may take part in the reinforcement of the genetic isolation between them (Gerhardt, 2013). The resulting interpopulation divergence of male signals is often the first detected sign of the process of divergence. Thus, the examination of acoustic signals in morphologically similar populations led to the discovery of 'cryptic' species in several taxa of animals (Henry, 1994; Jones, 1997; Ng *et al*., 2016; Heller *et al*., 2017). However, to estimate the significance of signal divergence regarding reproductive isolation and to get closer to the understanding of the evolutionary process causing the divergence, we also need to examine the receiver side of the communication system: female preferences against male signal variants. If females prefer the male song variant of their own population, then it is reasonable to presume that female preferences are the dominant drivers of male signal divergence (Gray & Cade, 2000; Grace & Shaw,

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2011; Barbosa, Rebar & Greenfield, 2016) and the differences in acoustic communication are expected to be the significant components of prezygotic isolation between the populations. However, if females do not show definite preference for the male signal variants of their own population, then strongly directional sexual selection, sensory exploitation (Ryan & Rand, 1993) or processes other than intersexual selection are likely to have significant roles in the male signal divergence (Simmons, Zuk & Rotenberry, 2001; Velásquez *et al*., 2015). In those cases, the divergence in acoustic communication would not bar interpopulation crossings in case of their secondary contact.

Acoustic signalling is a characteristic behaviour of many species in the insect order Orthoptera (Robinson & Hall, 2002). Males emit their acoustic signals spontaneously to attract distant females or to stimulate their acoustic response. The majority of studies in which female preferences for male song patterns were experimentally investigated found definite preferences for conspecific male signals (e.g. Perdeck, 1958; Stumpner & von Helversen, 1994; Guerra & Morris, 2002). However, in some species, the behavioural response of females against different male song variants has proved to be non-discriminative (Heller, von Helversen & Sergejeva, 1997; Bush & Schul, 2010). Thus, the high level of interspecies acoustic diversity is paired with a hidden variation in female response specificity (Schul, von Helversen & Weber, 1998; Kowalski & Lakes-Harlan, 2011). Female response specificity may be even more variable during speciation. In this study, we examined female preferences for subspecies-specific male songs and heterospecific calling songs in the two subspecies of the bush-cricket *Isophya kraussii* Brunner von Wattenwyl.

Isophya is the second most species-rich genus within the subfamily Phaneropterinae in Europe (Heller *et al*., 1998). Most species in the genus show subtle morphological differences, but differ conspicuously in their male calling songs (Heller *et al*., 2004; Chobanov *et al*., 2013; Zhantiev, Korsunovskaya & Benediktov, 2017), suggesting that acoustic diversification is a dominant component of the evolution of species richness in this genus. In spite of the conspicuous acoustic diversity of *Isophya*, experimental studies testing the effectiveness of male song differences as premating barriers in the genus are rare (Zhantiev & Dubrovin, 1977; Zhantiev & Korsunovskaya, 1990; Orci, 2007). As it is usual in the subfamily of Phaneropterinae, *Isophya* males and females perform an acoustic duet during their mate-finding behaviour: females emit short acoustic signals (a single click or group of a few impulses) in response to the male calling songs (Heller, 1990). The impulse repetition pattern of those short response signals is variable, but females emit their response during a species-specific time window in relation to the male signal. Among the European species of the genus, *I. kraussii* has the largest distribution range (Heller *et al*., 2004; Cigliano *et al*., 2017), and recently, Iorgu & Heller (2013) described a new subspecies, *I. kraussii moldavica*, near the eastern border of the area of the nominotypic subspecies. The two taxa differ mainly in the rhythmic features of the male calling songs: *I. kraussii moldavica* males produce significantly shorter syllables (see Material and Methods for the bioacoustic terminology applied in this study) containing a smaller number of impulses in comparison to the male song of the nominotypic subspecies (Iorgu & Heller, 2013). In addition to that difference, the male calling songs are very similar in oscillographic structure in the two subspecies: calling males produce long sequences of evenly repeated syllables (Fig. 1A, C), where each syllable is composed of a main impulse series and a few after clicks (Fig. 1B, D). Male songs of the two subspecies have similar, wide-band power spectra containing dominant frequency components between 20 and 40 kHz (Iorgu & Heller, 2013). The main aim of our study was to examine how much the attractiveness of subspecies-specific male calling songs differs for females in both subspecies. The examination of that question may help us to estimate whether the divergence of acoustic communication could cause a non-random mating pattern in case of a secondary contact of the two taxa. Furthermore, our results provide some important pieces of information about the signaller–receiver coevolution in this insect. Another question examined in this study was whether females in the two subspecies of *I. kraussii* discriminate against the heterospecific calls of *Isophya camptoxypha* (Fieber), a species sympatric with one subspecies, but allopatric with the other (Kenyeres & Bauer, 2005; Bauer & Kenyeres, 2006; Iorgu & Heller, 2013). The male calling song in that species is composed of the same oscillographic elements as in *I. kraussii* (Fig. 1E, F) and has a similar, wide-band frequency spectrum (15–45 kHz), but differs in syllable duration, syllable repetition rate and in the number of impulses per syllable (Orci, 2007). The attractiveness of that heterospecific signal was examined to test acoustic heterospecific discrimination in *I. kraussii* and to see whether the two subspecies differ in that.

MATERIAL AND METHODS

Study animals

In both subspecies of *I. kraussii*, females were collected as large nymphs from their natural habitats. *Isophya k. kraussii* females originated from meadows and forest clearings in the Pilis Mountains (Hungary), and *I. k. moldavica* females were collected in the hillside meadows of Suceava county (Romania). All of them

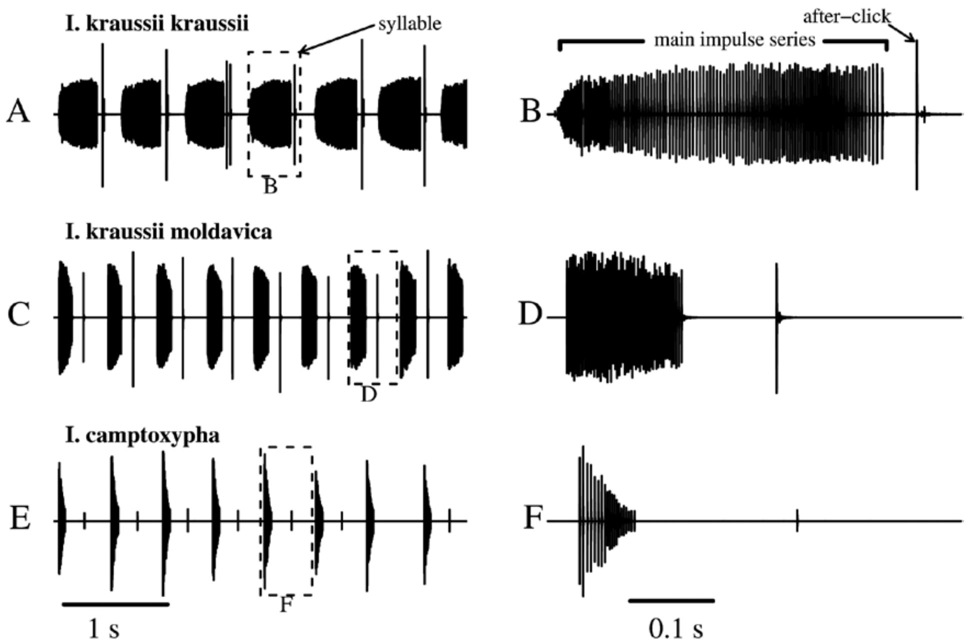

Figure 1. Oscillograms showing the rhythmic patterns of male calling songs in the two subspecies of *Isophya kraussii* (A–D) and in *I. camptoxypha* (E, F) at two time scale resolutions. A, C, E, series of syllables; B, D, F, single syllables. Oscillograms on the right are time axis magnified portions (as indicated by dashed line rectangles) of the left ones. Time scale bars at the bottom are for all oscillograms in the given column of oscillograms.

were reared to adults without any contact with males, nor could they hear any male signal. That way we tested virgin females with high motivation level concerning mate finding. Playback tests were done successfully with 16 females in *I. k. kraussii* and with 16 females in *I. k. moldavica*. During the whole period of the study, females were fed with dicotyledonous plants *ad libitum*.

BIOACOUSTIC TERMINOLOGY

The bioacoustic terminology used in this study follows Ragge & Reynolds (1998) with some additional terms that we found useful when describing the acoustic signals of phaneropterine bush-crickets (Heller *et al*., 2004).

Calling song: spontaneous song produced by an isolated male or the song produced by a male singing a duet with a female. Generally, this term is used in the literature only for the spontaneous song of an isolated male, but in the *Isophya* species examined in this paper, males use the same song type when calling alone and when duetting with a female.

Syllable: the song produced by one opening–closing movement cycle of the tegmina.

Syllable repetition period (SRP): one syllable and the following silent intersyllable interval.

Duration of syllable (DS): the time elapsed from the first to the last impulse of the main impulse series of a syllable.

Impulse: a simple, undivided, transient train of sound waves (here: the highly damped sound impulse arising as the impact of one tooth of the stridulatory file).

Click: an isolated, distinct impulse.

PLAYBACK STIMULI

Playback stimuli were made from original male calling song recordings. Male song recordings originated from the same population from which females were collected in the two subspecies of *I. kraussii*. Song samples of *I. camptoxypha* males were recorded from populations living in the Bieszczady Mountains (Poland) and in the Mecsek Mountains (Hungary). Male song stimuli (96 kHz sample rate, 16 bit) were modified with minimal acoustic processing: all recordings were high pass filtered (cut-off frequency: 3 kHz) and normalized to 90% of the available 16-bit amplitude range. Sound recordings from five males in both subspecies of *I. kraussii* and in *I. camptoxypha* were chosen (altogether, samples of 15 males were included). Each stimulus contained a series of 30 syllables from a single male. Those 15 playback stimuli were arranged in three stimulus series with a randomized stimulus order. In each stimulus series, every stimulus was present in one copy and stimuli were separated from each other by a silent interval of 45 s.

PLAYBACK PROTOCOL

Each female was tested separately. Stimulus series composed of original male song samples were played back to the tested female and her acoustic response songs were recorded together with the playback stimuli. To measure the attractiveness of the tested male song samples, the number of syllables answered vs. not answered by the tested female was counted by examining the sound recording of the playback trial (Fig. 2). We applied a no-choice (single-stimulus) playback design. In comparison to two-choice playback, this design gives less-sensitive results regarding the difference of attractiveness of the tested stimuli (Tauber *et al*., 2001), but provides a more rigorous test of the ability of females to choose a consubspecific/conspecific reproductive partner on the basis of its acoustic signal (Gerhardt & Huber, 2002). Each examined female was placed in a cylinder-shaped mesh cage (diameter 8 cm, height 12 cm). The cage was enclosed in an acoustic isolator box made of 18-mm-thick sheets of laminated wood. The interior of the box was lined with a 5-cm-thick acoustic foam. The box contained a speaker (Alpine SPS-130A) emitting the playback stimuli and a microphone (Brüel & Kjaer, type: 4191) for recording the acoustic response of the female. The microphone was placed between the speaker and the cage. Both the microphone and speaker were directed towards the cage of the tested female from a distance of 5 and 20 cm, respectively (Supporting Information, Fig. S1). Playback stimuli were broadcasted to the experimental animal from a Zoom H4n digital recorder through a Mac Audio (MPX 2000) amplifier driving the speaker [Alpine SPS-130A, frequency response flat (±3 dB) between 150 and 30 000 Hz] within the isolator box. The amplitude of playback stimuli was 69 (±2) dB SPL at the centre of the cage of the experimental animal, based on the root mean square amplitude of the main impulse series of the syllables. The acoustic response of the tested female was recorded using the software Avisoft Recorder 4.2.15 (Avisoft Bioacoustics, Germany) running on a computer connected to a National Instruments data acquisition card (NI USB 6122), which received its input from a 1/2-inch Brüel & Kjaer microphone (type: 4191) through a signal conditioning amplifier (Brüel & Kjaer, Nexus). All three stimulus series were presented to each tested female in a random order so that we could examine whether or not a female response was elicited in the case of 30*15*3 syllables (1350 observations per female) contained by the three stimulus series. We succeeded in performing the playback experiment with 16 females in *I. k. kraussii* and 16 females in *I. k. moldavica*. The software Adobe Audition 1.5 (San Jose, CA, USA) was used to count the number of female responses elicited by the playback stimuli. Oscillograms presented in this paper were produced using the R-package ‘seewave’ (Sueur, Aubin & Simonis, 2008).

STATISTICAL ANALYSIS

Our main question was whether male song type (consubspecific, heterosubspecific, heterospecific) has an effect on female response probability in the two subspecies of *I. kraussii*. To examine that question, a mixed-effect logistic regression model was calculated using the lme4 package (Bates *et al*., 2015) running in the R statistical environment (R Core Team, 2014). A logistic regression model describes the behaviour of a categorical, dependent variable in response to

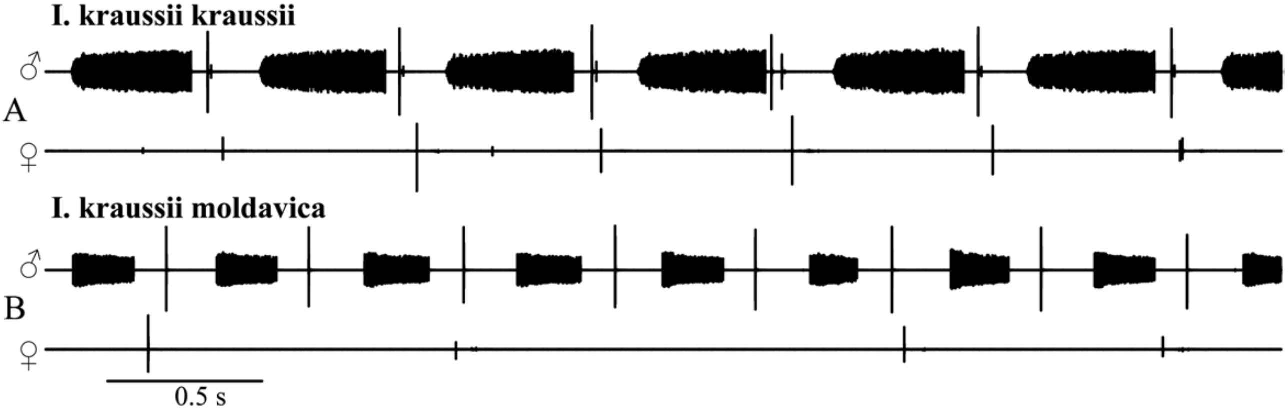

Figure 2. Two-traced oscillograms of male–female pair-forming duets: A, *Isophya kraussii kraussii*; B, *I. kraussii moldavica* (in order to make the duet structure easier to observe, male song was removed from the female tracks).

one or more explanatory (independent) variables. In its basic version, the examined dependent variable is binary and can be given as a series of values 0 and 1 or the proportion of those two outcomes (Hilbe, 2009). The dependent variable of our model was the ratio of answered vs. not answered syllables in the playback stimuli. Our observations (whether a female response song occurred) were not independent of each other because of the repeated observations of females, the repeated presentation of the same playback stimuli and the repeated use of the same three randomized stimulus series. Therefore, a mixed-effect model was calculated where random factors can account for the grouped pattern of observations (Faraway, 2016). The calculated mixed-effect logistic regression model included two fixed-effect variables and their interaction: (1) subspecies identity of female and (2) male song type as a factor with three levels such as consubspecific, heterosubspecific and heterospecific. Furthermore, the following random factors were included in the model: specimen identity of the tested females, specimen identity of the males from which the playback stimuli were recorded and the identity of the stimulus series.

RESULTS

A mixed-effect logistic regression model calculated on the basis of our playback results (Supporting Information, Table S1) showed that females of both subspecies responded to the male song of their own subspecies with significantly higher probability than to heterosubspecific and heterospecific male songs (Table 1; Fig. 3). Response proportion (syllables responded/all presented syllables) for consubspecific songs was similar in the two subspecies (Fig. 3), and the female's subspecies identity had no significant effect on the probability of response to own-subspecies male song (Table 1). No significant interaction between female subspecific identity and the effect of heterosubspecific male song on response probability was detected (Table 1). In contrast, interaction between female's subspecies identity and the effect of heterosubspecific male song on response probability was significant: *I. k. kraussii* females discriminated against heterospecific (*I. camptoxypha*) songs more strongly than *I. k. moldavica* females did.

DISCUSSION

Females in both subspecies of *I. kraussii* responded with significantly higher probability to consubspecific male calling songs than to heterosubspecific signals. However, with lower probability, heterosubspecific male songs were also effective in eliciting female response (Fig. 3; Table 1). These findings suggest that the divergence of male–female acoustic communication would cause an assortative mating pattern in the case of secondary contact of the two taxa, but acoustic divergence alone could not maintain a complete reproductive isolation between them. Therefore, our results are in accordance with the present subspecific treatment of the two taxa.

Sometimes, male signals vary significantly between populations, but their conspicuous differences are not important to females. For example, in the case of the frog *Pleurodema thaul*, a remarkably high, interpopulation call divergence was detected (Velásquez *et al*., 2013). That divergence seems to be unimportant for females and is likely to be the outcome of inter-male competition (Velásquez *et al*., 2015). New features of male signals may evolve whether or not those are

Table 1. Fixed-effect estimates of a mixed-effect logistic regression model testing the effect of male song taxonomic identity on the probability of acoustic response in females of the two subspecies of *Isophya kraussii*

Fixed effect	Estimate	SE	*z*	*P*
Intercept	0.217	0.295	0.733	0.464
Male song is heterosubspecific (reference level: male song is consubspecific)	**–2.118**	**0.274**	**–7.741**	**<0.0001**
Male song is heterospecific (ref. consubspecific male song)	**–4.576**	**0.286**	**–16.027**	**<0.0001**
Female's subspecies identity: *I. kraussii moldavica* (ref. *I. kraussii kraussii*)	0.075	0.417	0.18	0.857
Interaction: male song heterosubspecific * female's subspecies identity is *I. k. moldavica*	0.241	0.543	0.443	0.657
Interaction: male song heterospecific * female's subspecies identity is *I. k. moldavica*	**3.456**	**0.288**	**12.001**	**<0.0001**

Model estimates are given in logits. Model formula: probability of female acoustic response ~ male song type * female subspecific identity + (1 | female_id) + (1 | male song sample_id) + (1 | male song sample series_id). $N_{\text{observations}} = 1440$, $N_{\text{females}} = 32$, $N_{\text{male song samples}} = 15$, $N_{\text{male song sample series}} = 3$; male song type is a factor with three levels: consubspecific, heterosubspecific and heterospecific; one observation means a data pair: the number of answered vs. not answered syllables in case of a stimulus containing 30 syllables.

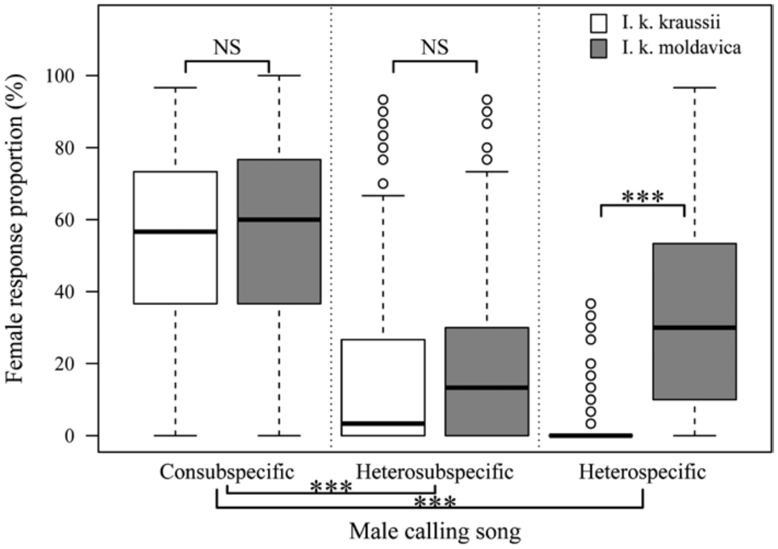

Figure 3. Variation of female response ratio measured during single-stimulus playback experiments in the females of the two subspecies of *Isophya kraussii* in response to consubspecific, heterosubspecific and heterospecific (*I. camptoxypha*) male song samples. Statistical significance of differences (NS, not significant, $***P < 0.001$) is indicated based on the results of a mixed-effect logistic regression model calculated on the basis of the playback results (Table 1). The figure is based on the playback trials of 32 females (16 females in each subspecies).

important to their females, as in the bush-crickets *Neoconocephalus retusus* and *N. maxillosus*, where females show no preferences for the derived, double-pulsed calls of the conspecific males (Bush & Schul, 2010). Another possibility is that the variation in male signals significantly affects their attractiveness, but female preferences mismatch male signal variation. For example, in the frog species group *Physalaemus pustulosus*, females show preferences for signal traits not produced by their males, but those are present in some of the closely related species in the group, suggesting that the evolution of male signals is driven by pre-existing female sensory biases (Ryan & Rand, 1993). Our study shows the most frequently reported pattern, where variation in male signals matches the variation of female preferences when closely related taxa are compared (e.g. Henry, 1985; Barth & Schmitt, 1991; Rodríguez, Ramaswamy & Cocroft, 2006; Boumans & Johnsen, 2014). Our results provide only a snapshot of the evolution of male–female acoustic communication in the two subspecies of *I. kraussii*, and therefore, we cannot make well-supported inferences about the evolutionary history of signaller–receiver divergence in this taxon pair; nevertheless, the supported concordance between female preferences and male signal pattern is most likely to result from tight, gradual coevolution of male song and female preferences.

Reproductive isolation between closely related allopatric or parapatric populations is often asymmetric, which may help us to make inferences about the sequence of ancestry (Kaneshiro, 1980), the progress of the speciation process (Arnold, Verrell & Tilley, 1996) and gene flow during hybridization (Hochkirch & Lemke, 2011). Our results suggest that reproductive isolation associated with acoustic communication is not strongly asymmetric in the case of this pair of subspecies (Tab. 1; Fig. 3). A quantitative genetic model (Arnold *et al*., 1996) predicts that high levels of asymmetry in reproductive isolation are most likely to occur during the intermediate phase of speciation, suggesting that the two subspecies examined here are at an initial or at a highly progressed phase of speciation. Morphology of the stridulatory files suggests that the latter may be the case, since the stridulatory files show clear differences in the two subspecies (Iorgu & Heller, 2013), indicating that behavioural divergence [which often appears to be faster than morphological differentiation (Henry, 1994; Jones, 1997)] is accompanied by the divergence of stridulatory organs.

Location-specific interaction with sympatric species is a potential driving force of speciation. Concerning the evolution of mate recognition, the presence or absence of closely related species is of special importance (Hamao, 2016). We found that females of the two subspecies of *I. kraussii* showed significantly different levels of discrimination against the song of *I. camptoxypha*, a closely related species (Fig. 3). *Isophya camptoxypha* is sympatric with *I. k. kraussii*, but allopatric with *I. k. moldavica*. As expected, *I. k. kraussii* females discriminated more strongly against the male song of *I. camptoxypha* than females of *I. k. moldavica* did. A possible explanation for this is that females are not exposed to a selection pressure favouring discrimination against a signal type of an allopatric relative, since they have no chance to meet and hybridize. Similarly, weaker discrimination against allopatric relatives than against sympatric ones has been reported in a wide range of animals (Barth & Schmitt, 1991; Honda-Sumi, 2005; Braune, Schmidt & Zimmermann, 2008; Bewick & Dyer, 2014). Another possible explanation, not excluding the previous one, for the weak discrimination of *I. k. moldavica* females against *I. camptoxypha* song is that the male signals of the two taxa are similar to each other (see the oscillograms in Fig. 1). The male songs of *I. k. kraussii* and *I. camptoxypha* differ conspicuously in the DS, and *I. k. moldavica* is intermediate regarding this signal parameter. Therefore, even with the same accuracy of signal recognition, *I. k. moldavica* females may be more responsive to *I. camptoxypha* songs than *I. k. kraussii* females are.

Our study is one step towards a better understanding of the evolution and behaviour of these insects. A number of interesting questions arise and await for further examination. Which signal traits are important and which ones are unimportant regarding mate recognition or sexual selection in the two subspecies? The chemical communication channel may also be involved in mate recognition, as in other orthopterans (e.g. Ritchie, 1990; Tyler *et al*., 2015; Finck & Ronacher, 2017); furthermore, postzygotic isolation may develop simultaneously with prezygotic isolation in allopatric closely related taxa (Gray *et al*., 2016). Geographic patterns in acoustic signalling and molecular genetic divergence (Pecsenye, Vadkerti & Varga, 2003) could provide us information needed to answer further interesting questions regarding the evolution of these subspecies. For example, is the larger male song difference between *I. k. kraussii* and *I. camptoxypha* a result of character displacement? We hope that our study draws attention to this species, which may be a suitable subject for further research on speciation, sexual selection and the relationship between those two processes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. A schematic drawing showing the configuration of playback devices within the isolator box from above. S, speaker; M, microphone; A, cage of the tested bush-cricket female; arrows show the direction of speaker and microphone.

Table S1. The results of playback experiments examining the response rate of females to consubspecific, heterosubspecific and heterospecific male songs in the two subspecies of *Isophya kraussii*.