



A narrow hybrid zone between the grasshoppers *Stenobothrus clavatus* and *Stenobothrus rubicundus*: courtship song analysis

VARVARA VEDENINA^{1*}, JAN SRADNICK^{2‡}, ANJA KLÖPFEL² and NORBERT ELSNER^{2†}

¹*Institute for Information Transmission Problems, Russian Academy of Sciences, Bolshoy Karetny pereulok 19, 127994 Moscow, Russia*

²*Department of Neurobiology, Johann-Friedrich-Blumenbach-Institute, Berliner Strasse 28, D-37073 Göttingen, Germany*

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The closely related grasshopper species *Stenobothrus rubicundus* and *Stenobothrus clavatus* are known to hybridize in a very narrow contact zone on Mt. Tomaros in northern Greece. These species produce very different and complex courtship songs accompanied with visual display. We analyzed the courtship songs and underlying stridulatory movements of the hind legs in natural hybrids from Mount Tomaros. The two species were also hybridized in the laboratory and their songs were compared with the songs of the natural hybrids. Some hybrid songs were shown to have intermediate features between parental songs, whereas other hybrid songs comprised completely new elements. The *clavatus*-like song elements were found to dominate in hybrid songs. These song features may influence the mating success of hybrid males in the contact zone. A comparison of hybrid songs with the song pattern of the north European *S. rubicundus* populations allowed us to suggest a scenario of *S. rubicundus* and *S. clavatus* origin. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, 107, 383–397.

ADDITIONAL KEYWORDS: courtship behaviour – grasshopper – hybrid zone – sexual selection – visual display.

INTRODUCTION

Hybrid zones, where genetically distinct groups of organisms interact and leave some offspring of mixed ancestry, are found in all major groups of higher organisms (Harrison, 1986, 1993; Barton & Hewitt, 1989; Jiggins & Mallet, 2000). Hybrid zones are considered to comprise excellent natural laboratories because hybridizing taxa offer good experimental material for studying the evolution of reproductive barriers. Interspecific reproductive barriers can be classified into pre-mating, post-mating prezygotic,

and post-zygotic isolation mechanisms according to the time when they occur during the life cycle (Dobzhansky, 1937; Mayr, 1963; Coyne & Orr, 2004). Intrinsic post-zygotic incompatibilities such as hybrid inviability and sterility have been considered as the classical driving force behind speciation (Coyne & Orr, 2004). At the same time, more intriguing processes occur when pre-mating isolation evolves faster than post-zygotic barriers. It is assumed that pre-mating isolation may be achieved by assortative mating evolving as a result of sexual selection, when a parallel change in mate preference and secondary sexual traits takes place (West-Eberhard, 1983; Andersson, 1994; Panhuis *et al.*, 2001). However, the explicit function of sexual selection in rapid speciation remains a question of debate because it is difficult to reveal and convincingly demonstrate this process.

Grasshoppers of the subfamily Gomphocerinae (Insecta: Orthoptera: Acrididae) are an excellent

*Corresponding author. E-mail: vedenin@iitp.ru

‡Current address: Division of Nephrology, Department of Internal Medicine III, University of Technology Dresden, Germany.

†We announce with deep regret the death of Norbert Elsner, initiator and inspirer of the present study, on 23 June 2011.

model for studying the establishment of premating isolation barriers. In these insects, the song is suggested to be the most important component of reproductive isolation. The males produce calling songs for searching conspecific mates at a distance, and courtship songs are produced in the immediate vicinity of a female. The courtship songs in some species can reach a high complexity and can be accompanied by conspicuous visual display (Otte, 1970; Helversen, 1986; Ragge & Reynolds, 1998). There are relatively few well documented examples of hybrid zones between singing grasshopper species. One of the examples is the hybrid zone between *Chorthippus brunneus* and *Chorthippus jacobsi* in northern Spain (Bridle, Baird & Butlin, 2001; Bridle & Butlin, 2002; Bailey, Thomas & Butlin, 2004; Saldamando *et al.*, 2005; Bridle *et al.*, 2006). These species are very similar in morphology but differ in their calling songs. Premating isolation (not only behavioral, but also spatial and seasonal isolation) was shown to play an important role in generating the genotypic composition of the zone. In addition, hybrid males were shown to have reduced fitness because their songs were unattractive to both parental and hybrid females. At the same time, no post-zygotic intrinsic barriers have been found between these species. Another example of the hybrid zone between singing grasshopper species is a wide contact zone between *Chorthippus albomarginatus* and *Chorthippus oschei* in Ukraine and Moldova (Vedenina & Helversen, 2003; Vedenina, Kulygina & Panyutin, 2007a; Vedenina, Panyutin & von Helversen, 2007b; Vedenina, 2011). These species are also quite similar in morphology and even in their calling songs, although they produce quite different courtship songs. In these species, premating ethological isolation was shown to be predominant; however, some post-zygotic barriers have also been found. Hybrid offspring obtained from the reciprocal crosses demonstrated a different degree of survival. Asymmetry was also found in the mating success of the hybrid males.

A recently described hybrid zone between related grasshopper species *Stenobothrus rubicundus* and *Stenobothrus clavatus* in Greece is of special interest (Elsner, Klöpfel & Sradnick, 2009; Sradnick, 2010). The species hybridize in a very narrow contact zone on Mount Tomaros in northern Greece. These species are remarkably different in several morphological characters, as well as in both calling and courtship songs. *S. clavatus* has conspicuous spatulate and darkened tips of antennae, whereas *S. rubicundus* has filiform antennae. The wings of the two species are also different: their forewings are of different shape, and the hind wings in *S. rubicundus* are dark and have heavily sclerotized leading edges (Elsner &

Wasser, 1995a), whereas, in *S. clavatus*, the hind wings are lighter and without sclerotized edges (Elsner *et al.*, 2009). It is remarkable that these species use either antennae or wings in visual display during courtship behaviour. When courting, *S. rubicundus* stridulates not only with the hindlegs, but also with the wings, and *S. rubicundus* from Greece alternate these two mechanisms in a specific order (Elsner & Wasser, 1995a). *S. clavatus* demonstrates a characteristic visual display with the antennae when producing a particular phase of courtship (Ostrowski *et al.*, 2009). Additionally, the courtship songs of the two species are quite different and very complex (Elsner & Wasser, 1995a; Ostrowski *et al.*, 2009).

The hybrid zone between *S. rubicundus* and *S. clavatus* is notable because the occurrence of both species on Mount Tomaros is limited by the particular altitude: they do not occur lower than 1300 m a.s.l. (Elsner *et al.*, 2009). An area of distribution of these species on Tomaros covers no more than 16 km². Thus, this hybrid zone can be identified as an island zone, where processes such as the dispersal of parental genotypes into the contact zone, exchange of gene flow, and rate of hybridization may run in a special way. Second, the hybridizing species *S. rubicundus* and *S. clavatus* may offer a striking example of sexual selection in rapid speciation. Not only song evolution, but also evolution of morphological traits (e.g. the shape of antennae and wing venation) may be faster in *S. rubicundus* and *S. clavatus* than in other grasshopper species. Because wings and antennae are actively used by these species during courtship behaviour, these traits may play an important role in an assortative mating.

In the present study, we analyzed the courtship songs of the natural hybrids between *S. rubicundus* and *S. clavatus* and compared them with the songs of the laboratory hybrids. We analyzed not only the sound patterns, but also the underlying stridulatory movements of the hind legs. Because *S. rubicundus* stridulates not only with hindlegs, but also with wings, the analysis of the leg-movement patterns in hybrid grasshoppers allowed us to find both intermediate song patterns and novel elements compared to the parental songs. The hybrid song analysis allowed us to suggest a different mating success of hybrid males on Mount Tomaros.

MATERIAL AND METHODS

SPECIMENS

All specimens used for the song recordings and song analysis were collected in northern Greece. Allopatric *S. rubicundus* was collected on Mount Mitsikeli

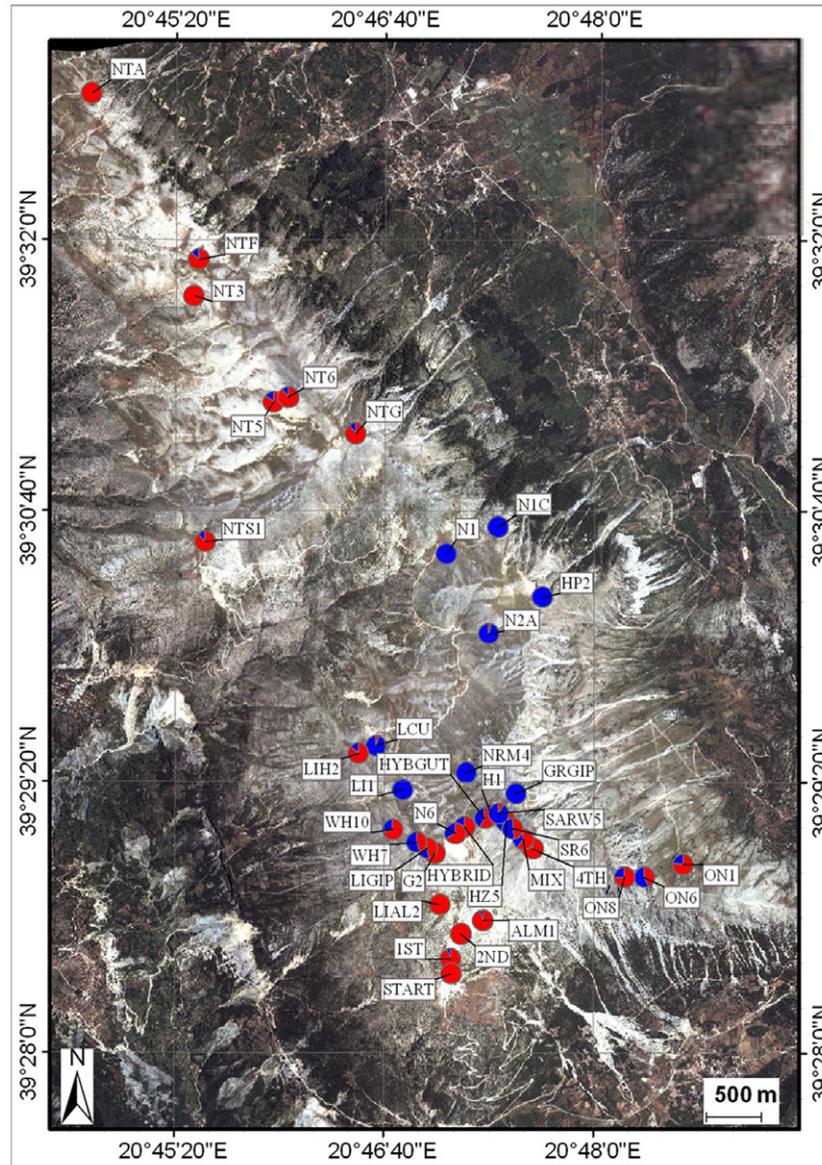


Figure 1. Map showing sample sites on Mt. Tomaros. Pie charts represent mean song phenotypes based on PC1 (details shown in Fig. 6); red, *clavatus*-song phenotypes; blue, *rubicundus*-song phenotypes.

(23 km north-east of Mount Tomaros) during the summer seasons of 2005–2009 and on Mount Vernon (150 km north from Mount Tomaros) in 2006. Allopatric *S. clavatus* was only found on Mount Xerovouni (16 km south-east from Mount Tomaros) during the summer seasons of 2007–2009. On Mount Tomaros, where the two species meet and hybridize, the specimens were collected from 37 sites during the summer seasons of 2005–2009 (Fig. 1, Table 1). *S. rubicundus* was also collected in Slovenia, Brje. The courtship songs of the latter specimens were only used for the comparison with the hybrid songs.

LABORATORY CROSSES

Laboratory crosses were conducted in 2009–2010. An F_1 hybrid generation was obtained from the crosses of allopatric *clavatus* females with allopatric *rubicundus* males from Mount Mitsikeli and of allopatric *rubicundus* females from Mount Valtou (20 km south-east from Mount Tomaros) with allopatric *clavatus* males. We also obtained a F_2 generation and backcrosses with both parental species. To obtain hybrids, a single virgin female was placed with a single male in a separate cage and kept together for 1–2 weeks. All

Table 1. Sampling sites on Mount Tomaros

Name of locality	Longitude (N)	Latitude (E)	Height (m)	Number of males recorded
1ST	39°28'27,7"	20°47'05,5"	1368	5
2ND	39°28'35,0"	20°47'09,4"	1425	1
4TH	39°29'00,2"	20°47'36,8"	1876	2
ALM1	39°28'38,9"	20°47'17,8"	1514	3
G2	39°28'58,9"	20°46'59,4"	1795	6
GRGIP	39°29'16,4"	20°47'30,3"	1918	3
H1	39°29'10,2"	20°47'20,9"	1854	16
HP2	39°30'14,5"	20°47'40,4"	1401	1
HYBGUT	39°29'09,2"	20°47'19,9"	1833	11
HYBRID	39°29'06,6"	20°47'10,8"	1790	4
HZ5	39°29'08,3"	20°47'26,6"	1888	7
LCU	39°29'30,5"	20°46'36,9	1545	4
LI1	39°29'17,7"	20°46'47,2"	1661	3
LIAL2	39°28'43,7"	20°47'01,3"	1543	1
LIGIP	39°29'00,3"	20°46'56,4"	1803	17
LIH2	39°29'20,3"	20°46'29,4"	1581	4
MIX	39°29'03,0"	20°47'33,1"	1880	2
N1	39°30'27,3"	20°47'03,6"	1438	1
N1C	39°30'35,2"	20°47'23,3"	1398	2
N2A	39°30'03,7"	20°47'19,7"	1595	1
N6	39°29'04,7"	20°47'07,3"	1793	4
NRM4	39°29'22,6"	20°47'11,3"	1811	3
NT3	39°31'43,4"	20°45'26,9"	1516	1
NT5	39°31'12,1"	20°45'57,4"	1667	4
NT6	39°31'13,5"	20°46'03,0"	1720	1
NTA	39°32'43,2"	20°44'47,7"	1267	2
NTF	39°31'54,1"	20°45'28,7"	1456	3
NTG	39°31'02,8"	20°46'28,7"	1765	1
NTS1	39°30'30,7"	20°45'31,3"	1540	3
ON1	39°28'55,7"	20°48'34,3"	1313	1
ON6	39°28'51,9"	20°48'19,5"	1497	2
ON8	39°28'51,9"	20°48'11,9"	1576	2
SARW5	39°29'10,6"	20°47'23,7"	1884	7
SR6	39°29'06,1"	20°47'29,2"	1885	14
START	39°28'23,3"	20°47'05,6"	1326	1
WH10	39°29'05,8"	20°46'43,2"	1710	2
WH7	39°29'02,1"	20°46'52,5"	1784	4

females were supplied with moist sand for oviposition. Egg pods were collected and placed in Petri dishes containing moist sand. These dishes were stored for 6 months in a refrigerator at 4 °C for diapause. To start hatching, egg pods were transferred from the fridge to room temperature; after 2–3 weeks, nymphs started to hatch. Grasshoppers were bred at 25 °C daytime/16 °C night-time, under a 12 : 12 h light/dark cycle in mesh cages (10 × 10 × 15 cm). Grasshoppers were fed with different species of *Festuca* grass, which was changed every 2–3 days. On the first day after imaginal moult,

females were isolated from males. All specimens were marked with paint on the back of the pronotum.

The crosses between *clavatus* females and *rubicundus* males gave a viable and fertile offspring comparable to offspring from the pure species. By contrast, the reciprocal crosses resulted in a smaller number of offspring because very few or no nymphs hatched from the majority of eggs. At the same time, F_1 hybrids between *rubicundus* females and *clavatus* males that reached adulthood were viable and fertile.

SONG RECORDINGS

All song recordings were made in the laboratory. To record the courtship song, a male was placed nearby a female on a round, rotatable and temperature controlled heating plate. The ambient temperature near a singing male varied between 35 and 41 °C. Not only the sound, but also the movements of the hind legs during stridulation were recorded with an optoelectronic device (Helvesen & Elsnér, 1977). This technique allowed us to conduct a more comprehensive analysis of the elaborate song pattern. A small piece of light-reflecting foil was attached to the distal end of each hind femur. A light spot sent through the camera's optics illuminated the foil and generated voltage in a photosensor linearly correlated to the position of the light spot on the hind leg. Stridulatory sounds were recorded with a piezo microphone connected to a flexible rod. All data were digitally stored on a computer via a data acquisition card (National Instruments) with the software LAB-VIEW, version 7 (National Instruments) and visualized later with DIADEM, version 9.1 (National Instruments) and TURBOLAB, version 4.0 (Bressner Technology). The sampling rate was 40 kHz for recording both sound and stridulatory movements. Courtship song recordings were made from 213 males. In particular, recordings were conducted from 12 males of allopatric *S. rubicundus*, 13 males of allopatric *S. clavatus*, 150 males from 37 sites of Mount Tomaros, nine males of F_1 hybrids obtained from one direction of the cross (*clavatus* females × *rubicundus* males) and four males of F_1 hybrids obtained from the reverse direction the of cross, two males of F_2 hybrids, nine males of backcrosses with *rubicundus* and 14 males of backcrosses with *clavatus*.

RESULTS

COURTSHIP SONGS OF PURE SPECIES

Courtship song of *S. clavatus* (Ostrowski *et al.*, 2009) comprised four phases (Fig. 2A; see also Supporting information, Video S1). The initial phase I could last for up to 15 min and was characterized by the

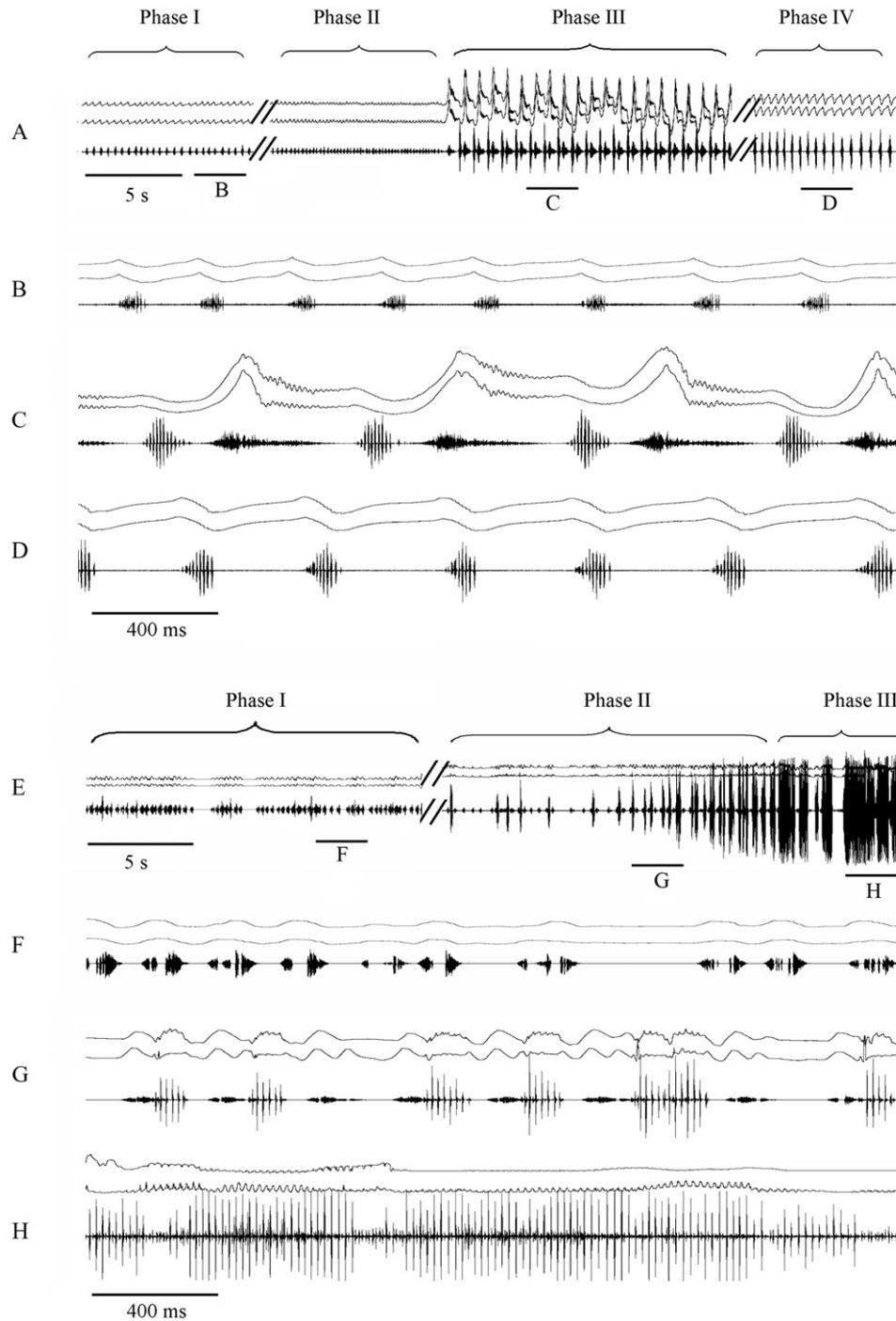


Figure 2. Oscillograms of the courtship songs (the two upper lines are recordings of hind leg movements and the lower line is the sound recording) in *Stenobothrus clavatus* (A–D) and *Stenobothrus rubicundus* (E–H). (B–D) Sections of *S. clavatus* song and (F–H) sections of *S. rubicundus* song at an expanded time scale of the indicated parts of the songs shown in (A) and (E), respectively.

small-amplitude up and downstrokes of the hindlegs. Very quiet pulses were produced during downstrokes only (Fig. 2B). In the relatively short (10–20 s) phase II, up and down leg movements followed at a much

higher rate than in phase I. The sound produced was similar to that in phase I and only occurred during the downstroke. Phase III followed phase II with no obvious transitional elements and comprised, on

Table 2. Song characters chosen for calculation of a hybrid index score

Song character	<i>clavatus</i> (<i>N</i> = 13)	Males from Mount Tomaros (<i>N</i> = 150)	<i>rubicundus</i> (<i>N</i> = 12)
1* Relative sound amplitude of pulses produced by upstroke and downstroke in phase I	0	0.35 (0.15; 0.58)	0.70 (0.58; 0.78)
2 Duration of sound pulse produced by downstroke in phase I (s)	0.067 (0.062; 0.077)	0.056 (0.047; 0.064)	0.039 (0.035; 0.050)
3 The number of the wing beats in <i>rubicundus</i> -like phase II	0	0 (0; 12.5)	45 (17.6; 68.6)
4* Relative sound amplitude of pulses produced by wing and leg movements in <i>rubicundus</i> -like phase II	0	0 (0; 3.15)	3.08 (2.67; 4.00)
5† The relative number of simple series to series of pulses in <i>rubicundus</i> -like phase II	0	0.6 (0; 2.33)	2.38 (2.24; 3.80)
6 Duration of <i>rubicundus</i> -like phase III produced by wing beats (s)	0	1.3 (0; 1.37)	5.2 (2.5; 6.7)
7 The number of strokes produced by the legs in <i>clavatus</i> -like phase III	20.7 (19.3; 24)	9 (0; 21)	0
8 The number of series of pulses produced by stepwise leg movements in <i>clavatus</i> -like phase IV	10.5 (5; 17)	2 (0; 7.8)	0

Medians and the lower/upper quartiles (in parenthesis) are shown.

*Relative sound amplitude was calculated from measurements of signal on oscillograms; †We were not concerned with whether series of pulses were produced by the wing or leg movements.

average, 22 high-amplitude strokes with the hind femora. During the upstroke and the beginning of the downstroke, an uninterrupted quiet noise was produced. At the end of the downstroke, the femora were moved in a stepwise manner, which resulted in the emission of a loud series of short pulses (Fig. 2C). These high-amplitude leg movements were accompanied by conspicuous antennal movements. During phase IV, approximately ten low-amplitude movements of the hind legs were produced and each movement comprised a simple upstroke and a stepwise downstroke. Sound was only produced during the downstroke and was similar to the series of short pulses emitted in phase III (Fig. 2D).

The courtship song of *S. rubicundus* (Elsner & Wasser, 1995a) typically consisted of three phases (Fig. 2E; see also the Supporting information, Video S2). Phase I comprised a long series of relatively quiet double pulses produced by simple up and down movements of the hindlegs (Fig. 2F). The sound produced at the downstroke has a slightly higher intensity than that at the upstroke. Phase II included pulses produced by two different mechanisms: leg and wing movements. Simple double pulses produced by the legs started to alternate with series of five to eight brief pulses produced by wing-beats (Fig. 2G). Initially, this happened occasionally but, later, it became more frequent and, towards the end of this phase, every first or second double pulse alternated with a

wing-beat series. The amplitude of the wing movement was so large that the pieces of reflecting foil at the tips of the hind femora regularly interfered with wings. As a result, the trace representing the leg movement was modulated in the rhythm of the wing-beats, which could give the misleading impression of jerky leg movements. Phase III of the courtship was composed of only the wing stridulation performed first on the ground and then in flight (Fig. 2H).

SONGS OF MALES FROM MOUNT TOMAROS

Seventy-two of 150 (48%) males from Mount Tomaros produced either *clavatus*-like or *rubicundus*-like courtship songs. Another half of the specimens from Mount Tomaros produced songs more or less different from those of the pure species. In 23 (15%) males, the courtship songs contained elements clearly intermediate between *S. rubicundus* and *S. clavatus*. For example, during phase I, a sound that was different from that of *S. clavatus* was produced during up- and downstroke, although the upstroke sound was much quieter than that in *S. rubicundus* (Table 2). Intermediate songs also had combinations of different parental elements, and the order and duration of these elements were quite variable (Fig. 3; see also the Supporting information, Video S3). In particular, phase III of *clavatus* (the complex syllables produced by high-amplitude strokes of the legs) could precede

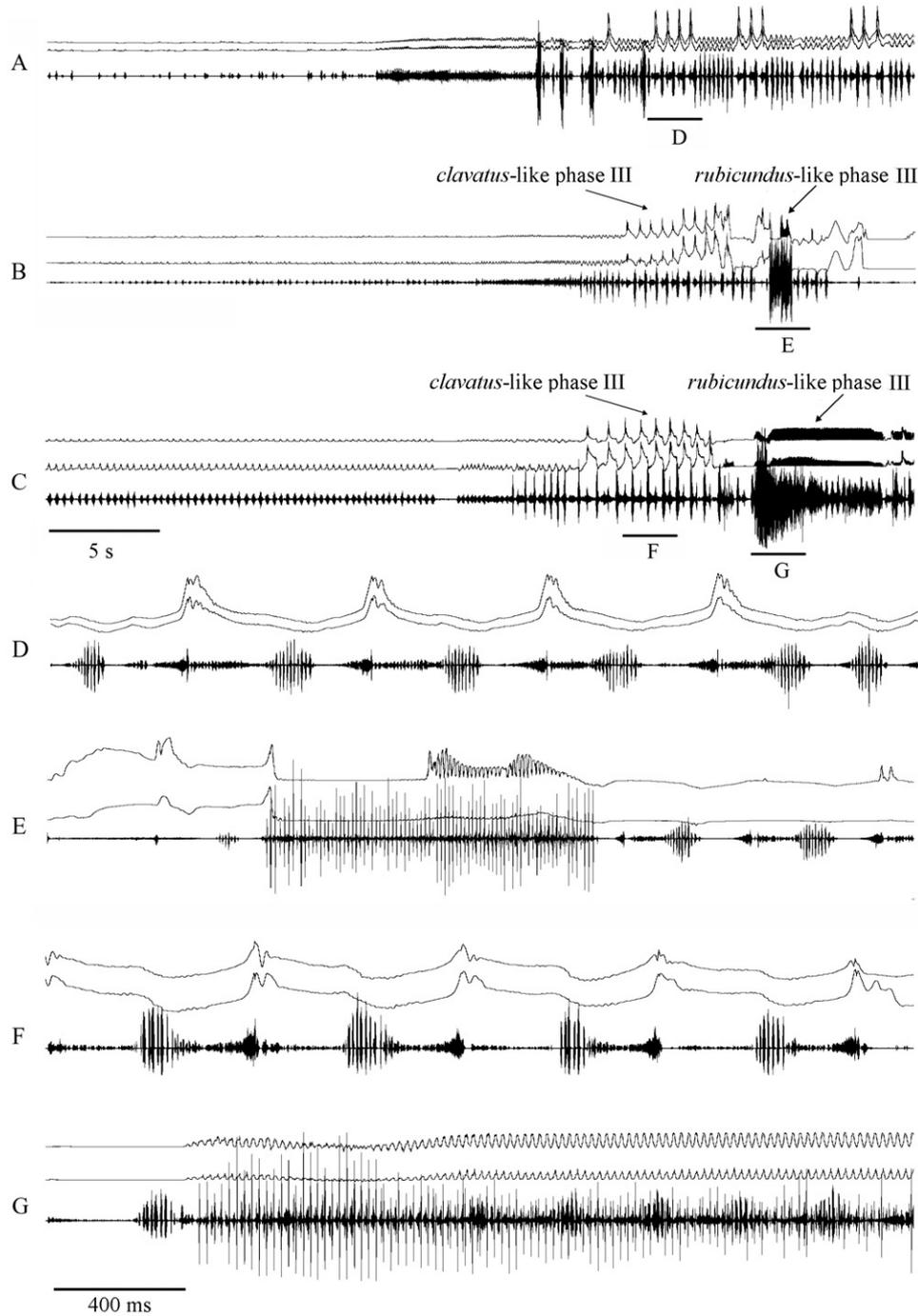


Figure 3. The courtship songs (hind leg movements and sound) of two natural hybrids from Mount Tomaros (A, B) and a laboratory-raised F_1 hybrid (C) illustrating the intermediate patterns between *S. clavatus* and *S. rubicundus*. (D–G) sections of hybrid songs at an expanded time scale of the indicated parts shown in (A) to (C).

phase III of *rubicundus* (the long sound produced by wing movements) (Fig. 3B). Phase III of *clavatus* could only comprise one to three strokes and alternate with a series of pulses typical for phase IV of *clavatus* (Fig. 3A) or phase II of *rubicundus*. In some cases, the

hybrid songs comprised elements that were not just intermediate but slightly or completely different from the parental ones. Seventeen (11%) males from Mount Tomaros produced a sound that was quite similar to phase II of *rubicundus*; however, the loud series of

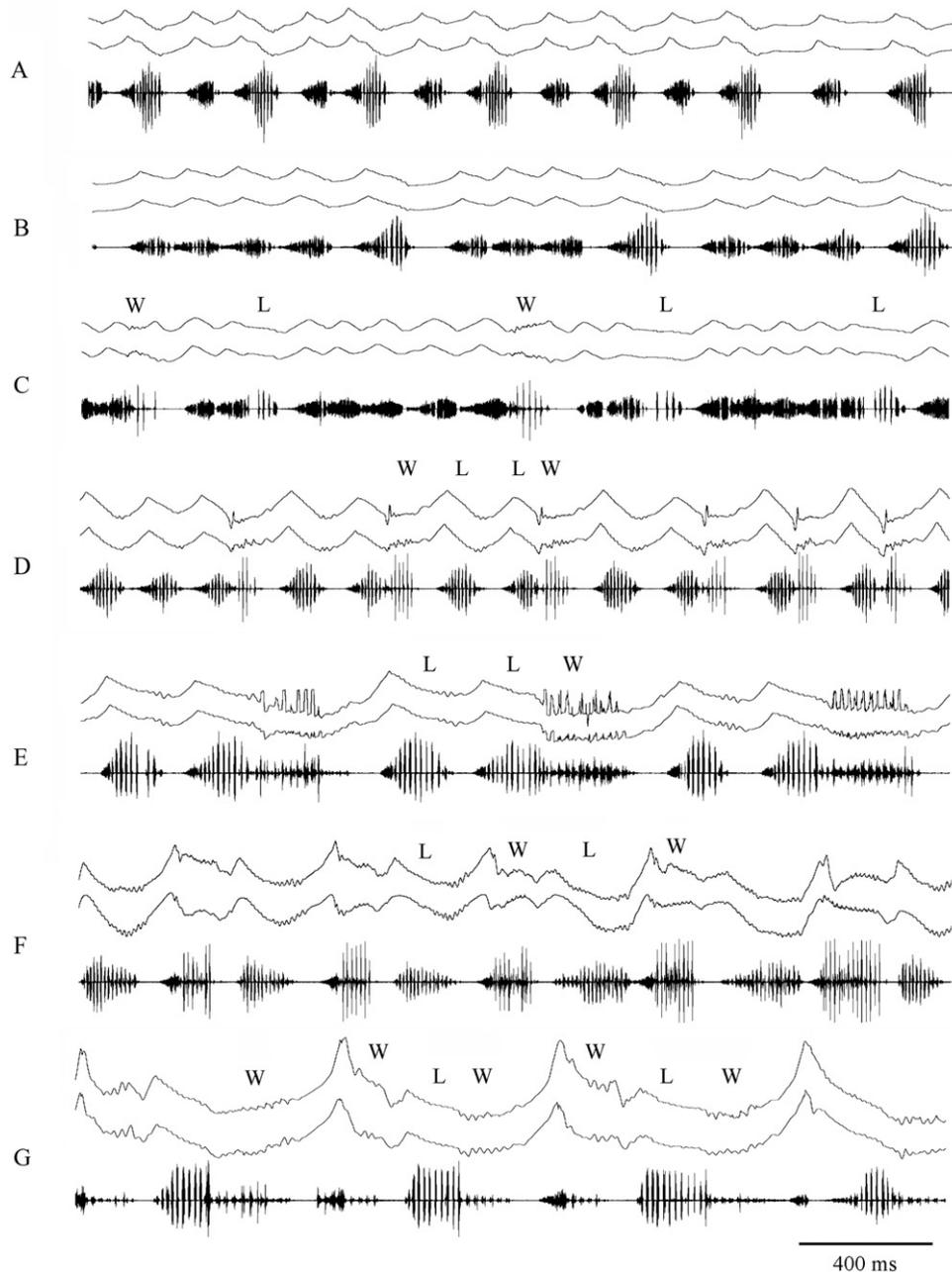


Figure 4. Novel courtship elements produced by natural hybrids (A, D, F), F_1 hybrids between *Stenobothrus clavatus* and *Stenobothrus rubicundus* (B, E, G) and the backcross to *S. rubicundus* (C). L and W indicate the song elements produced by leg movements and wing beats, respectively.

short pulses were produced by stepwise downstrokes of the femora (Fig. 4A), whereas, in *rubicundus*, they were generated by the wing movements. The pulse rate was similar in both types of series (mean of 60–70 Hz). Another widespread (9%) hybrid pattern was an alternation of the series of pulses produced by stepwise leg movements (typical for *clavatus*) with the series of pulses produced by the wing beats

(typical for *rubicundus*) (Fig. 4D). More rarely (3%), the wing beats were incorporated into phase III of the song (Fig. 4E).

To calculate a hybrid index score, we used eight song characters shown in Table 2. Because the values for most characters of the songs recorded from Mount Tomaros were not Gaussian distributed (Kolmogorov–Smirnov test, $D < 0.2$, $P < 0.01$), we calculated median

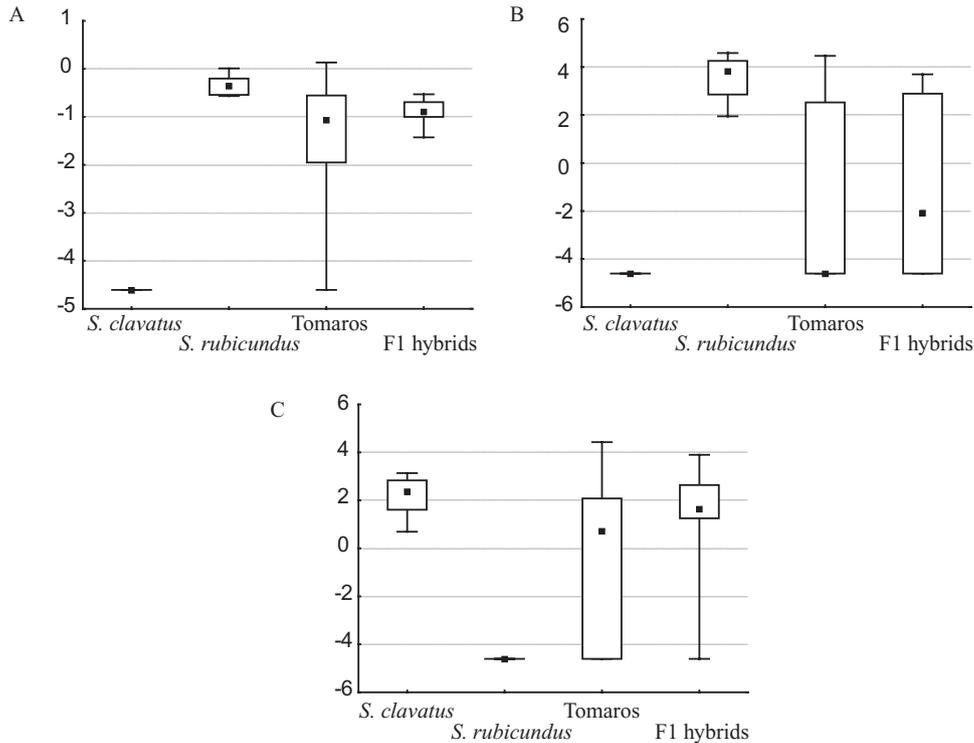


Figure 5. Distribution of three courtship song characters (medians, quartiles and minimum/maximum) in allopatric *Stenobothrus clavatus* ($N = 13$), allopatric *Stenobothrus rubicundus* ($N = 12$), specimens from Mount Tomaros ($N = 150$) and laboratory-raised F_1 hybrids ($N = 13$). (A) character 1, relative sound amplitude of pulses in phase I; (B) character 3, the number of wing beats in a *rubicundus*-like phase II; (C) character 8, the number of series of pulses in a *clavatus*-like phase IV. All values are \log_e -transformed.

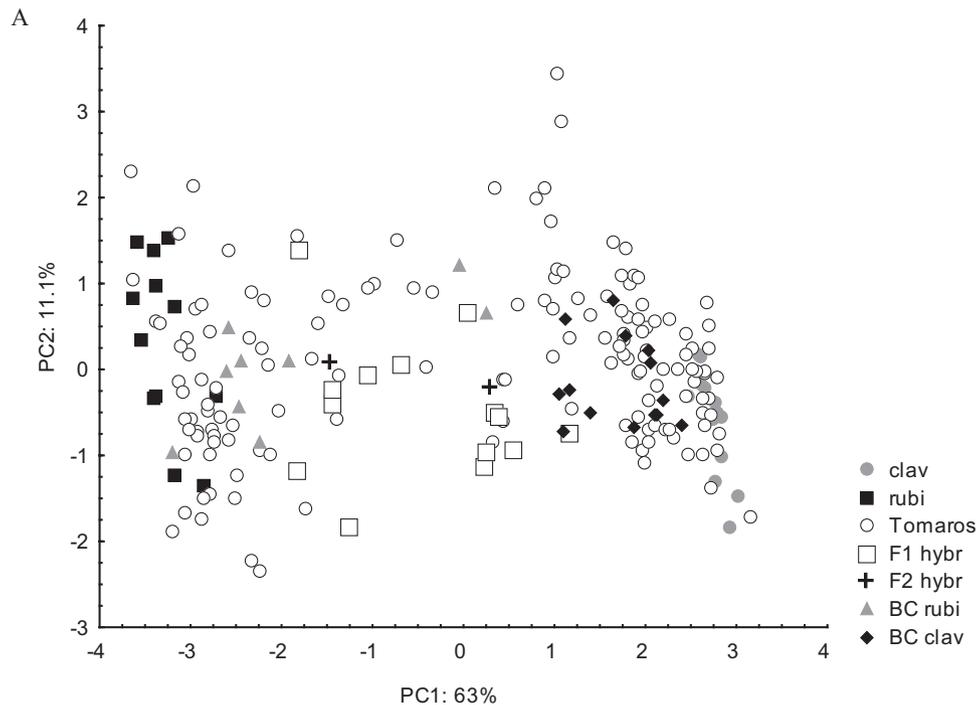
values. Subsequently, we designated all the males from Mount Tomaros as ‘natural hybrids’ to distinguish them from the hybrids obtained from the laboratory crosses. For most characters of the natural hybrid songs, median values were intermediate between the parental ones. However, characters 3 and 4 showed medians equal to 0, which indicated that the majority of males from Mount Tomaros did not produce the wing beats, much like *S. clavatus* males (Table 2).

SONGS OF LABORATORY HYBRIDS

Songs of laboratory-raised F_1 hybrid males comprised either intermediate elements between the parental songs (Fig. 3C, F, G) or novel traits, much like the songs of natural hybrids. Characters 1 and 2 describing phase I of the song had more or less intermediate values between *rubicundus* and *clavatus*: all F_1 hybrids produced double pulses during phase I, although the first pulse produced by the leg upstroke was usually very small in amplitude

(Fig. 5A). Characters 3, 4, and 6 showed bimodal distributions in F_1 hybrids. This means that some F_1 hybrids made the wing movements either in phase II (alternation of leg and wing stridulation) (Fig. 4E) or in phase III (continuous wing beats), whereas other hybrids did not produce the wing beats at all (Fig. 5B). Notably, this did not depend on the direction of crosses. Characters 7 and 8 were *clavatus*-like in all F_1 hybrids: the males always produced high-amplitude strokes with legs in the *clavatus*-like phase III and stepwise downward movements of the legs in phase IV (Fig. 5C). Finally, all F_1 hybrids showed alternation of simple sound pulses and complex series of pulses (character 5 was different from 0). A complex series of pulses was produced either by leg movements (Fig. 4B) or by wing movements (Fig. 4E, G).

Songs of all backcrosses with *S. clavatus* possessed features very similar to the *S. clavatus* song. By contrast, not all backcrosses with *S. rubicundus* lead to *rubicundus*-like song (Fig. 4C). One backcross with *rubicundus* did not produce wing movements during courtship at all; another one produced high-amplitude strokes with legs typical for the *clavatus*-like phase



B

Song characters	PC1	PC2
1. Relative up- to downstroke pulse amplitude in ph I	-0,76	0,21
2. Duration of downstroke pulse in ph II	0,44	-0,87
3. The number of the wing beats in ph II	-0,92	-0,09
4. Relative pulse amplitude produced by the wings and legs in ph II	-0,86	0,08
5. The relative number of simple to pulsed series in ph II	-0,81	-0,03
6. Duration of rubicundus-like ph III	-0,82	-0,18
7. The number of strokes in clavatus-like ph III	0,90	0,12
8. The number of series of pulses in ph IV	0,73	0,15

Figure 6. The values of the first and second principal components (PCs) using eight courtship song characters in allopatric *Stenobothrus clavatus* and *Stenobothrus rubicundus*, specimens from Mount Tomaros, F_1 , F_2 , backcrosses with *S. rubicundus* and backcrosses with *S. clavatus* (A) and loadings of different characters in PC1 and PC2 (B).

III. Finally, most of backcrosses with *rubicundus* (7 of 9) made stepwise downward movements with legs typical for the *clavatus*-like phase IV.

PRINCIPAL COMPONENT ANALYSIS (PCA) OF THE SONG PARAMETERS

To visualize and clarify the song differences between the allopatric populations, hybrid populations and laboratory hybrids, a PCA was applied to all eight song characters studied. When a character was equal to 0, we changed it to 0.01 by convention because we only used the logarithmic values for PCA. The contribution of most characters to principal component (PC) 1 was larger (64.4%) than to PC2 (11.1%) (Fig. 6). Only one

song character (i.e. the duration of sound pulse produced by a downstroke in phase I) largely contributed to PC2. This may be explained by the gradation values of this character in all of the songs analyzed, whereas other characters showed the same values in either of the parental-like songs (Table 2).

The PC1 scores of all F_1 and F_2 hybrids were intermediate between the scores of allopatric *S. clavatus* and *S. rubicundus* (Fig. 6). The backcrosses were concentrated close to their parental species; however, two backcrosses with *rubicundus* fell closer to the *clavatus*-like cluster. Figure 6 also shows that most of natural hybrids can be divided into two groups: the *clavatus*-like and *rubicundus*-like groups. To some degree, natural hybrids overlapped with

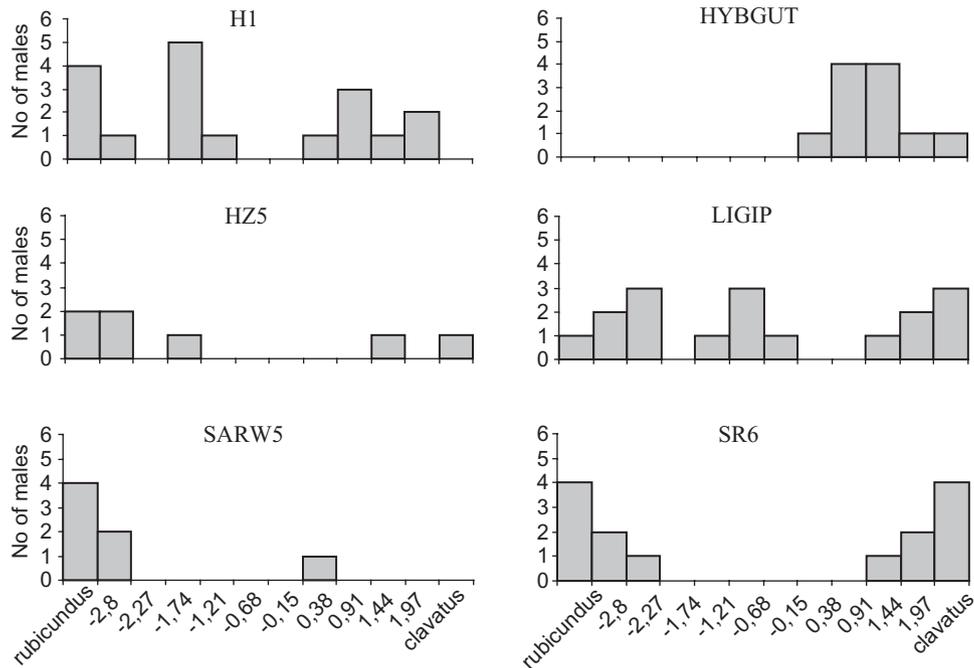


Figure 7. Distribution of the principal component (PC) 1 scores at six closely situated sites (map with sample sites shown in Figure 1).

allopatric specimens, although 52% of natural hybrids (78 of 150) fell into the cluster of the backcrosses with *clavatus*. Intermediate phenotypes in hybrid populations were in a minority.

The distribution of the PC1 scores in six closely situated sites shows a variety of song phenotypes (Fig. 7). In sites HZ5 and SR6, the distribution was typically bimodal: only parental song types and no intermediate song pattern were recorded. In sites H1 and LIGIP, both parental and hybrid phenotypes were found in an approximately equal number. Finally, one of the parental song phenotypes dominated in sites HYBGUT and SARW5.

DISCUSSION

HOMOLOGOUS ELEMENTS IN THE COURTSHIP SONGS OF *S. RUBICUNDUS* AND *S. CLAVATUS*

The courtship songs of the two species are remarkably different with respect to both sounds and the sound-producing mechanisms. Therefore, it is not surprising that only a few courtship elements appeared to be homologous in the songs of these species. The most evident homologous element is a prolonged phase I. The leg movements in the parental species are similar during this phase, and many hybrids produce clearly intermediate pattern. This prolonged song phase comprising rather simple leg movements,

however, is widespread among Gomphocerinae, being described in *Omocestus*, *Stenobothrus*, and *Myrmeleotettix* (Ragge & Reynolds, 1998; Berger, 2008).

The stepwise downstrokes of the legs found in the *clavatus*-like phases III and IV seem to be homologous to the wing movements typical for the *rubicundus*-like phase II. Both types of movements produce similar series of short pulses at the same pulse rate. One of the common hybrid patterns was the sound similar to that in phase II of the *rubicundus* song, although with the series of pulses produced by stepwise downstrokes instead of wing beats. Elsner (1974b) showed that bifunctional thoracic muscles can move both the wings and legs in the grasshoppers. Moreover, it was shown in *S. rubicundus* that simple changes in coordination can convert the movement patterns typical of legs and wings into one another (Elsner & Wasser, 1995b). In addition, the males of *S. rubicundus* from north European populations were shown to produce stepwise downstrokes instead of wing beats in phase II (Elsner & Wasser, 1995a). Therefore, we have many reasons to suggest a homology between these two elements produced by different mechanisms. On the other hand, the series of pulses produced both by the legs and by the wings were sometimes found in the same hybrid songs. The superposition of specific, independent parental elements in the hybrid song indicates

that these elements are nonhomologous. Our data coincide with the data obtained from the hybrids between *Ch. biguttulus* and *Ch. mollis*. These hybrids sometimes produced intermediate patterns, and sometimes both parental patterns were superimposed on each other (von Helversen & von Helversen, 1975).

Analysis of the courtship songs in hybrids between *Ch. albomarginatus* and *Ch. oschei* (i.e. the species that also demonstrate elaborate courtship behaviour) revealed almost all song elements to be homologous in the two species (Vedenina & Helversen, 2003). Despite many different features in the parental songs, it was possible to follow the transition from one pure song type to the other pure pattern through intermediate variants. In the case of *S. clavatus* and *S. rubicundus*, we have an example of a much stronger difference between the songs possessing non-homologous elements, which, however, do not prevent interspecific hybridization.

NOVELTY OF THE HYBRID SONGS

We showed in the present study that, in many hybrid songs, some parental elements were more or less independently superimposed on each other. Thus, the hybrid song pattern exhibited an increased degree of complexity compared to both parental songs. For example, phase III of *clavatus* alternated with phase III of *rubicundus* in some hybrid songs or the wing beats were incorporated into phase III of *clavatus* in the other songs. In both cases, the visual display of *S. clavatus* (high-amplitude strokes of the legs) was enhanced by the visual display of *S. rubicundus* (wing movements). These new elements might offer a potential material for sexual selection. According to the 'good genes' hypothesis of sexual selection (Zahavi, 1987; Maynard Smith, 1991), the females testing the quality of a male must prefer the most costly features of the signal. The production of high-amplitude strokes of the legs and wing beats in the same song phase is expensive in both production and coordination, and thus may be used by a female as an indicator of vigour.

The calling songs of hybrids between *Ch. biguttulus* and *Ch. mollis* also exhibited an increased degree of complexity compared to the songs of parental species because of the superposition of independent parental song elements. Based on revealed nonhomology of the parental elements, two pattern-generating neuronal networks were suggested to be formed in the central nervous system of the hybrids. The outputs of the two networks converge in a common final pathway, probably at the level of the motoneurons, and may lead to the superimposed pattern of the hybrid song (Helversen & Helversen, 1975).

DOMINANCE OF THE *CLAVATUS*-LIKE ELEMENTS IN THE HYBRID SONGS

Song analysis in the laboratory hybrids showed a dominance of some *clavatus*-like features. All F_1 hybrids produced the high-amplitude strokes with the hind legs typical for the *clavatus*-like phase III. Another *clavatus*-like element, stepwise movements during the downstroke of the hind legs, was found not only in all F_1 hybrids, but also in most of backcrosses with *rubicundus*. This element was produced by different hybrids in different phases, from II to IV. The stepwise movements in the downstroke are suggested to be a plesiomorphic element, which can be found in many Gomphocerinae species from different genera (Elsner, 1974a; Helversen & Helversen, 1994; Elsner & Wasser, 1995a; Vedenina & Mugue, 2011). Ancestral features are more likely to be dominant, whereas, at the first steps of speciation, mutations that cause the appearance of novel features are more likely to be recessive. In hybrids between *Ch. albomarginatus* and *Ch. oschei*, a dominance of *Ch. albomarginatus* that was clearly expressed in the hybrid courtship songs could also suggest a more ancient origin of this species (Vedenina *et al.*, 2007b). This suggestion is additionally supported by the fact that the courtship song of *Ch. oschei* is regarded to be more elaborate than the *Ch. albomarginatus* song. By contrast, the courtship songs of *S. rubicundus* and *S. clavatus* appear to have a similar degree of complexity (Berger, 2008).

Dominance of the *clavatus*-like elements in the hybrid songs might have implications for mating success of hybrid males in the contact zone. It is highly probable that the hybrid songs may be more attractive to females of *S. clavatus* than to females of *S. rubicundus*, which may result in producing more backcrosses with *clavatus*. The analysis of the songs from Mount Tomaros supports this idea. In addition, we found an asymmetry in the hybrid viability depending on the direction of crosses: the hybrid offspring obtained from *clavatus* females demonstrated a higher survival than the offspring obtained from *rubicundus* females. Thus, one can suggest a stronger introgression of *rubicundus* alleles into *clavatus* genom than vice versa, and therefore a displacement of the hybrid zone in favour of *S. clavatus*. On the other hand, because both species do not occur lower than 1300 m a.s.l., the hybrid zone could not move significantly, similar to a narrow hybrid zone between chromosomal races of grasshopper *Podisma pedestris* in the Alpes Maritimes (Hewitt, 1975; Barton & Hewitt, 1981).

Sradnick (2010) studied transitions in two morphological characters, forewings and antennae, across the hybrid zone on Mount Tomaros. Because both

morphological characters are actively used during courtship, selection of a similar strength on the morphological characters and courtship songs can be expected. However, the comparison of cline width and position across the hybrid zone did not show concordant and coincident clines. Clines for wings and antennae were displaced to different sides of the hybrid zone, whereas the cline for courtship song was steeper than the clines for both morphological characters. It is very likely that some other factors may influence differential introgression of the relevant alleles, such as habitat use or dispersal capabilities. Individuals of *S. rubicundus* showed a better flying capacity than *S. clavatus* individuals, which means that the occasional dispersal of single individuals into a strange, closely situated habitat may be asymmetrical.

MOSAIC STRUCTURE OF THE HYBRID ZONE

We found a different distribution of song phenotypes at closely situated hybrid sites. This pattern may be indicative of a mosaic hybrid zone. Mosaic hybrid zone models predict the persistence of parental populations throughout the hybrid zone in ecologically divergent patches that are to a great degree relative to the mean dispersal of the organisms (Harrison, 1993). On Mount Tomaros, *S. clavatus* and *S. rubicundus* were shown to prefer different habitats (Sradnick, 2010): *S. clavatus* prefers stony places, whereas *S. rubicundus* more likely occurs in grassy habitats. On the other hand, both species, especially *S. rubicundus*, can fly away over tens of metres when disturbed, which suggests a dispersal comparable with the width of the hybrid zone. It is likely that deviations from a smooth cline are explained by habitat-genotype effects, as in the cricket species *Gryllus firmus* and *G. pennsylvanicus* hybridizing in North America (Harrison, 1986; Harrison & Rand, 1989) or the grasshopper species *Ch. albomarginatus* and *Ch. oschei* (Vedenina, 2011). Mosaic pattern on Mount Tomaros could be also explained by other factors, as shown for the hybrid zone between *Ch. brunneus* and *Ch. jacobsi* in northern Spain (Bridle & Butlin, 2002). Further comparisons of morphological and song indices and a study of the associations between all phenotypic and habitat variations are required to explain the mosaic structure of the hybrid zone on Mount Tomaros.

SCENARIO OF THE ORIGIN OF *S. RUBICUNDUS* AND *S. CLAVATUS*

Stenobothrus clavatus was only found on two mountains of northern Greece. By contrast, *S. rubicundus* is known to inhabit a relatively large range in Europe (Harz, 1975; Berger, Chobanov & Mayer, 2010).

Courtship songs of *S. rubicundus* from the Alps, Slovenia and Croatia were recorded and analyzed by Elsner & Wasser (1995a, b). During phase II of the courtship, the males from these populations produce complex series of pulses using stepwise downward leg movements where Greek populations of *S. rubicundus* produce wing beats. The wing beats are only produced in phase III of the courtship. A comparison of the hybrid songs recorded from Mount Tomaros with the songs of *rubicundus* from the northern populations revealed a high similarity of the song patterns. In many hybrids, a complex series of pulses during phase II is often produced by stepwise downstrokes of the hind legs.

Two possible scenarios can be suggested that explain the similarities between the song of northern *rubicundus* and that of the hybrid between *clavatus* and Greek *rubicundus*. It might be tempting to regard the northern populations of *rubicundus* to be the result of hybridization between *S. clavatus* and Greek *S. rubicundus*. However, taking into account the very local distribution of *S. clavatus*, this scenario is unlikely. Moreover, the stepwise downstroke of the legs is suggested to be the ancestral pattern. It is most likely that the northern populations of *rubicundus* are more close to the ancestral species, whereas the Greek populations of *rubicundus* represent a diverged form. The same idea was proposed by Elsner & Wasser (1995a) when comparing different populations of *S. rubicundus* from Alps, Slovenia, Croatia, and Greece.

The examples from other groups of Gomphocerinae, such as the *Ch. biguttulus* group (Willemse, von Helversen & Odé, 2009) and the *Ch. albomarginatus* group (Vedenina & Helversen, 2009), showed a great species diversity in Greece. This diversity is suggested to have originated rather recently, probably during the Pleistocene period. During interglacial periods, populations in southern refugia were split into many subpopulations when ascending the mountains (Hewitt, 1996; Çiplak, 2004). These subpopulations were mixed again in the subsequent cold period, when they descended the mountains, and the process was repeated over several ice ages. In this process, the subpopulations could diverge, form hybrid zones, and speciation could accelerate. At the same time, the most northern expansions were driven extinct by each ice age. Such a scenario explains why, in many groups of insects, there are only one or a few species occurring in the central and northern Europe and many more species inhabiting the southern peninsulas, especially Greece. One can infer that the ancestral species from which *S. rubicundus* and *S. clavatus* originated was widespread and invaded all the Balkans. It is very likely that this ancestral species produced a stepwise downstroke with the legs during

courtship, which diverged into phase II of the northern *rubicundus* song (produced only with legs) and into phase III of the *clavatus* song. Alternation of the wing and leg movements in phase II of the Greek *rubicundus* song is very likely to be the derivative of an ancestral pattern. Such increasing complexity by a combination of auditory and visual signals in courtship could be driven very fast by sexual selection (Andersson, 1994). Evolution of phase III in the *clavatus* song, which is accompanied by the visual signals comprising not only the high-amplitude strokes of the legs, but also the spectacular movements with antennae (Ostrowski *et al.*, 2009), could similarly be a result of sexual selection. Unfortunately, we did not document the antennae movements in the hybrids. It is very likely that this parameter could correlate with the antennae morphology (Elsner *et al.*, 2009) because a fast raising of the antennae with their dark spatulate tips should be better recognized by females and affect their behaviour.

In a future study, female preferences for the courtship songs in playback experiments will be analyzed and allow us to further discuss the processes occurring in the hybrid zone. In particular, we aim to discuss divergence in mating behaviour and reinforcement.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Video S1. *Stenobothrus clavatus* courtship.

Video S2. *Stenobothrus rubicundus* courtship.

Video S3. Courtship of F1 hybrid between *Stenobothrus clavatus* × *Stenobothrus rubicundus*.

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