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Speciation in gomphocerine grasshoppers: molecular phylogeny versus bioacoustics and courtship behavior

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Abstract

To understand the driving forces of speciation in grasshoppers of the subfamily Gomphocerinae, we compared a molecular phylogenetic tree with the distribution of the song pattern complexity and courtship behavior. In 50 grasshopper species of Gomphocerinae, a barcoding region of mitochondrial gene COI was sequenced and analyzed. A plesiomorphic pattern of calling song was shown to be predominant in the species of the most basal clusters, including the tribes Chrysochraontini, Dociostaurini and Arcypterini. In the tribes Stenobothrini and Gomphocerini, plesiomorphic pattern of the calling song was found in less than half of the species studied; others demonstrated increasing song complexity. The species of the basal cluster of the phylogenetic tree did not show complex courtship songs. Courtship songs that are different and more complex than the calling songs were only found in about half of species belonging to the tribes Stenobothrini and Gomphocerini. Most species with complex courtship (species of *Stenobothrus* genus, of *Chorthippus albomarginatus* group) were shown to be recently diverged species. Comparison of visual displays accompanying complex courtship song, revealed similarities between the species from different groups. According to the molecular phylogeny, complex courtship behavior in Gomphocerinae evolved independently and convergently. Since the courtship song can be used by a female to judge mate quality, sexual selection is suggested to be the main driving force of rapid speciation in these young species.

Key words

Gomphocerinae, mtDNA, stridulatory leg movements, complex song, visual display, sexual selection

Introduction

Gomphocerine grasshoppers comprise perhaps the most diverse and species-rich subfamily of Acrididae, occurring on all continents except for Australia and Madagascar. Members of Gomphocerinae can be distinguished from other subfamilies of Acrididae by a characteristic structure of the acoustic apparatus: namely by the presence of stridulatory pegs on the inner surface of the hind femur (Uvarov 1966). Among Acrididae subfamilies, acoustic communication in Gomphocerinae is most developed in terms of complexity of stridulatory leg movements, the number of sound elements and mating strategies (*e.g.*, Otte 1970, Helversen & Helversen 1994, Ragge & Reynolds 1998).

The song is produced by stroking a stridulatory file of each hind femur across a raised vein on the ipsilateral wing. In most species, the sound has a broad frequency spectrum, so that the specificity of the songs lies not in their frequency band but almost always in the pattern of amplitude over time. Using both hindlegs, the grasshoppers have two separate sound-producing devices, which must be co-ordinated with one another. The stridulatory movements of the two legs often differ in amplitude and form, and the legs can exchange roles from time to time (Elsner 1974a, Helversen & Elsner 1977, Elsner 1994). Various species demonstrate different degrees of song complexity. Song pattern diversity can arise from an increase in the complexity of individual pattern units themselves, as well as from the combination of different units to form a sequence (Helversen & Helversen 1994). The song in Gomphocerinae also varies according to the behavioral situation. A solitary male produces a calling song, listening for the response song of a female that is ready to mate. The response of a female initiates a duet, during which the female usually sits still while the male approaches her. When a male finds a female, in many species the male begins a special courtship song, which may reach a high complexity and may be accompanied by conspicuous movements of different parts of the body such as abdomen, head, antennae or palps (Faber 1953, Otte 1970, Helversen & Helversen 1994).

Despite extensive systematic investigations of Gomphocerinae, using morphological (*e.g.*, Bei-Bienko & Mistshenko 1951, Jago 1971, Harz 1975, Vickery & Kevan 1985) and acoustical (Otte 1970; Helversen & Helversen 1975, 1994; Helversen 1986; Bukhvalova 1993; Ingrisch 1995; Ragge & Reynolds 1998; Vedenina & Bukhvalova 2001; Savitsky 2000, 2002; Tishechkin 2008; Berger 2008; Vedenina & Helversen 2009) data, molecular phylogenetic relationships remain poorly studied. To date, three phylogenetic studies involving mitochondrial DNA (mtDNA) analysis have been published. Guliaeva *et al.* (2005) tried to reveal relationships between Nearctic and Palaearctic Gomphocerinae species based on the analysis of the mt16S rRNA gene. Bugrov *et al.* (2006) used two genes, cytochrome b (Cytb) and cytochrome oxidase subunit I (COI), to test the hypothesis of Eurasian Gomphocerinae monophyly and assess the validity of the different tribal classifications. In the study of Contreras and Chapco (2006), four genes (Cytb, CO I and II, NADH subunit V) were sequenced to test the monophyletic status of different tribes of Gomphocerinae, as well as to test the theory of multiple incursions of Gomphocerinae from Eurasia to North America. Recently, analysis of the mt COI gene has also been conducted on several closely related species of 78 *Stenobothrus* genus (Berger *et al.* 2010).

In the present study, we compare molecular phylogeny based on COI gene analysis, with acoustic and courtship behavior to understand the driving forces of speciation in Eurasian Gomphocerinae. We analyze not only distantly, but also closely related species. In gomphocerine grasshoppers, the song is suggested to be the most important component of reproductive isolation. There are many sibling species that show morphological similarity but have very different songs. For example, the songs in the *Chorthippus biguttulus* group (*e.g.*, Ragge & Reynolds 1998, Ingrisch 1995, Willemse *et al.*

Table 1. List of species used, GenBank accession numbers and localities of the specimens used in the phylogenetic analysis and analyzed acoustically.

Species	Locality of specimen used in the phylogenetic analysis	Accession No. (COI)	Locality of the song recording
Chrysochraon dispar	GenBank	AY738345	Russia, Kostroma region
Euthystira brachyptera	GenBank	AY738367	Russia, Kostroma region
Podismopsis poppiusi Russia 1	Russia, Karelia	HQ652511	
Podismopsis poppiusi Russia 2	Russia, Irkutsk region	HQ652513	Russia, Irkutsk region
Podismopsis genicularibus	Russia, Maritime province	HQ652512	
Podismopsis u. ussuriensis	Russia, Maritime province	HQ652509	
Podismopsis ussuriensis micra	Russia, Khabarovsk province	HQ652510	
Dociostaurus kraussi	Russia, Volgograd region	HQ738950	
Dociostaurus brevicollis	Ukraine, Kherson region	HQ738949	Ukraine, Cherkassy region
Dociostaurus anatolicus	Dagestan, 20 km W Makhachkala	HQ738948	
Docioustaurus albicornis	Ukraine, Crimea	HQ738947	Ukraine, Crimea
Arcyptera fusca	GenBank	AY738368	Bulgaria, 30 km SW Pazardzhik
Arcyptera microptera	Russia, Saratov	HQ738920	Ukraine, Lugansk region
Ramburiella bolivari	Russia, Astrakhan region	HQ738959	
Omocestus viridulus	Russia, Leningrad region	HQ738958	
Omocestus rufipes	Greece, N. Kerkyra	HQ738957	
Omocestus haemorrhoidalis	GenBank	AY738364	Russia, Kostroma region
Omocestus petraeus	GenBank	AY738362	
Omocestus minutus	Ukraine, Odessa region	HQ738956	Russia, Saratov region
Stenobothrus lineatus	Russia, Moscow region	HQ738963	Russia, Saratov
Stenobothrus stigmaticus	Germany, Hesse	HQ738967	Germany, Hesse
Stenobothrus fischeri	Greece, Ipiros, Ioannina	HQ738962	Russia, Saratov
Stenobothrus nigromaculatus	Moldova, Bolshaya Khalakhora	HQ738965	Russia, Saratov
Stenobothrus miramae	Ukraine, Crimea	HQ738964	
Stenobothrus e. eurasius	Ukraine, Kherson region	HQ738961	Ukraine, Kherson region
Stenobothrus e. hyalosuperficies	Russia, Saratov region	HQ738960	
Stenobothrus rubicundulus	Greece, Ipiros, Ioannina	HQ738966	Greece, Makedhonia, Florina
Myrmeleotettix maculatus	Ukraine, Nikolaev region	HQ738955	Ukraine, Nikolaev region
Myrmeleotettix antennatus	Ukraine, Kherson region	HQ738954	Ukraine, Kherson region
Euchorthippus pulvinatus	Russia, Saratov	HQ738952	Ukraine, Poltava region
Euchorthippus declivus	Bulgaria, Burgas region	HQ738951	Ukraine, Chernovtzy region
Chorthippus parallelus	Hungary, Budapest	HQ738946	Russia, Kostroma region
Chorthippus montanus	Ukraine, Ivano-Frankovsk region	HQ738940	Ukraine, Ivano-Frankovsk region
Chorthippus dichrous	GenBank	AY738354	Bulgaria, 20 km SW of Varna
Chorthippus dorsatus	Russia, Moscow region	HQ738930	Russia, Moscow
Chorthippus macrocerus	Ukraine, Kherson region	HQ738937	Ukraine, Nikolaev region
Chorthippus apricarius	GenBank	AY738351	Russia, Moscow
Chorthippus albicornis	Italy, province Abruzzi	HQ738921	Italy, province Abruzzi
Chorthippus intermedius	GenBank	AY738352	
Chorthippus biguttulus	Russia, Kostroma region	HQ738926	Russia, Kaliningrad region
Chorthippus maritimus	Abkhazia, near Sukhumi	HQ738938	Abkhazia, near Sukhumi
Chorthippus bornhalmi	Bulgaria, 20 km SE of Sofia	HQ738927	
Chorthippus brunneus	Bulgaria, 20 km SE of Sofia	HQ738928	
Chorthippus brunneus	Russia, Saratov	HQ738929	
Chorthippus mollis	Russia, Moscow region	HQ738939	Russia, Saratov region
Chorthippus albomarginatus	Germany, Bavaria	HQ738922	Germany, Bavaria
Chorthippus albomarginatus	Poland, 85 km S of Krakov	HQ738923	
Chorthippus albomarginatus	Ukraine, Poltava region	HQ738925	
Chorthippus albomarginatus	Russia, Moscow, Bitza	HQ738924	
Chorthippus o. oschei Greece 1	Greece, Ipiros, Ioannina	HQ738942	Greece, Ipiros, Ioannina
Chorthippus o. oschei Greece 2	Greece, Makedhonia, Grammos	HQ738941	
Chorthippus oschei pusztaensis Hungary 1	Hungary, province Bekes, Kistompopuszta	HQ738944	
Chorthippus oschei pusztaensis Hungary 2	Hungary, province Bekes, Csikopuszta	HQ738945	
Chorthippus oschei pusztaensis	Bulgaria, 30 km SW Pazardzhik	HQ738943	
Chorthippus k. karelini	Ukraine, Kherson region	HQ738933	

Table 1. Continued.

Species	Locality of specimen used in the phylogenetic analysis	Accession No. (COI)	Locality of the song recording
Chorthippus k. karelini Russia 1	Russia, Volgograd region	HQ738934	
Chorthippus k. karelini Russia2	Russia, Tuva	HQ738935	
Chorthippus karelini bruttius	Italy, province Calabria	HQ738932	
Chorthippus lacustris	Greece, Ipiros, Thesprotia	HQ738936	
Chorthippus ferdinandi	Greece, Peloponnesus	HQ738931	
Stauroderus scalaris	GenBank	AY738360	Bulgaria, betw. Mezdra & Vratza
Aeropus sibiricus	GenBank	AY738358	
Gomphocerippus rufus	Italy, province Abruzzi	HQ738953	Ukraine, Poltava region
Schistocerca gregaria	GenBank	GQ491031	

2009) and in the *C. albomarginatus* group (Helversen 1986, Vedenina & Helversen 2009) provide the only reliable key of species identification. In the current paper, we analyze the degree of complexity of the calling and courtship song patterns. To more entirely describe the songs, we analyze not only the sound, but also the underlying stridulatory movements of the hind legs, since many grasshopper species reach an extremely high complexity in the motor pattern of the leg movements. (During the courtship behavior, a male may demonstrate conspicuous leg movements without producing the sound.) Finally, we take into account the whole visual display (movements of head, antennae or abdomen) accompanying the courtship song in some species.

Methods

Molecular analysis.— Fifty-three specimens of 39 species were collected from natural populations (Table 1). Total DNA was isolated from hind femora of either a grasshopper fixed in 96% alcohol or from a dry grasshopper. One half of hind femora was cut into several pieces and digested with proteinase K overnight. DNA was extracted according to the protocols described in Aljanabi & Martinez (1997).

A PCR amplification of the COI gene (653 bp) was performed with the universal primers LCOI 1490 and HCOI 2198 (Folmer *et al.* 1994). PCR was performed in 25-μl reaction volumes, including 1 × PCR buffer (Sileks), 2.5 mM MgCl2, 0.8 mM of each dNTP, 10 pM of each primer, 1-μl template DNA solution and 1.25 U of Taq-polymerase (Sileks-M, Moscow). Reactions were cycled on an MJResearch PTC200 thermocycler, starting with a 2-min denaturing step at 95°C, followed by 35 cycles of 95°C for 30 s, 48°C for 1 min and 72°C for 1 min 30 s. Amplifications ended with a single 72°C, 10-min elongation step. PCR products were cleaned using Wizard PCR Preps (Promega, Madison, WI, USA). Sequencing was performed from the PCR primers in both directions with the ABI PRISMs BigDye Terminator v. 1.1 kit on an ABI PRISM 3100, according to the protocol of the manufacturer.

The newly collected sequences were edited, contigs were assembled, and sequence proofreading performed using the SeqMan and EditSeq modules of the DNAStar package (DNAStar, http://www.dnastar.com/). Accession numbers for the newly obtained sequences deposited in GenBank are shown in Table 1. For the phylogenetic analysis, 11 additional sequences were obtained from GenBank (Table 1). Sequences were aligned with MUSCLE (v3.7) (Edgar 2004), alignment was straightforward because of the absence of gaps in protein-coding sequence. Sequence pairwise distances were calculated with MegAlign module of DNAStar package.

Phylogenetic trees were reconstructed based on neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference methods using PAUP 4.0b10 (NJ and MP, Swofford 2003), PhyML (Guindon & Gascuel 2003) and MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) respectively. Statistical support for the MP and ML trees was evaluated from 1000 replicates of bootstrap analysis. GTR+I model was chosen as the model of sequence evolution in the ML and Bayesian analysis, using the following parameters: nst = 6, rates = invgamma. Four Markov chains were run for 10^5 generations, sampling every 100 generations, and a consensus tree was built after burning 2500 trees.

Analysis of the songs and courtship behavior.—Calling songs were recorded from an isolated male; each courtship song was recorded when a male was sitting nearby a female. The ambient temperature near a singing male was 30 to 35° C. During stridulation, not only the sound but also the movements of the hind legs, were recorded with a custom-built opto-electronic device (Helversen & Elsner 1977, Hedwig 2000). A piece of reflecting foil was glued to the distal part of each hind-leg femur of a male and two opto-electronic cameras focused on the illuminated reflecting dots. Each camera was equipped with a position-sensitive photodiode that converted the upward and downward movements of the hind legs into voltage changes. These signals, together with the microphone (1/2" Brüel and Kjaer) recordings of the sounds, were A/D-converted with a custom-built PC card. The sampling rate was 2 kHz for recording the stridulatory movements and 100 kHz for sound recordings. The recordings were analyzed with a Turbolab 4.0 program (Germany, Bressner Technology). Courtship behavior was also recorded with a Sony DCR-TRV 355E digital video camera; the video signals were transferred to a PC for analysis of visual display.

We described the song elements on the basis of the leg-movement pattern. For the song description, we used the following terms: *pulse* – the sound produced by one uninterrupted upward or downward movement of a hind leg; *gap* – the interval between pulses; *element* – the sound produced by the same leg movements.

Results

Phylogenetic analysis.— After trimming, multiple alignment of 582 bp region of COI region for 64 taxa had 189 parsimony-informative characters. Phylogenetic trees obtained by different methods had similar topology except for the two positions (Fig. 1). The most basal clusters of the phylogenetic tree comprise the tribes Chrysochraontini (*Euthystira*, *Chrysochraon* and *Podismopsis*), Dociostaurini (*Dociostaurus*) and Arcypterini (*Ramburiella* and *Arcyptera*). The tribes Arcypterini and Dociostaurini are not monophyletic: *R. bolivari* is associated with *D. brevicollis,* while *Arcyptera* is placed apart. Another three species of *Dociostaurus* comprise a separate cluster. The tribe Chrysochraontini represents a separate clade relative to Arcypterini and Dociostaurini. The difference between various species of one genus is relatively large in this cluster: sequence divergence between the species of *Dociostaurus* varies from 8 to 14.7%; between the species

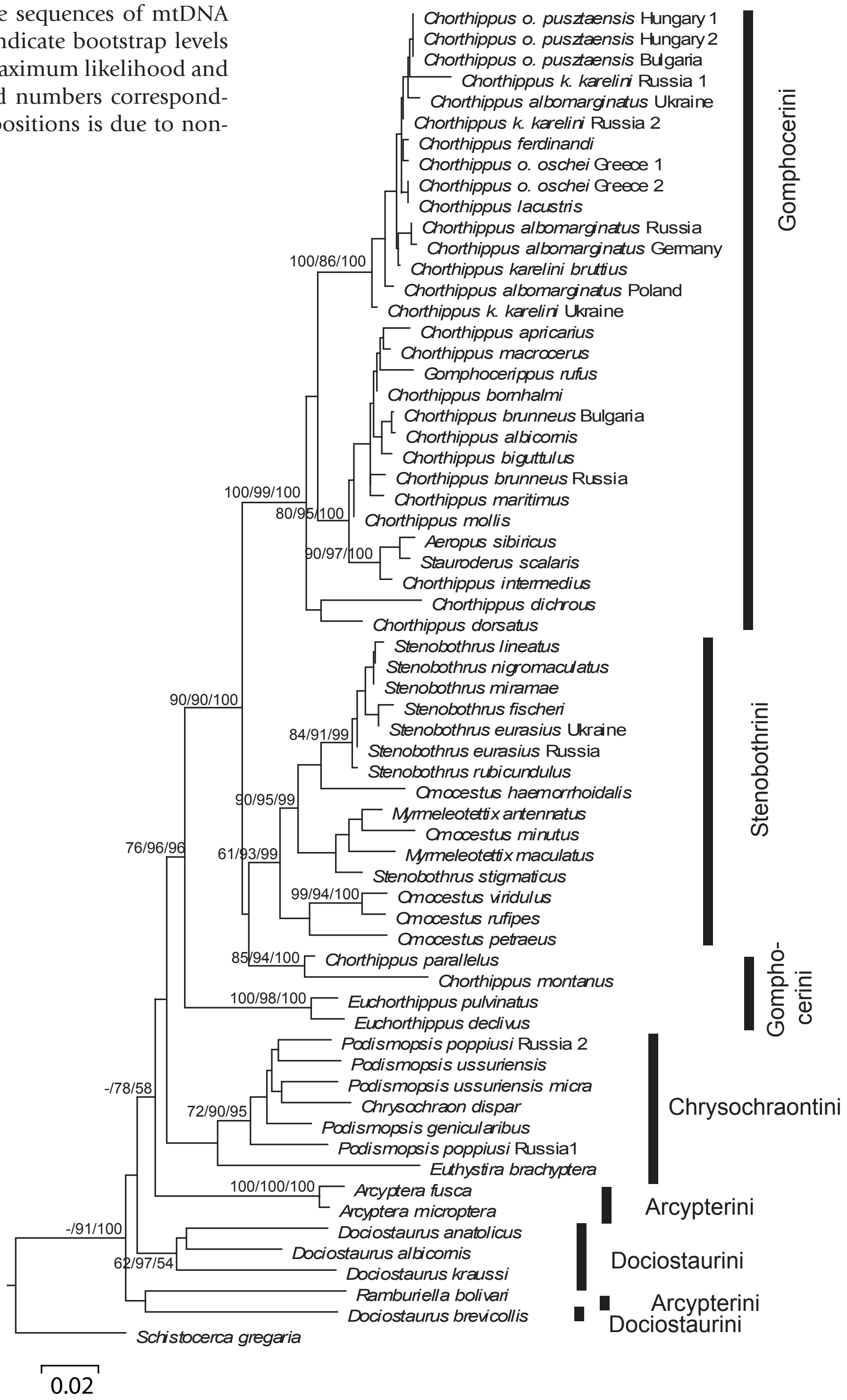
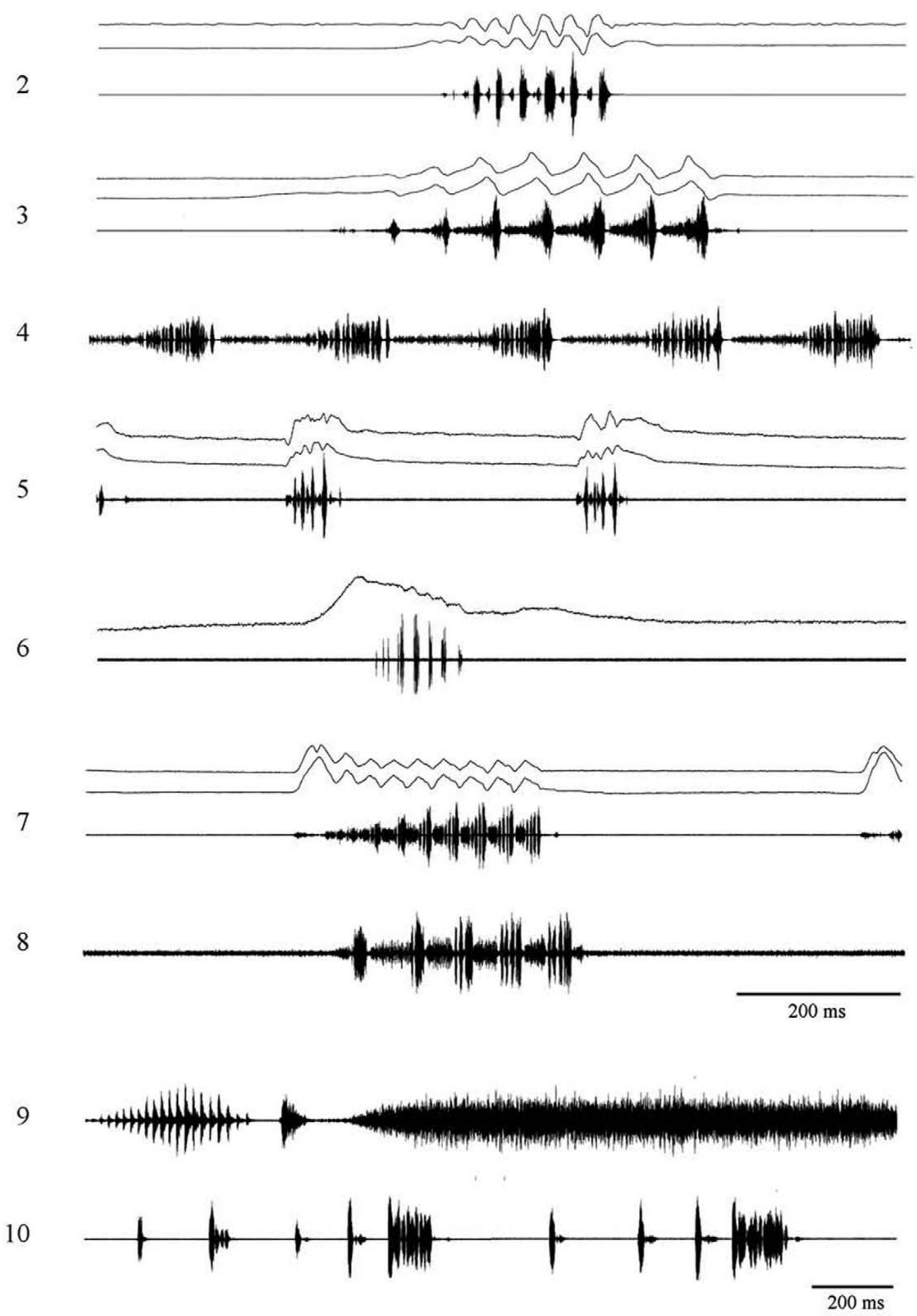


Fig.1. Neighbor-joining tree based on the sequences of mtDNA COI gene fragment (653 bp). Numbers indicate bootstrap levels of support using maximum parsimony, maximum likelihood and Bayesian methods (first, second and third numbers correspondingly). Absence of the first figure in two positions is due to non-congruence of the trees.

of *Podismopsis* it varies from 3.6 to 5.5%. The least interspecific variation in this cluster is found between two species of *Arcyptera* (1.2%).

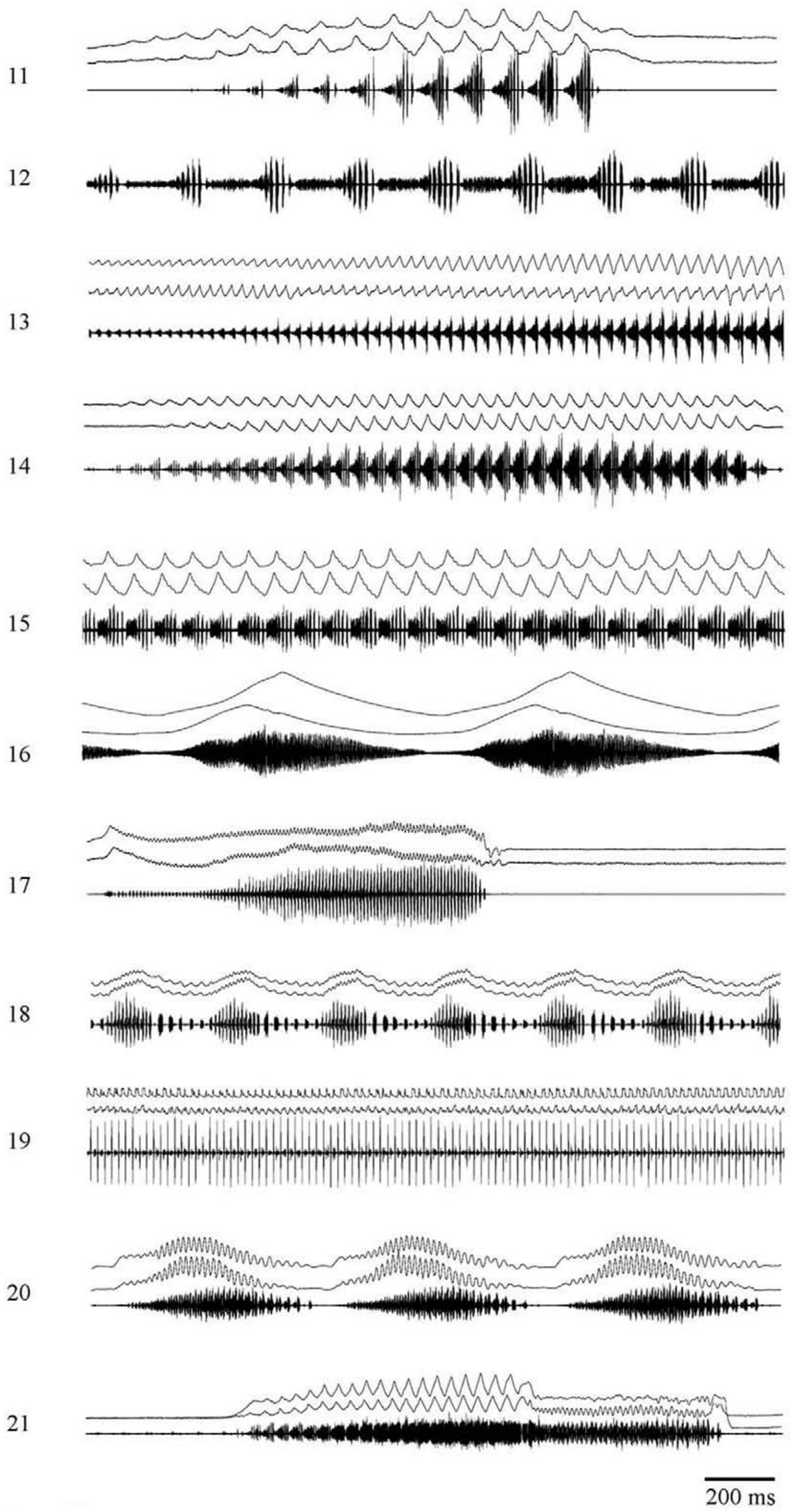
Other clusters contain the species belonging to the two tribes: Stenobothrini (*Stenobothrus*, *Omocestus* and *Myrmeleotettix*) and Gomphocerini (*Gomphocerippus*, *Aeropus*, *Stauroderus*, *Chorthippus* and *Euchorthippus*) according to subdivisions of Harz (1975) and Storozhenko (1986). The genus *Euchorthippus* lies apart from all other members of Stenobothrini and Gomphocerini, which indicates a polyphyletic status for the tribe Gomphocerini. The species of the *C. parallelus* group lie basally to the tribe Stenobothrini, which again indicates a polyphyletic status of the tribe Gomphocerini. The tribe Stenobothrini is divided into two groups, and it is remarkable that the species from the same genera are divided into these two groups as well. However, the species of *Omocestus* and *Myrmeleotettix* genera dominate in one group and the species of *Stenobothrus* genus dominate in another group. The difference between various *Omocestus* and *Myrmeleotettix* species is larger (1.6-7 %) than that between the majority of *Stenobothrus* species (0.5-1 %).

Another cluster including only the members of Gomphocerini is divided into several groups. *C. dichrous* and *C. dorsatus* comprise



Figs 2-10. Oscillograms of calling songs of the species that comprise the basal clusters of the phylogenetic tree: 2. *Euthystira brachyptera*; 3. *Chrysochraon dispar*; 4. *Podismopsis poppiusi*; 5. *Dociostaurus brevicollis*; 6. *D. albicornis*; 7. *Euchorthippus pulvinatus*; 8. *E. declivus*; 9. *Arcyptera fusca*; 10. *A. microptera*. In figures 2-3 and 5-7, the two upper lines are recordings of hind leg movements and the lower line is the sound recording. Oscillograms in figures 2-8 and in figures 9-10 are at different speed.

a basal group to this cluster, and demonstrate a relatively large interspecific difference (5.6 %). Another group includes different *Chorthippus* species: in particular, the species relative to *C. biguttulus* (*C. biguttulus, C. bornhalmi, C. brunneus, C. maritimus* and *C. mollis*). These species are linked with *G. rufus*, *A. sibiricus* and *S. scalaris*. However, three species, *C. intermedius*, *S. scalaris* and *A. sibiricus*, lie apart and show a substantial sequence divergence (3.5-5.5%) from the other members of this cluster. The least difference is found between the species of *C. biguttulus* group (0.3-1.8 %). The species relative to *C. albomarginatus* (*C. albomarginatus, C. oschei, C. karelini, C. lacustris* and *C. ferdinandi*) are separated into the third group. The interspecific difference in this group is comparable to that between the species of *C. biguttulus* group and varies from 0.3 to 1.9 %. The only exception is *C. k. karelini* from the Volgograd region (indicated as 'Russia 1'), with 2-3 % sequence divergence from other members of this group. It is notable that the difference between some species of this group is smaller than between the single individuals from the different populations of one species. For example, *C. o. oschei*



Figs 11-21. Oscillograms of the calling songs of species belonging to the tribes Stenobothrini and Gomphocerini: 11. *Chorthippus parallelus*; 12. *C. montanus*; 13. *Omocestus haemorrhoidalis*; 14. *O. minutus*; 15. *Stenobothrus stigmaticus*; 16. *S. lineatus*; 17. *S. nigromaculatus*; 18. *S. eurasius* Ukraine; 19. *S. rubicundulus*; 20. *Myrmeleotettix maculatus*; 21. *M. antennatus*. In all figures except fig. 12, the two upper lines are recordings of hind leg movements and the lower line is the sound recording.

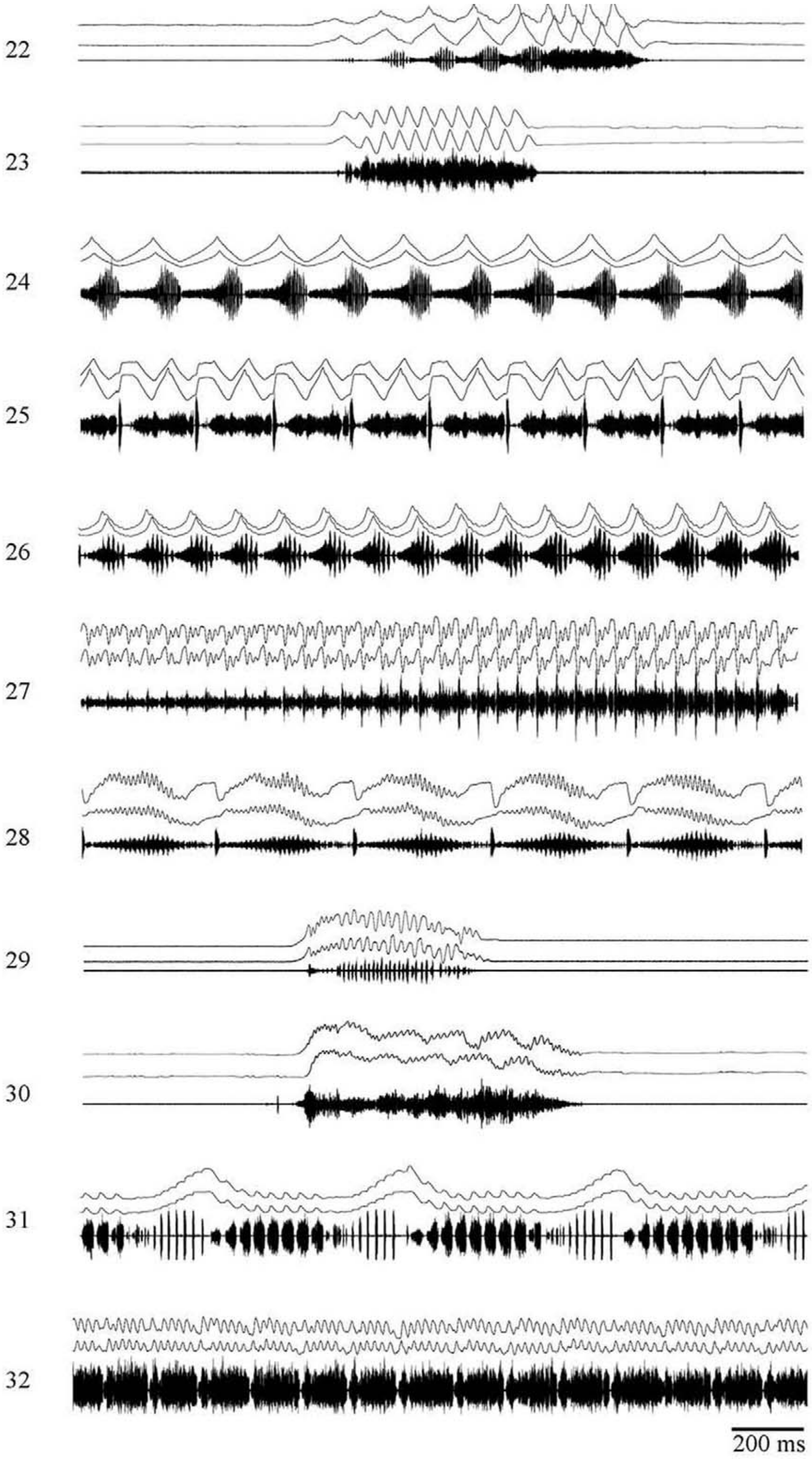
from Greece appears to be closer to *C. lacustris* and *C. ferdinandi* than to *C. o. pusztaensis*.

Calling songs in Gomphocerinae.— One of the simple song patterns found in Gomphocerinae may be characterized as a sound produced by straight upward and downward movements of the hind legs. The first pulse produced by an upstroke is usually quieter, whereas the second pulse is loud, often with gradually increasing amplitude. These movements are performed at rates varying in the range of 5-30 Hz, and the two legs are moved synchronously or with a slight phase shift. The species of the tribes Chrysochraontini (*E. brachyptera* and *C. dispar*) and Dociostaurini (*D. brevicollis*) demonstrate such a simple leg-movement pattern (Figs 2-3, 5). In *D. brevicollis*, the legs are, however, moved at a higher rate and in an irregular manner. It is very likely that the sound produced during calling signaling in *P. poppiusi* (tribe Chrysochraontini) and *R. bolivari* (Arcypterini) (Savitsky 2002) is also generated by simple up and downstrokes of the legs: in the songs of both species, one may distinguish alternation of low- and high-amplitude pulses.

Such pattern is also found in Stenobothrini, *e.g.*, *O. viridulus* (Elsner 1974a) and *O. haemorrhoidalis* (Fig. 13) and Gomphocerini, *e.g.*, *C. macrocerus* (Fig. 24). In these species, two hind legs perform slightly different movement patterns, one with a lower and one with a higher amplitude.

The next step of increasing complexity is a stepwise downstroke, which produces a series of pulses separated by gaps. The pulse rate in such series varies in a range of 60-150 Hz. In *D. albicornis*, the sound is produced during the stepwise downstroke of only one leg at a slow rate of about 0.5 Hz (Fig. 6). More often the rate of the up and downstrokes is similar to that in the simplest pattern (on average from 5 to 20 Hz in different species). Such widespread pattern is found in the tribes Stenobothrini, *e.g.*, *O. minutus* (Fig. 14), *S. stigmaticus* (Fig. 15), *S. fischeri* (Fig. 35B) and Gomphocerini, *e.g.*, *Euchorthippus* (Figs 7-8), *C. parallelus* and *C. montanus* (Figs 11-12), *C. maritimus* (Fig. 26), *A. sibiricus* (Elsner 1974a). Further, we refer all the above song patterns to plesiomorphic patterns.

In the tribes Arcypterini, Stenobothrini and Gomphocerini, one may find increased complexity of the leg movements beyond simple upstroke and simple/stepwise downstroke. In *S. lineatus*, the legs produce simple upward and downward movements, but at an exceptionally slow rate (about 1.2 Hz) (Elsner 1974a) (Fig. 16). In *M. maculatus*, leg vibrations of about 60 Hz produced at antiphase are superimposed on the slow up- and down-movements repeated at a rate of about 1.5 Hz, which form arcs in the recorded trace (Heinrich *et al.* 2001) (Fig. 20). In a subsequent step of increasing complexity, both upstroke and downstroke may be produced in a stepwise manner, each at a different frequency. Such pattern is found in *S. eurasius* from Ukraine (Fig. 18) and S. *scalaris* (Fig. 31). In the songs of these species, two elements contain pulses of different amplitude and repetition rate. In *M. antennatus*, the two legs are moved in a similar, relatively basic, pattern, at a rate of about 25 Hz in the first part of the song, while in the second part of the song only one leg is moved at a much faster rate of about 75 Hz (Fig. 21).



Figs 22-32. Oscillograms of the calling songs of species belonging to the tribe Gomphocerini: 22. *Chorthippus dorsatus*; 23. *C. dichrous*; 24. *C. macrocerus*; 25. *C. apricarius*; 26. *C. maritimus*; 27. *C. biguttulus*; 28. *C. mollis*; 29. *C. albomarginatus*; 30. *C. o. oschei*; 31. *Stauroderus scalaris*; 32. *Gomphocerippus rufus*. In all figures the two upper lines are recordings of hind leg movements and the lower line is the sound recording.

Two elements in a song may also be produced by changing the phase shift between the two legs. In *C. dorsatus*, the first 4-6 elements contain distinct pulses, whereas a subsequent buzzing element lacks distinct pulses, which results from the change in the phase shift between the legs in the course of the song (Stumpner & Helversen 1994) (Fig. 22). In some species of Gomphocerini, such as *C. dichrous* (Fig. 23), *C. brunneus*, species of *C. albomarginatus* group (Figs 29-30), only one or several first up and downstrokes are produced by synchronous leg movements, while a remaining song part is generated by alternating leg movements (Helversen & Helversen 1994, Vedenina & Helversen 2009). In *G. rufus*, each element of the song starts with synchronous up and downstroke of the legs, but the next four to five strokes are produced by alternating leg movements (Elsner 1974a) (Fig. 32).In *C. biguttulus*, each element is produced by one large-amplitude and usually two small-amplitude up and down movements, which results in the alternation of loud and quieter pulses (Fig. 27). In *C. mollis* one leg produces a large-amplitude downstroke, followed by a slow up and down movement, which forms a broad curve in the recorded trace and repeats at about 2.5 Hz 247 (Fig. 28). Superimposed on these slow movements are small-amplitude vibrations of 50- 60 Hz. This pattern recalls the pattern produced by *M. maculatus*. It is notable that in *C. biguttulus* and *C. mollis*, the two legs are moved according to different programs (Elsner 1974a; Helversen & Helversen 1975, 1994).

Some species of *Stenobothrus* use another stridulatory mechanism, distinct from the leg stridulation. In *S. rubicundulus*, the wings with heavily sclerotized veins are clapped together and generated a very loud stridulatory sound, not while flying but during sitting on the ground (Elsner 1974b) (Fig. 19). Eastern subspecies of *S. eurasius* use a similar way of wing stridulation (Bukhvalova & Vedenina 1998).

Courtship behavior in Gomphocerinae.— Males of most species belonging to the tribes Chrysochraontini, Dociostaurini and Arcypterini, do not produce specific courtship songs (Ragge & Reynolds 1998; Savitsky 2000, 2002). Their courtship songs are similar to their calling songs — the courtship song may differ only in being somewhat longer. Thus, no new elements appear in the courtship songs of these species: only temporal characteristics change slightly. In many species of the tribe Gomphocerini males also produce courtship songs that are not different from their calling songs in the number of elements. According to our observations and to the data of Ragge & Reynolds (1984, 1998), the courtship song is similar to the calling song in all species of *Euchorthippus*, in *C. parallelus*, *C. montanus*, *C. macrocerus*, and most species of *C. biguttulus* group.

In Stenobothrini, almost all species produce courtship songs that are either slightly or significantly more complex than the calling song. In most *Omocestus* species, the first part of the courtship song is almost identical to the calling song; however, it is followed by additional elements (Elsner 1974a, Ragge 1986, Ragge & Reynolds 1998). For example, in *O. minutus*, the first part of the courtship sequence is similar to the calling one, while the second part contains a much quieter element. After that, one leg gradually rises and produces several up and down strokes. The single pulse produced

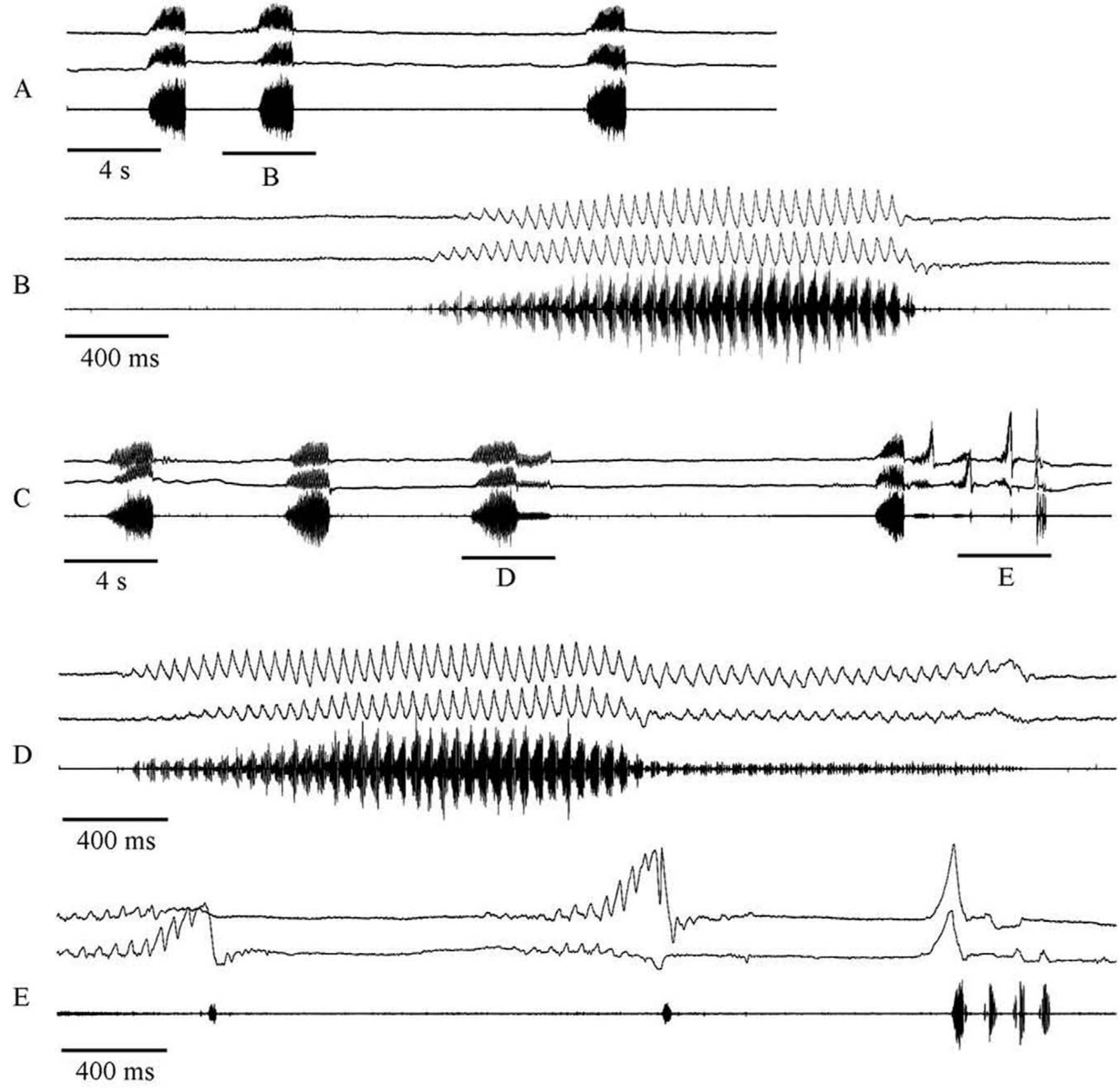


Fig. 33. Oscillograms of the calling (A-B) and courtship (C-E) songs of *O. minutus.* In all figures the two upper lines are recordings of hind leg movements and the lower line is the sound recording.

by these strokes is not loud (Fig. 33). In *O. viridulus*, the first part of the courtship song, which is similar to the calling song, is followed by leg shaking. One leg performs large-amplitude up and down movements, while another leg produces movements with a smaller amplitude. Then the legs generate simultaneous strokes producing the loud pulses (*e.g.* Elsner 1974a, Hedwig & Heinrich 1997, Heinrich *et al*., 1997).

In the majority of *Stenobothrus* species, the complexity is further increased by assembling different elements to form a courtship song (Ragge 1987, Ragge & Reynolds 1998, Berger 2008). The two or three more elements in addition to the calling song element may be distinguished in the courtship song of *S. nigromaculatus* and *S. fischeri*. In both species, the calling song pattern is incorporated into the courtship song so that the calling song element alternates with other elements in a highly specific order. In *S. nigromaculatus*, usually four quiet sequences of the calling song type alternate with a loud and more complex sequence. In this loud sequence, two alternating elements contain pulses of different repetition rate; one of these elements is similar to the calling-song element. This loud part is accompanied by high-amplitude strokes of the hind femora (Fig. 34).

In *S. fischeri*, courtship starts with small-amplitude movements of hind legs producing quiet pulses. Then the legs generate movements of the calling song type. However, the sound produced by this movement pattern is similar to the calling sound in the first half of the sequence; in the second half of the sequence, another sound element is produced. Then the high-amplitude leg movements follow and produce a fourth sound element. During these high-amplitude strokes of the legs, the tibiae are slightly taken away at an angle of about 20°. This phase of the courtship is accompanied by side to

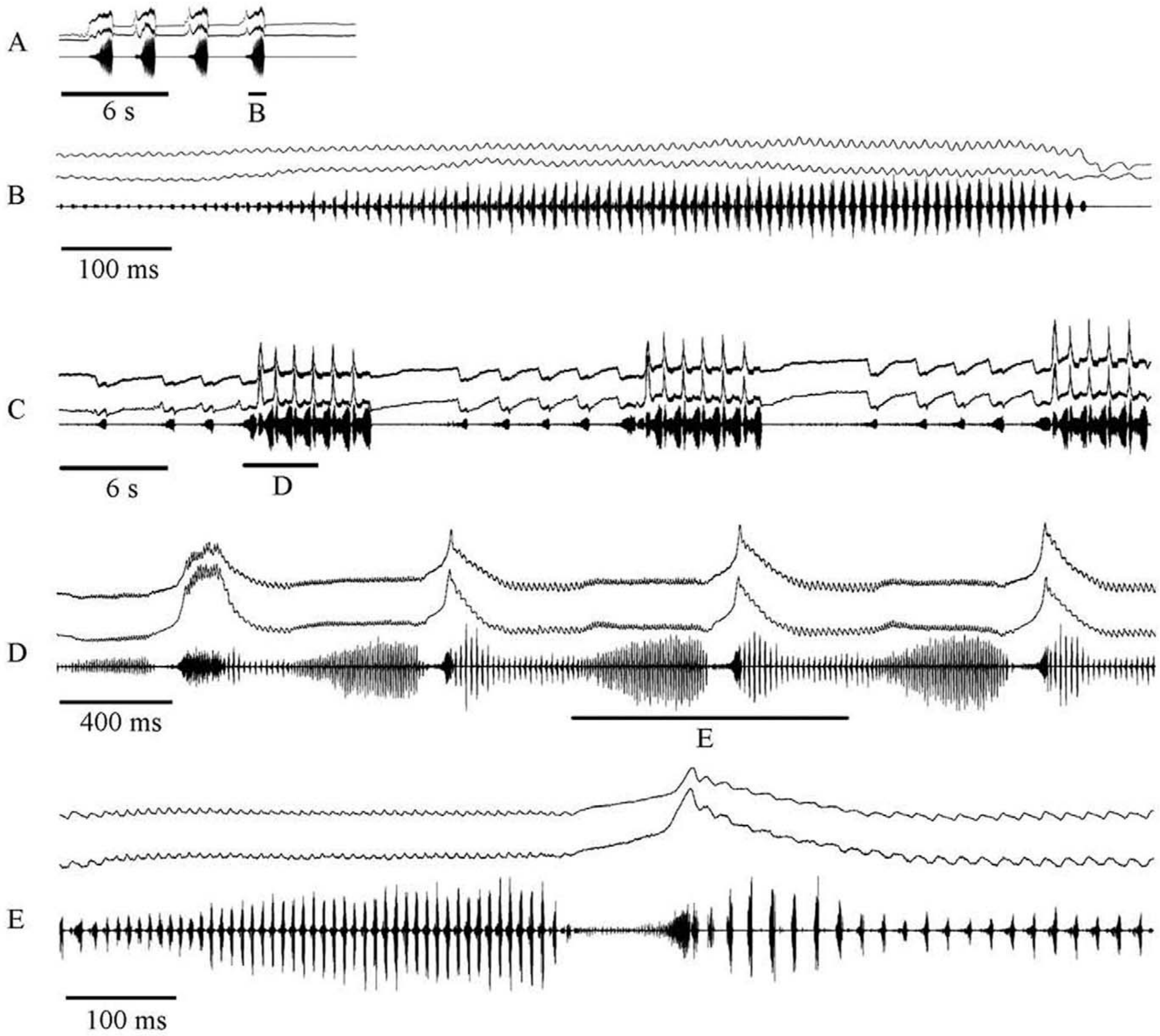


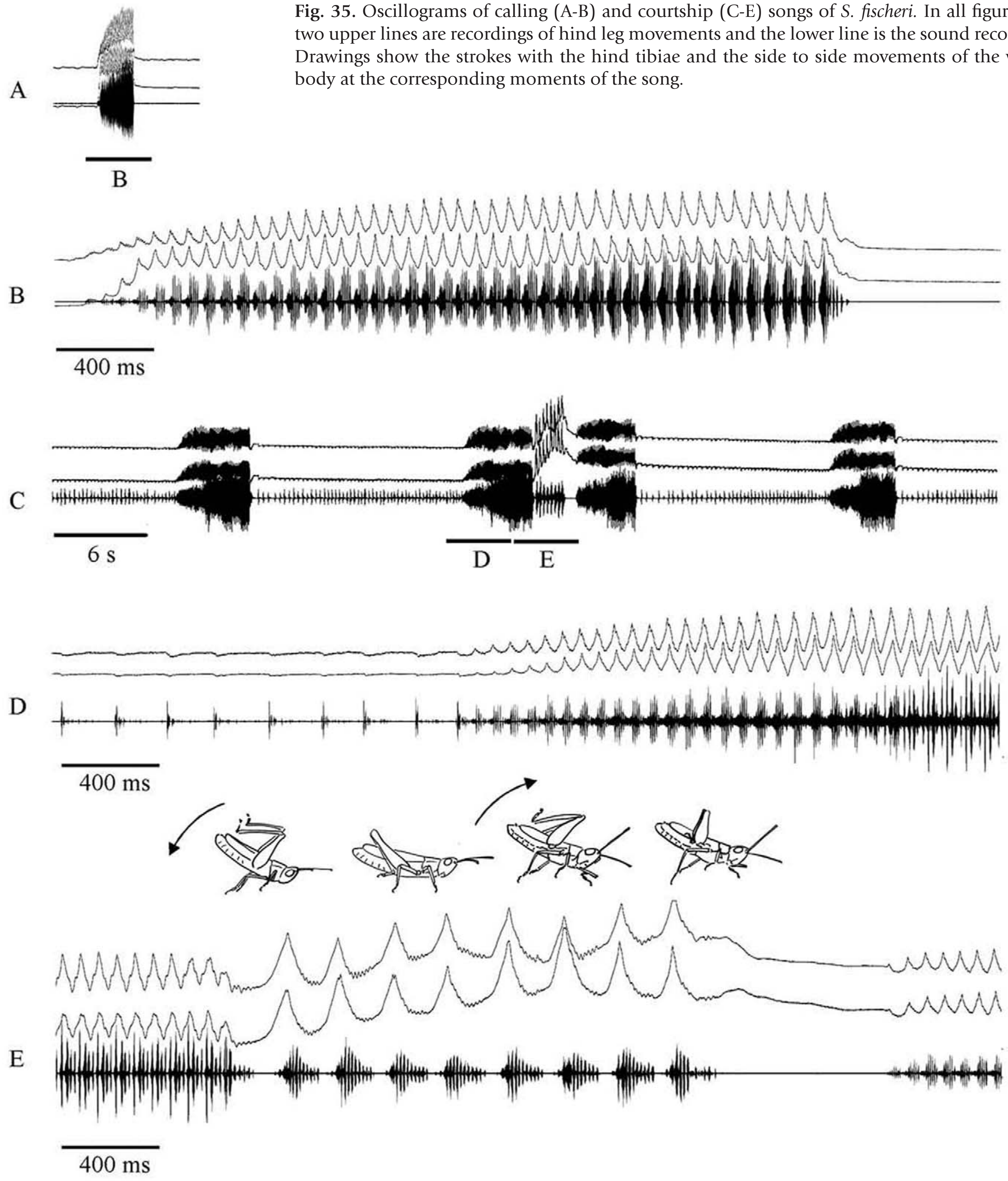
Fig. 34. Oscillograms of the calling (A-B) and courtship (C-E) songs of *S. nigromaculatus*. In all figures the two upper lines are recordings of hind leg movements and the lower line is the sound recording.

side movements of the whole body (Fig. 35).

In *M. maculatus*, the courtship song starts with an alternation of the element similar to the quiet calling element and a short, louder pulse (Ragge & Reynolds 1998, Heinrich *et al.* 2001). Then this sequence is sharply interrupted by a high-amplitude stroke of the femora and characteristic movements of the antennae. A subsequent loud sequence contains two elements that are produced by leg vibrations of different amplitude. These sequences gradually become quieter and appear to again, like the calling sequences, alternate with the short pulses. Then the cycle may repeat, starting with the stroke of legs and antennae or transform to another type: the leg movements repeated at a rate twice as high as before (Fig. 36). When producing the latter sequence, a male also performs swinging movements with its whole body, turning the head from side to side. In *M. antennatus*, the courtship starts with small-amplitude movements of hind legs producing very soft pulses. After that, the two elements of the calling song are repeated two or three times (Berger & Gottsberger 2010). When producing high-frequency vibrations, the two legs are moved by turns. At the end of each sequence produced by low-frequency movements, a male performs a characteristic stroke with his antennae (Fig. 37).

Within the most speciose genus of Gomphocerinae, *Chorthippus*, there are some species that do not produce any specific courtship song; others perform a courtship song that is slightly more complex than the calling song; and finally, some species demonstrate a very different and complex courtship behavior. In some other species of the *C. biguttulus* group, for example in *C. biguttulus*, a second element appears in the courtship song: the sequence of the calling song type is followed by very soft sound sequences (Ragge & Reynolds 1998). The latter ones are produced in the course of lifting the hind legs and generation of the high-amplitude leg movements (Fig. 38). In the courtship of species from another group of *Chorthippus*, *C. albicornis*, the buzzing sequences of the calling song type described by Fontana & La Greca (1999) are alternated with three to four series of pulses of another type accompanied by a visual display. The calling sequence is produced by high-frequency, low-amplitude leg vibrations, while additional courtship elements are produced by high-amplitude strokes with the hind legs, and simultaneously

Fig. 35. Oscillograms of calling (A-B) and courtship (C-E) songs of *S. fischeri*. In all figures the two upper lines are recordings of hind leg movements and the lower line is the sound recording. Drawings show the strokes with the hind tibiae and the side to side movements of the whole body at the corresponding moments of the song.



the whole body is slowly moved from side to side. Similarly to *S. fischeri*, the tibiae of *C. albicornis* are periodically taken away at an angle of about 20° during the high-amplitude strokes (Fig. 39).

All species of the *C. albomarginatus* group demonstrate an extremely complex courtship behavior that is completely different from the calling song and contains up to five sound elements. In the four species of this group, the particular courtship song elements are accompanied by lifting of the abdomen and strokes of the hind tibiae (Helversen 1986, Vedenina & Helversen 2009). *G. rufus* demonstrates a very spectacular visual display during courtship described previously by several authors (Elsner 1974a, 1994; Riede 1983; Ragge & Reynolds 1998). Between the sound sequences of the calling song type, a male produces movements with the head, palps, clubbed-shaped antennae and hind legs.

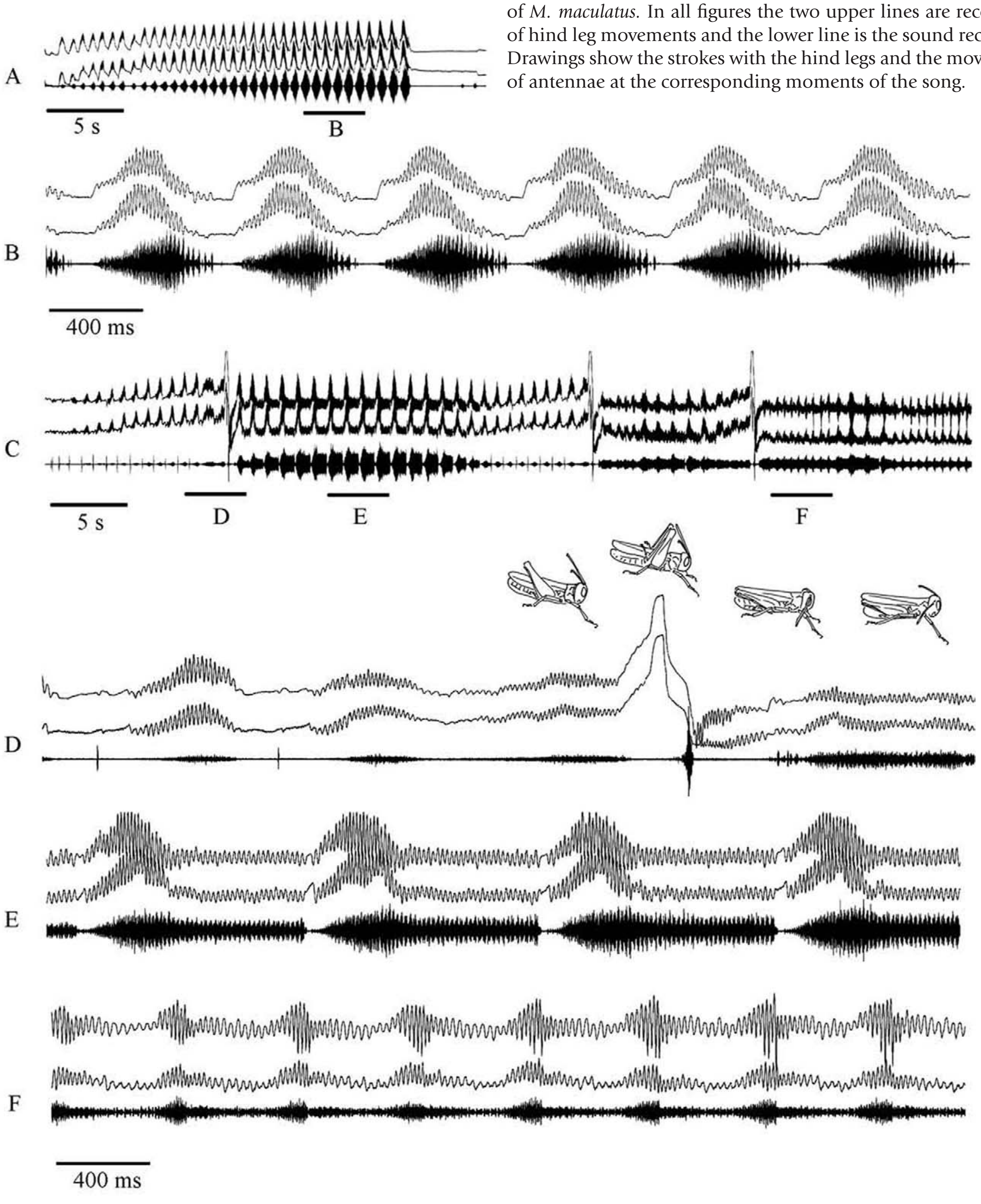


Fig. 36. Oscillograms of calling (A-B) and courtship (C-F) songs of *M. maculatus*. In all figures the two upper lines are recordings of hind leg movements and the lower line is the sound recording. Drawings show the strokes with the hind legs and the movements of antennae at the corresponding moments of the song.

Discussion

Our results mainly support previous phylogenetic studies on Gomphocerinae. Thus, a polyphyletic origin of the tribe Arcypterini is also demonstrated by Contreras & Chapco (2006). Based on analysis of the four mtDNA genes, they show that *Ramburiella* occupies a basal position relative to almost all other species, whereas *Pararcyptera* is associated with *Dociostaurus*.

Polyphyletic origin of the genus *Chorthippus* is shown by Contreras & Chapco (2006) and Guliaeva *et al.* (2005). These authors show the very basal position of *Euchorthippus* (close to Chrysochraontini); we however, have the species of *Euchorthippus* lying basally to all other Gomphocerini and Stenobothrini. The proximity of *Gomphocerippus rufus* to the *C. biguttulus* group is also demonstrated by the same authors. Our results on the proximity of *C. parallelus* to Stenobothrini are similar to the data of Contreras & Chapco (2006). A very little sequence divergence between several species of *Stenobothrus*, such as *S. lineatus*, *S. fischeri*, *S. eurasius* and *S. rubicundulus*, is also

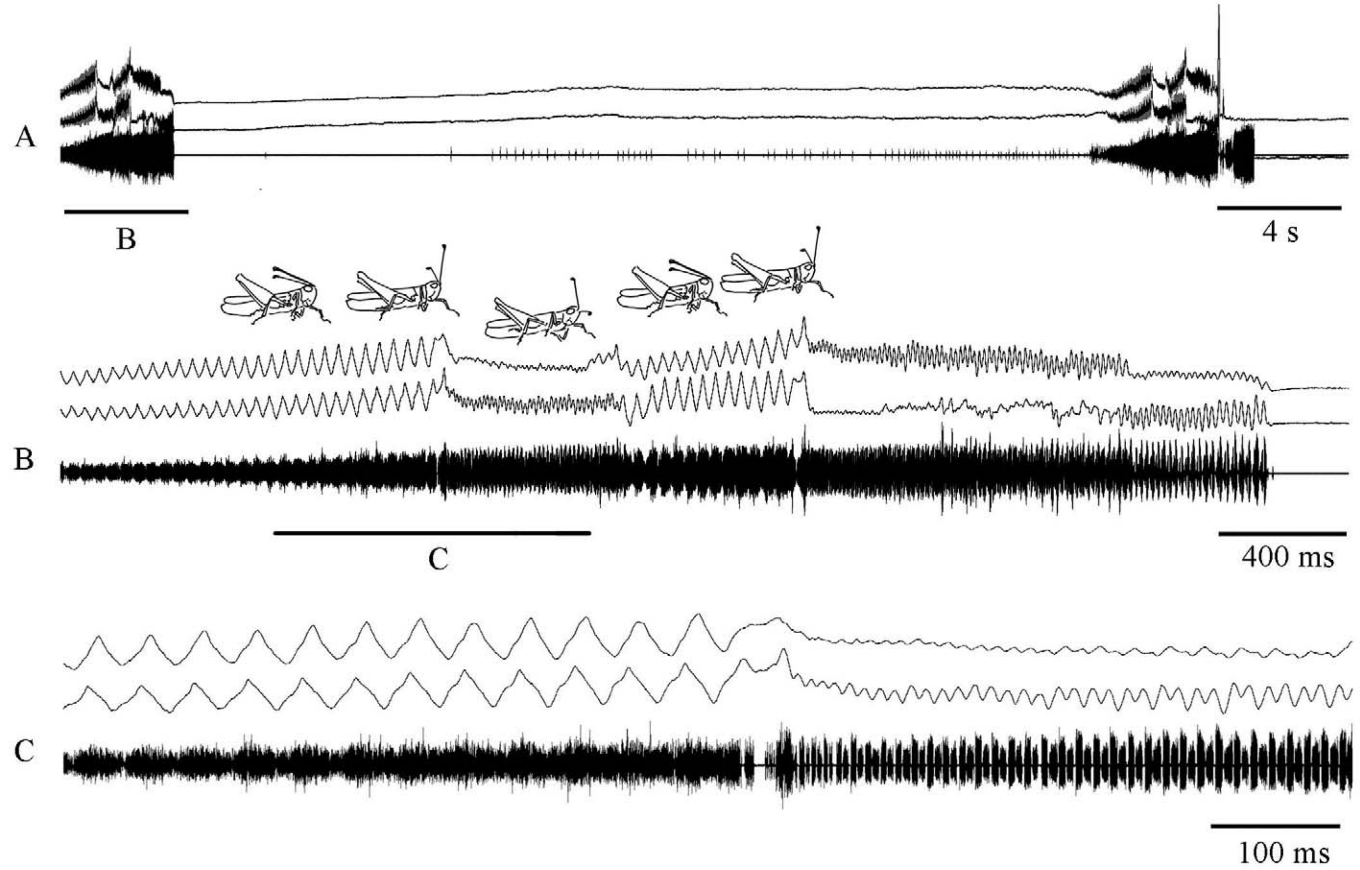


Fig. 37. Oscillograms of the courtship song of *M. antennatus*. Drawings show the movements of antennae at the corresponding moments of the song.

shown by Berger *et al.* (2009).

In our molecular analysis, we show the polyphyletic origin of the tribe Arcypterini, and the genera *Omocestus*, *Stenobothrus* and *Chorthippus*. The most substantial interspecific sequence divergence is found between the species of *Podismopsis* and *Dociostaurus*, which indicates the old origin of these species. Interestingly, the difference between the individuals from the two *P. poppiusi* populations is relatively large (5.5 %). We therefore suggest that these specimens may be attributed to different species.

In the cluster including the genera *Omocestus*, *Myrmeleotettix* and *Stenobothrus*, one may distinguish two groups: the more distantly related species of *Omocestus* and *Myrmeleotettix* and the very young flock of most *Stenobothrus* species studied. Our analysis also indicates the quite recent divergence of many species belonging to the genus *Chorthippus*, especially the *C. biguttulus* and *C. albomarginatus* groups. In the *C. albomarginatus* group, the interspecific difference between some species may be smaller than between single individuals from the different populations of one species. This can be explained by a common ancestral polymorphism, which supports the recent origin of these species.

The recent divergence of the species indicates a rapid speciation in particular groups of Gomphocerinae. How can one explain the rapid speciation in certain gomphocerine grasshoppers, and the relative conservatism in other groups, such as Dociostaurini and Chrysochraontini? Below, we try to find the answer from a comparative analysis of bioacoustics and courtship behavior of Gomphocerinae.

Evolution of the song pattern.— It was suggested by Helversen & Helversen (1994) that the most primitive sound in Gomphocerinae is produced by straight upward and downward movements of the hind legs. However, they named the next level of song complexity (straight up and stepwise downstroke) as a plesiomorphic pattern. The reason for such a suggestion is based on the prevalence of the latter pattern in Gomphocerinae and on the relatively simple explanation of its production.

The plesiomorphic pattern of the grasshopper song can be produced by relatively slow up- and down- leg movements (5-20 Hz at 30° C) with a downstroke modulated by a higher frequency of about 50-150 Hz. Rapid vibratory movements of 50-70 Hz frequency may be derived from flight (Elsner 1994). This hypothesis is supported not only by the similarity in movement frequencies, but also by the bifunctionality of some thoracic muscles that can move both the wing and the leg.

In *Stenobothrus rubicundulus*, it has been shown that simple changes in coordination can convert the movement patterns typical of legs and wings into one another (Elsner 1974b, Elsner & Wasser 1995). Lower (25-30 Hz) or higher (120-140 Hz) vibration frequencies could originate from the half or double rates of the wing beat. Slow repetition movements of 1-10 Hz could originate from the walking or breathing rhythms (Heinrich & Elsner 1997). This possibility is supported by the finding of a close coupling between the respiratory rhythm and the rhythm of the song subunits. However, speculation about the origin of a slow rhythm has less support.

A comparison between the distribution of the two simplest song patterns and the molecular tree does not allow us to conclude which

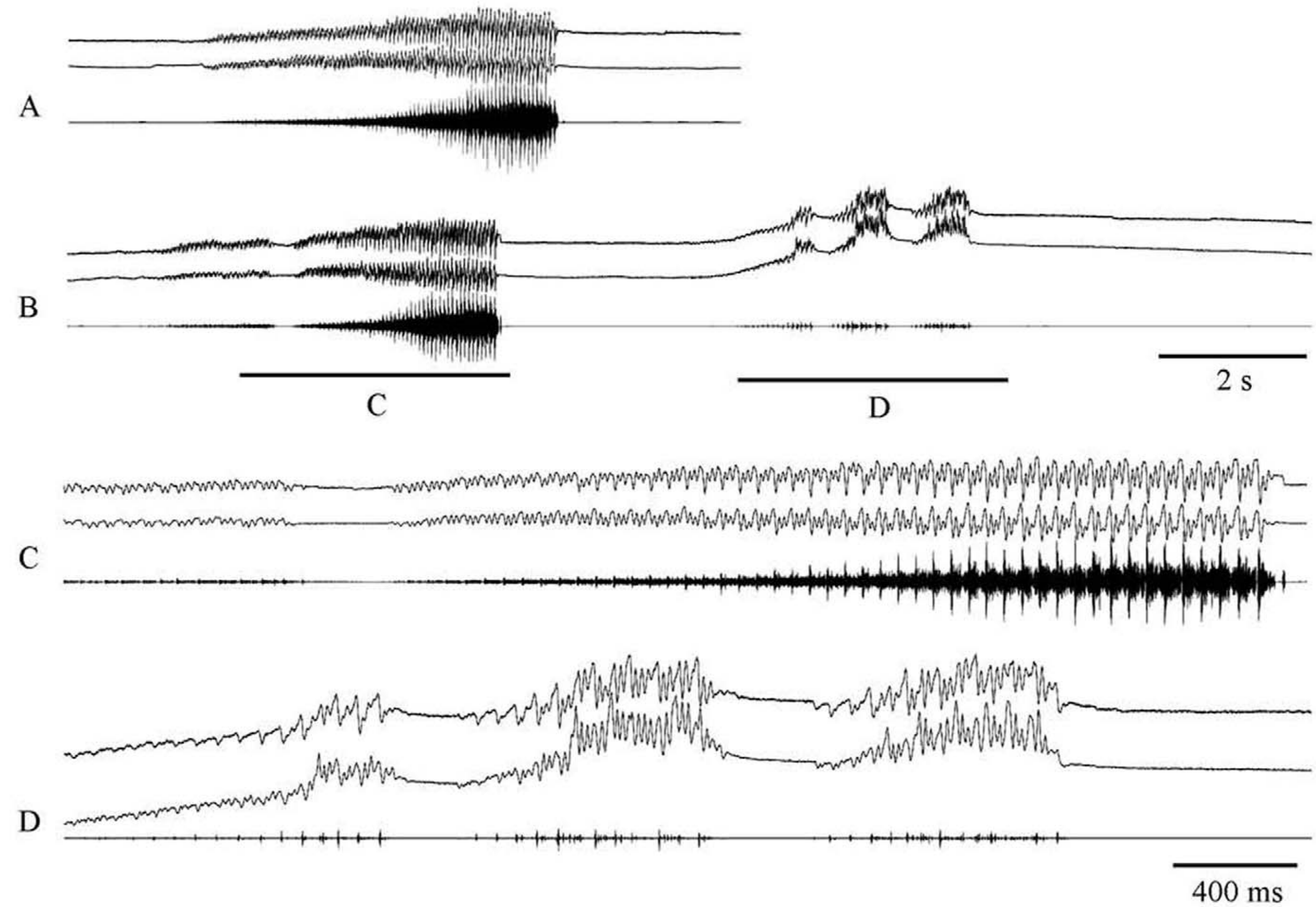


Fig. 38. Oscillograms of the calling (A) and courtship (B-D) songs of *C. biguttulus*. In all figures the two upper lines are recordings of hind leg movements and the lower line is the sound recording.

of two patterns could be more ancient. According to the molecular analysis, they evolved at about the same time. Thus, we refer both these 'simplest patterns' to a plesiomorphic pattern.

The plesiomorphic song pattern is found in almost all clusters of the phylogenetic tree (Fig. 40). In the most basal clusters including Chrysochraontini, Dociostaurini, Arcypterini, the species of *Euchorthippus* and *C. parallelus* group (Gomphocerini), the plesiomorphic pattern is, however, predominant. Moreover, there is a very little interspecific variation in the calling songs between the species of *Euchorthippus* (Ragge & Reynolds 1984), *Podismopsis* (Tishechkin 2008) and *Dociostaurus* (Ragge & Reynolds 1998; Savitsky 2000, 2007). The exception is *A. fusca* and *A. microptera,* that demonstrate a more complex song pattern comprising alternation of the two elements. It is remarkable that genetic distance between these two species is smaller than between the other species of the basal clusters. In Stenobothrini, the plesiomorphic pattern is predominant among the *Omocestus* species, but is found as often as the complex pattern among the *Stenobothrus* species (Ragge & Reynolds 1998, Berger 2008). Within the genus *Chorthippus*, the plesiomorphic pattern may be found in the minority of species (Ragge & Reynolds 1998, Willemse *et al.* 2009).

In the more recently diverged species, especially in the species of *Stenobothrus* and *Chorthippus*, the number of song rhythms increases and coordination of the two legs becomes more complex. A phase shift between the leg movements may change in the course of the song, thus they can moving synchronously at the beginning of a song or even of an element, while move in antiphase at the end of a song or an element. The legs can be also moved according to different programs, and the programs may be exchanged between the legs in the course of the song.

Complex song pattern evolved independently and convergently.—Species from different clusters sometimes demonstrate a similar way of increasing song complexity. For example, a song with two elements produced by a different manner of stepwise leg-movements is found in the genera *Stenobothrus* (*S. eurasius*) and *Chorthippus* (*S. scalaris*). High-frequency (60-80 Hz), low-amplitude leg vibrations are found in *S. nigromaculatus* and *C. albicornis*, whereas high-frequency leg vibrations of a higher amplitude are found in *M. maculatus* and *C. mollis*. In the two latter species, the song element is produced by high-frequency vibrations of 50-60 Hz, and the amplitude of the leg movements gradually increases, reaching maximum intensity in the middle of the element's duration and decreasing by the end of the element. As a result, one can see a spindle-shaped emission in the oscillogram as it was described by Ragge & Reynolds (1998). In both species, two legs are moved strongly in antiphase, so that the upstroke produced by one leg coincides with the downstroke produced by the other leg (Figs 20, 28). All these different ways of increasing the song complexity are fully demonstrated in the courtship songs of most species of the genera *Stenobothrus*, *Myrmeleotettix* and *Chorthippus*.

Increasing complexity in courtship behavior appears to happen similarly in different grasshopper groups (Fig. 40). Very often one can find the courtship song sequences containing very faint and

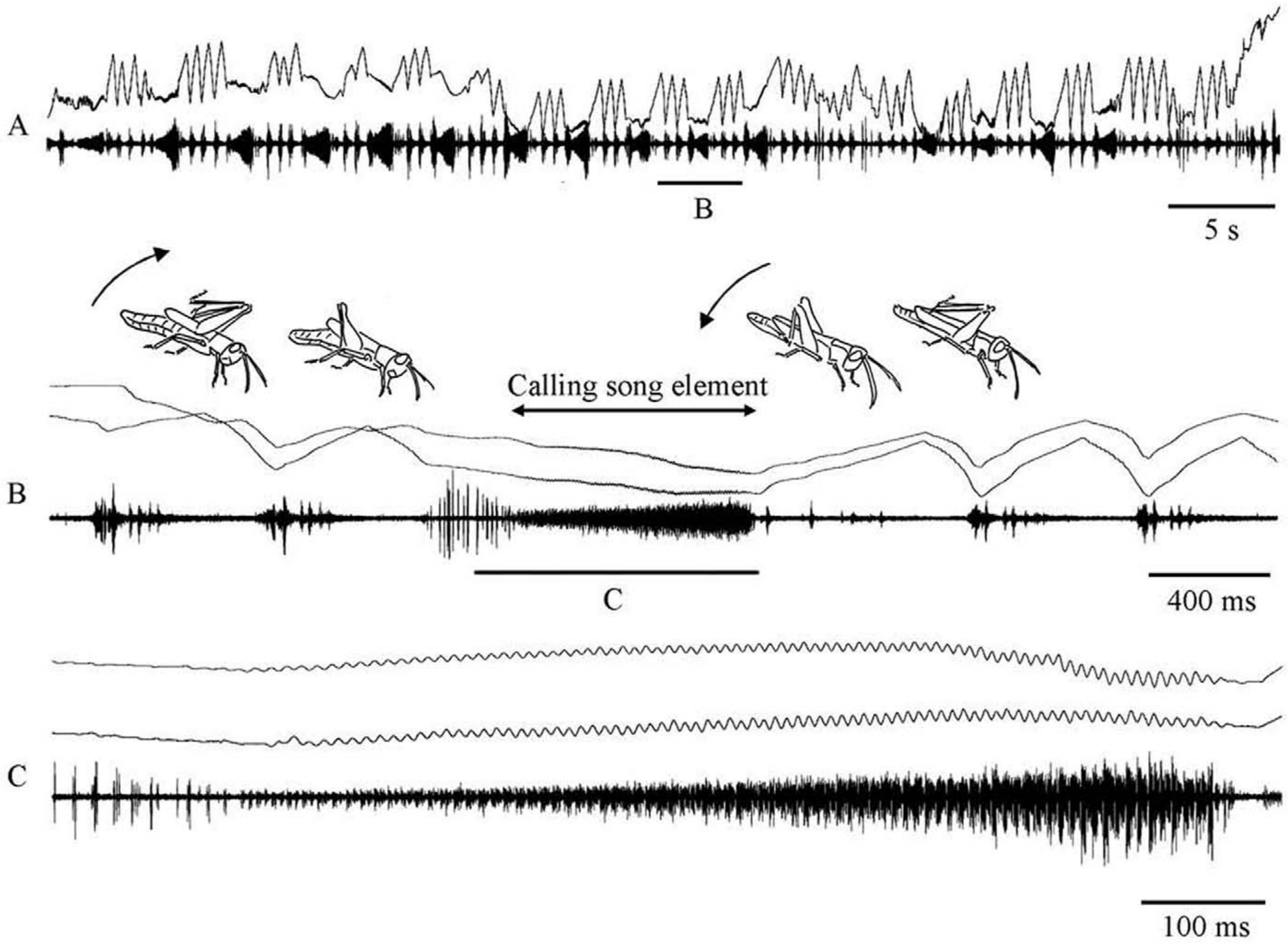


Fig. 39. Oscillograms of the courtship song of *C. albicornis.* In figures B-C, the two upper lines are recordings of hind leg movements and the lower line is the sound recording; in figure A, recordings of only one leg movements are shown. Drawings show the strokes with the hind tibiae and the side to side movements of the whole body at the corresponding moments of the song.

simple pulses alternating with loud complex sequences, as in *M. antennatus*, *S. rubicundulus*, *S. fischeri*, *C. lacustris*. The species from all groups demonstrate incorporation of the calling song pattern into the courtship song. Usually the calling song element is followed by or alternated with, one or more completely different elements, as in *O. viridulus*, *S. nigromaculatus*, *S. fischeri* and *C. albicornis*. The remarkable exception is represented by the species of the *C. albomarginatus* group that have developed two completely different courtship elements, neither being similar to the calling song.

A similarity in visual display accompanying the courtship song is also observed in different groups. The high-amplitude strokes of the hind legs without a pronounced sound are produced by *O. minutus*, *M. maculatus*, *G. rufus* and *C. biguttulus*. Some species of *Stenobothrus* such as *S. fischeri* and *S. clavatus*, *C. albicornis*, and the species of the *C. albomarginatus* group (Vedenina & Helversen 2009), demonstrate the strokes with the hind tibiae at a particular phase of courtship. The strokes with antennae are performed by *M. maculatus*, *M. antennatus*, *S. clavatus*, *G. rufus* and *C. lacustris*. All these species have either clubbed-shaped antennae or antennae with colored tips. Movements of the whole body from side to side are found in *S. fischeri*, *C. albicornis* and *C. lacustris*.

Rapid speciation of courting species: a result of sexual selection?.— Calling and courtship songs have undoubtedly different functions (Vedenina 2005, Fitzpatrick & Gray 2001). Calling songs in Gomphocerinae, as in other Orthoptera, are used to search for conspecific mates at a distance. The calling song has to be reliably detected against a background of other simultaneously singing species. In addition, in a long distance communication system the effects of the acoustic environment on sound propagation are much more important than in a close-range system (Römer 1993). Fine temporal elements of the song may be lost due to reverberations and scattering. Thus, in acoustic long-range systems it is much easier to extract information about the species of a signaller than about his quality. It is shown that the calling song pattern in crickets and bushcrickets is under a stabilizing selection (Ferreira & Ferguson 2002, Heller 2006, Zuk *et al.* 2008). The song patterns in most Ensifera are generally very simple: trills, chirps and double syllable patterns are most common.

In grasshopper communities usually not more than 10 species may sing simultaneously in one biotope. To distinguish between the calling songs of syntopic grasshopper species, it is enough for the songs to be different in only two or three characters. One song character, an element repetition rate, was measured in different species in several biotopes, and it was shown that its range of variability remains unchanged in one and the same species occurring in different biotopes (Bukhvalova & Zhantiev 1994, Bukhvalova 2006, Tishechkin & Bukhvalova 2009). This indicates that such a parameter is also under a stabilizing selection.

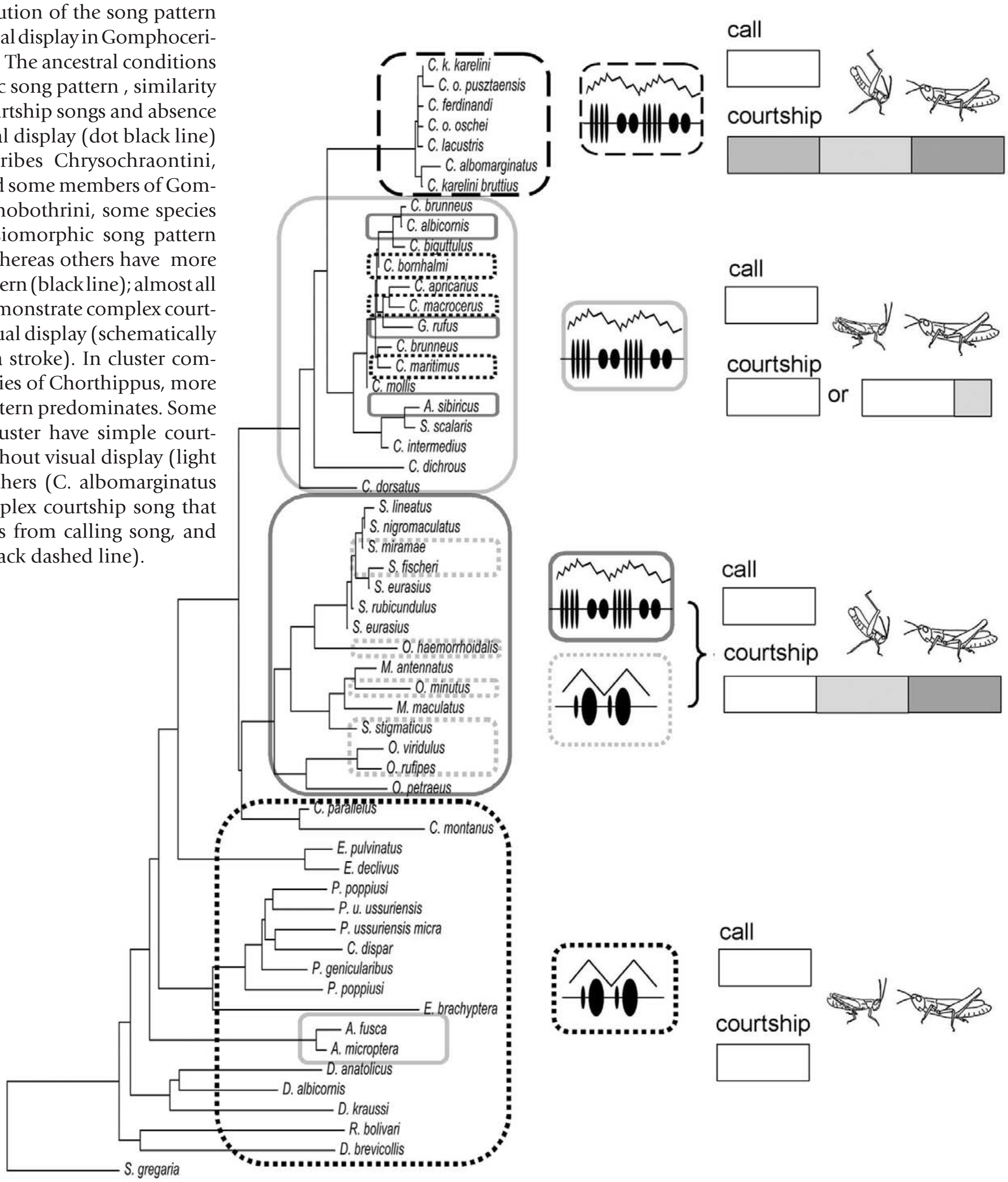


Fig. 40. The evolution of the song pattern and courtship visual display in Gomphocerinae grasshoppers. The ancestral conditions are plesiomorphic song pattern , similarity of calling and courtship songs and absence of courtship visual display (dot black line) typical for the tribes Chrysochraontini, Dociostaurini and some members of Gomphocerini. In Stenobothrini, some species demonstrate plesiomorphic song pattern (dot grey line), whereas others have more complex song pattern (black line); almost all Stenobothrini demonstrate complex courtship song and visual display (schematically indicated by tibia stroke). In cluster comprising most species of Chorthippus, more complex song pattern predominates. Some species of this cluster have simple courtship behavior without visual display (light grey line), but others (C. albomarginatus group) have complex courtship song that completely differs from calling song, and visual display (black dashed line).

Courtship songs are produced by the males in a close proximity to a female. Some courtship parameters can be used for species recognition, whereas others may serve for the evaluation of mate quality. Evolution of male traits and female preferences in the courting grasshopper species, due to a different mating strategy, may be faster than in the noncourting species. During courtship, several competing males often sing for many hours around a female that can hear and compare different males in the field. This may facilitate female choice and favor competition among males, similar to a "lek" situation.

Kirckpatrick & Ryan (1991) called it the "paradox of the lek", when females prefer elaborate male mating displays just in those species in which they receive little more from males than sperm. The long and complex courtship is characteristic for many species of *Omocestus*, *Stenobothrus*, *Myrmeleotettix*, some species of *Chorthippus* and *G. rufus* (Elsner 1974a, Bull 1979, Riede 1983, Elsner & Wasser 1995, Ragge & Reynolds 1998, Berger 2008, Ostrowski *et al.* 2009, Vedenina & Helversen 2009, Berger & Gottsberger 2010). In our phylogenetic tree, all these species belong to the more recently diverged groups than those in the tribes Chrysochraontini, Dociostaurini and Arcypterini, as well as the species of *Euchorthippus*, where the courtship behavior is generally absent or, in only a few of species, poorly developed (Ragge & Reynolds 1998; Savitsky 2000, 2002). This could indicate that the mating strategy without courtship is an ancestral strategy in gomphocerine grasshoppers, whereas the complex courtship has evolved later, independently and convergently (Fig. 40).

We have shown that the genetic difference between related species of *Stenobothrus* or between the species of the *C. albomarginatus* group is much less than between non-courting species of Dociostaurini or Chrysochraontini. This evidences the rapid speciation of the courting species. We suggest that sexual selection could be the main driving force of speciation in these young species.

On the other hand, the species of the *C. biguttulus* group, *C. apricarius*, *C. macrocerus*, and *S. scalaris*, also represent a young species cluster. These species lack complex courtship songs. In general, their mating strategy may be compared with the strategy of *Dociostaurus* or *Arcyptera*. This raises a question— what was a driving force for rapid speciation in the *Chorthippus* genus?

One possible explanation could be that the mating strategies nevertheless differ in these groups. In *Dociostaurus* species, a male leaves a female in the case of her refusal and immediately begins to search for another female (Helversen, pers. comm.). In the *C. biguttulus* group a male may pursue a female after her refusal and thus, the species of this group are well on the way to switching to another mating strategy, the strategy with a complex courtship. The presence of an additional element in the courtship song of *C. biguttulus* (a very soft sound produced by the high-amplitude leg movements) is in accordance with this hypothesis.

Another possible explanation of rapid speciation in the *Chorthippus* genus might be that a rather simple calling song structure in the species of the basal tribes and the diversity of the calling song complexity in the species of *Chorthippus* is a result of different nervous control of stridulatory behavior in grasshoppers. The neuromuscular patterns determining the stridulatory movements, as well as central nervous control, were studied in detail in various *Omocestus*, *Stenobothrus* and *Chorthippus* species (Elsner 1974a, b, 1994; Ronacher 1989; Hedwig 1992; Heinrich & Elsner 1997; Heinrich *et al.* 2001), but not in species of Dociostaurini or Chrysochraontini. We speculate that in the species of the basal tribes, the central nervous control of stridulation may be organized so that only a simple song pattern is produced. In the species belonging to the tribes Stenobothrini and Gomphocerini, evolution of the neuronal network controlling stridulation resulted in the possibility of increasing calling song complexity. Thus, complexity of the courtship song structure increased as well and hence, sexual selection could be the main driving force of rapid speciation in the *C. biguttulus* group and some other species of Gomphocerini.

Acknowledgments

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