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ORIGINAL ARTICLE

Evolution of calling songs in the grasshopper subfamily Gomphocerinae (Orthoptera, Acrididae)

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Abstract

The evolution of the calling songs in Gomphocerinae was evaluated via estimating a phylogenetic signal of the song characters and an ancestral character state reconstruction. Analyses of the calling songs in 80 palearctic gomphocerine species allowed us to define 24 characters describing the temporal pattern of the sound and the stridulatory leg-movement pattern. The ancestral song of Gomphocerinae was shown to consist of numerous short echemes lasting on average 0.9 s; each echeme comprised only one syllable produced by movements of only one leg. The next step of the song evolution could be producing longer echemes or longer echeme-sequence. Later, echeme duration again decreased, but this was accompanied by increasing of echeme or syllable complexity. The characters describing the echeme structure were found to be conservative in their evolution. By contrast, most characters of the syllable temporal structure were shown to be relatively labile and more likely under natural or sexual selection. Our study shows that the song evolution in Gomphocerinae implied not only increasing but also decreasing complexity of the syllable temporal structure.

KEYWORDS

ancestral character state reconstruction, grasshopper, phylogenetic signal, sexual selection, song, speciation, stridulation

1 | INTRODUCTION

In many species of animals, acoustic communication is one of the important components of reproductive isolation. Acoustic communication systems with their diversity of song patterns and song recognition mechanisms have long served as a model for many questions on speciation (e.g. Gerhardt & Huber, 2002; Greenfield, 2002). For example, how did the diversity of species, forms, adaptations and behaviours that we find today evolve? Signal diversity resulted from natural selection may be constrained by (1) habitat acoustics, (2) acoustically oriented predators or parasites and (3) masking of signals by those of distantly related species. Reproductive interactions between closely related species are another potential source of selection for song divergence. Songs also diverge under sexual selection when competition over mates takes place, either by male contest or by mate choice (Andersson, 1994; West–Eberhard, 1983).

A long-distance acoustic signal (an advertisement signal or a calling song) shows a clearly species-specific structure, as this type of signal is often the only way to attract conspecific females. However, calling songs can also be used in the context of sexual selection, for females to discriminate among males on the basis of certain properties of calling songs for mating. Various studies on insects and anurans show that the characteristics of calling songs may vary to different degrees. It has been suggested that stable parameters are used for the process of recognition, and variable parameters are important for intraspecific

competition (Gerhardt, 1991; Popov & Shuvalov, 1977). The differential role of song parameters in communication (species recognition vs. mate choice) can thus promote distinct evolutionary patterns. For example, one might expect that acoustic traits constrained by a morphological trait will evolve slowly, whereas those depending on behavioural or neurological processes would be more labile (Gerhardt & Huber, 2002).

The relative importance of stochastic forces or selection in the evolution of communication traits can be investigated by estimating the phylogenetic signal of such traits, that is the degree of congruence between traits and the topology of a phylogenetic tree that summarizes the evolutionary relationships among species (Blomberg et al., 2003; Münkemüller et al., 2012). A high phylogenetic signal is expected for a given trait if it has evolved under the effects of random genetic drift or fluctuating directional evolution (Ord & Martins, 2006). By contrast, a low phylogenetic signal for a given trait implies that a trait is more responsive to natural or sexual selection.

Among animal groups that exhibit acoustic communication, the insect order Orthoptera is considered a good model for both large-scale macroevolutionary studies and microevolutionary surveys in small groups of lower taxa. For example, Song et al. (2020) tried to elucidate how both hearing and sound production evolved and affected diversification in Orthoptera. They showed that in the suborder Ensifera (crickets and katydids), forewing-based stridulation and tibial tympanal ears co-evolved, but in the suborder Caelifera (grasshoppers), abdominal tympanal ears first evolved in a non-sexual context, and later co-opted for sexual signalling when sound-producing organs evolved. The evolutions of song frequency in Ensifera were evaluated based on an ancestral character state reconstruction (Li et al., 2018). The analysis indicated that the pathway of the song evolution is mainly from low-frequency pure tones to high-frequency broadband noise and, finally, to various types. Frederick and Schul (2016) reconstructed the evolutionary history of three call traits in the katydid genus *Neoconocephalus* including 17 species. The most likely ancestral call pattern has been suggested to be a continuous call with a fast pulse rate and single pulse pattern.

The evolution of acoustic communication in another suborder of Orthoptera, Caelifera, has been less studied than in Ensifera, especially in a phylogenetic framework. However, acoustic communication in one of the Acrididae subfamilies, Gomphocerinae, is most developed in terms of complexity of stridulatory leg movements, the number of sound elements and mating strategies (e.g. Otte, 1970; Ragge & Reynolds, 1998; von Helversen & von Helversen, 1994). The song is produced by stroking a stridulatory file of each hind femur across a raised vein on the ipsilateral wing. Using both hindlegs, the grasshoppers have two separate sound-producing devices that 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, 1994; von Helversen & Elsner, 1977). 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 (von Helversen & von Helversen, 1994). The courtship song may reach an extremely high complexity in the unit number; moreover, the song may be accompanied by conspicuous movements of different parts of the body such as the abdomen, head, antennae or palps (e.g. Berger, 2008; Elsner & Wasser, 1995; Otte, 1970; Vedenina et al., 2012, 2020; Vedenina & von Helversen, 2009).

It was suggested by von Helversen and von Helversen (1994) that the most 'primitive' sound in Gomphocerinae is produced by straight upward and downward movements of the hind legs. The authors suggested a linear gradual evolution of the calling song. A model of song evolution in Gomphocerinae was, however, proposed without a phylogenetic context. Vedenina and Mugue (2011) compared a molecular phylogenetic tree with the distribution of the song pattern complexity and courtship behaviour. They supported the hypothesis of Helversen & Helversen about the gradual increasing of the song complexity and predicted that the time of species divergence should correlate with the song complexity. They also showed that complex courtship behaviour in Gomphocerinae evolved independently and convergently. Hereby, the main driving force of rapid speciation in this subfamily was suggested to be sexual selection. Mayer et al. (2010) came to similar conclusions, emphasizing that evolution of complex species-specific songs contributed to independent radiations in different clades. By contrast, Nattier et al. (2011) observed no clear trend toward increasing song complexity and claimed that the calling song evolution in Gomphocerinae involved many parallel transformations and reversals. Thus, a pattern of transformations was described as 'dynamic'. Notably, so far only Nattier et al. (2011) used a strict parameterization of the song characters.

In this study, we evaluated evolution of the calling songs in Gomphocerinae via estimating a phylogenetic signal of the song characters and an ancestral character state reconstruction. Analyses of the calling songs in 80 gomphocerine species allowed us to define 24 characters describing the temporal pattern of the song. Compared to the characters used by Nattier et al. (2011), most of the song traits we have chosen also implied the stridulatory pattern of the leg movements. Because of the extreme complexity of the courtship songs in Gomphocerinae, we

could not conduct a strict parameterization of the courtship characters, and did not therefore consider this type of the songs in the current study. Estimation of the phylogenetic signal allowed us to reveal more evolutionary conservative and more labile song traits. This explained which characters evolved under the effects of random genetic drift, and which ones as a result of selection. The ancestral character state reconstruction allowed us to reveal the ancestral calling song pattern, and whether the song evolution in Gomphocerinae implied not only increasing but also decreasing complexity of the song temporal pattern.

2 | MATERIAL AND METHODS

2.1 | Song recording and analysis

Calling songs were recorded from an isolated male. Field recordings of the songs were made using a Sony F-V610 microphone, or an Audio-technica ATR55 microphone, and an Elektronika-302-1 cassette recorder (the upper frequency limit 14 kHz; before 2001), or a Sharp MD-MT190H minidisk recorder (sampling frequency 44.1 kHz; before 2013). The signals were A/D converted with a PC card L-305 (L-Card Ltd.). The ambient temperature near a singing male in the field was 20–40°C.

During recordings made in the laboratory, both the sound and the movements of the hind legs were recorded with a custom-built opto-electronic device (Hedwig, 2000; von Helversen & Elsner, 1977). A piece of reflecting foil was glued to the distal part of each hind leg femur of the male and two opto-electronic cameras were 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 signals. These signals, together with the recordings of the sounds (a microphone type 4191, ½ inch; a conditioning amplifier type 2690; Brüel & Kjaer, Nærum, Denmark), were A/D converted with a custom-built PC card. The sampling rate was 3125 Hz for recording the stridulatory movements and 100 kHz for sound recordings. The ambient temperature near the singing male was 30–32°C.

We recorded the calling songs in 61 species (Table S1); in each species, the songs of three males were analysed on average. Most species were recorded in the laboratory, or both in the laboratory and in the field. For two species (*Dociostaurus kraussi*, *Notostaurus albicornis*), only field recordings were used. If the temperature of the field recordings was very different from that used in the lab (30–32°C), we used the change correction of continuous temporal parameters (Bauer & von Helversen, 1987; Tishechkin & Bukhvalova, 2009). The temporal parameters of the songs were analysed with a COOLEDIT (Syntrillium) and a TURBOLAB 4.0 (Bressner Technology) programs. The songs of 18 species were analysed on the basis of literature. Altogether, we recorded 111 males and analysed 158 songs (Table S1).

For description of the calling songs, we used 24 characters, which considered both leg-movement and sound (Table 1) patterns. We used two main terms for the song description, *syllable* and *echeme* (Figure 1). *Syllable* starts when the legs leave their initial position and ends when the legs return to this position. The syllable can be produced by one complete up and down leg movement (Figure 1d) or by several up and down leg movements (Figure 1h). In the latter case, the amplitude of the leg movements or phase shift between two legs within syllable greatly varies. Sometimes, we distinguished *pulses* within syllable. *Pulse* is a structural unit of the lowest level, which is produced by one stroke of a hind leg against a fore wing. We only distinguished pulses when the pulse duration did not exceed 15 ms, and the intervals between pulses varied in the range of 1.25–15 ms (Figure 1c,e). Pulses can be produced by stepwise up or down movements (Figure 1c,e,f) or by high-amplitude leg movement (Figure 1g). *Echeme* is a structural unit of the highest level, which represents a series of consistent syllables separated by pauses. In some cases (*Dociostaurus maroccanus*, *Notostaurus albicornis*), echeme may consist of only one syllable (Figure 1c). The calling songs contain only one echeme in some species (Figure 1a) or the echeme-sequence in others (Figure 1b). Sometimes, echeme contains two types of syllables; then, we distinguished *element* as a sequence of syllables of similar structure (Figure 1i).

For the parameterization of the calling songs of Gomphocerinae, we mainly used the two structural units of the calling songs, *syllable* and *echeme*. The unit of the lowest level, *pulse*, was used in only one character (character 21, Table 1). The pulses were only found in one third of the species studied. We did not analyse the amplitude modulation of the sound because it could vary depending on the recording equipment. Our field recordings and most recordings obtained from the literature were made by portable recorders with a frequency range not exceeding 12.5–15 kHz. At the same time, the sound produced by Gomphocerinae has a broad frequency spectrum with two peaks between 5 and 15 kHz and between 20 and 40 kHz (Meyer & Elsner, 1996), and various song elements may significantly differ in the carrier frequency (Ostrowski et al., 2009; Vedenina et al., 2007, 2020). The difference in the frequency spectra between the various song elements may influence the amplitude ratio on the oscillogram.

We distinguished the song parameters to be either discrete (e.g. presence or absence of pulses, complexity of the syllable structure) or continuous (e.g. echeme or syllable

TABLE 1 Description of the calling song characters in Gomphocerinae and the character states used in the song analysis.

Echeme	
1. *Echeme homogeneity*. All echemes have a similar structure – (0) or echemes of different structure are present – (1).	
2. *Syllable homogeneity*. Echemes consist of one (0) or two (1) types of syllables.	
3. *Echeme number*. Total number of echemes.	
The first type of echemes	***The second type of echeme (if present)***
4. *Echeme duration* (s).	4. …
5. *Syllable number*. Total number of syllables of all types per echeme.	5. …
6. *Echeme and syllable equality*. Echeme always consists of only one syllable: no – (0), yes – (1).	6. …
Syllable	
The first type of syllables	***The second type of syllable (if present)***
Continuous characters	
7. *Syllable number per echeme*.	7. …
8. *Syllable period* (ms).	8. …
9. *Syllable duration* (ms).	9. …
Discrete characters	
10. *Sound-producing mechanisms*. Sound is produced by only femoro-tegminal stridulation (0) or by other mechanisms of sound emission (1).	10. …
11. *Syllable complexity*. Complexity of the syllable structure: each syllable consists of one upward and one downward leg movements – (0), each syllable consists of several up- and down-movements – (1).	11. …
12. *Stepwise upstroke*. Stepwise leg movements during the upstroke: absent – (0), present – (1).	12. …
13. *Stepwise downstroke*. Stepwise leg movements during the downstroke: absent – (0), present – (1).	13. …
14. *Silent syllable part*. Sound is generated during both upstroke and downstroke (0) or only during the downstroke (1).	14. …
15. *Silent syllable part variation*. Character 14 is uniform while generating the entire syllable – (0), or character 14 varies for the different syllable parts – (1).	15. …
16. *Leg-movement pattern difference*. Two legs are moved with the same pattern (0) or different patterns (1).	16. …
17. *Phase shift variation*. Phase shift between the movements of two legs is constant – (0), variable – (1).	17. …
18. *Phase shift*. Two legs are moved synchronously or with a slight phase shift (less than 0.1 syllable period) – (0), with a large phase shift (between the 0.1 and 0.4 syllable period) – (1), alternatively (more than 0.4 syllable period) – (2).	18. …
19. *Usage of two legs*. Sound is generated by the movements of both legs – (0), by the movements of only one leg – (1).	19. …
20. *Variation in usage of two legs*. Character 19 is uniform while generating the entire syllable – (0), or character 19 varies for the different syllable parts – (1).	20. …
21. *Pulses*. Syllable contains no pulses (0), regular pulses in some part (1) or syllable consists of only regular pulses – (2).	21. …
Continuous characters for the leg-movement rate	
22. *Stepwise upstroke rate*. Rate during the stepwise upstroke (Hz).	22. …
23. *Stepwise downstroke rate*. Rate during the stepwise downstroke (Hz).	23. …
24. *Superimposed movements rate*. Rate of vibrations superimposed on the slower movements (Hz).	24. …

duration, echeme or syllable number). Most song characters describe the syllable structure, since there are many variations of this unit within the subfamily Gomphocerinae. In the current study, we used a non-canonical definition of the syllable. According to Ragge and Reynolds (1998), the syllable is produced by one up- and down-movement of the hind legs. We, however, distinguished between simple syllables (Figure 1d–f) corresponding to the description of Ragge and Reynolds (1998) and complex syllables that could be produced by several upward and downward movements of the legs with a different phase shift or a different movement pattern (Figure 1g,h). The syllable structure may reach an extremely high complexity. Thus, we sometimes could not describe the feature

state with a single character, and we had to use additional character (the character pairs 14–15 and 19–20, Table 1 and Tables S2–S4). The Table S2 includes encoding matrices of characters 1–3 that describe the song regardless of the number of syllable or echeme types. The Table S3 includes encoding matrices of characters 4–6 that describe the echeme structure. When we analysed these characters, we considered a species that produced two types of echemes (*Arcyptera fusca* or *Stenobothrus rubicundulus*) as two branches of the phylogenetic tree. The Table S4 includes encoding matrices of characters 7–24 that describe the syllable structure. During the analysis of these characters (similarly to characters 4–6), we considered a species that produced two types of syllables as two branches of the tree. The divergence time of the two types of echemes or syllables was estimated as half the time of the divergence of the species.

It was impossible to evaluate the character value in some cases. For example, we had no data for the leg-movement patterns for the song recordings taken from the literature. The two legs could be moved synchronously in one syllable part and with a large phase shift in another syllable part (character 18 in *Chorthippus biguttulus* or *Gomphocerippus rufus*). In all such cases, the character values were considered as missing data.

2.2 | Molecular analysis

For the DNA extraction, 52 specimens of 46 species were collected from different populations (Table S1). The total DNA was isolated from the hind femora of either a specimen fixed in 96% alcohol or of a dry specimen. Each hind femur was cleaned of cuticles and was grinded by a pestle. To extract DNA, we used Diatom™ DNAPrep 100 kit following the manufacturer's guidelines.

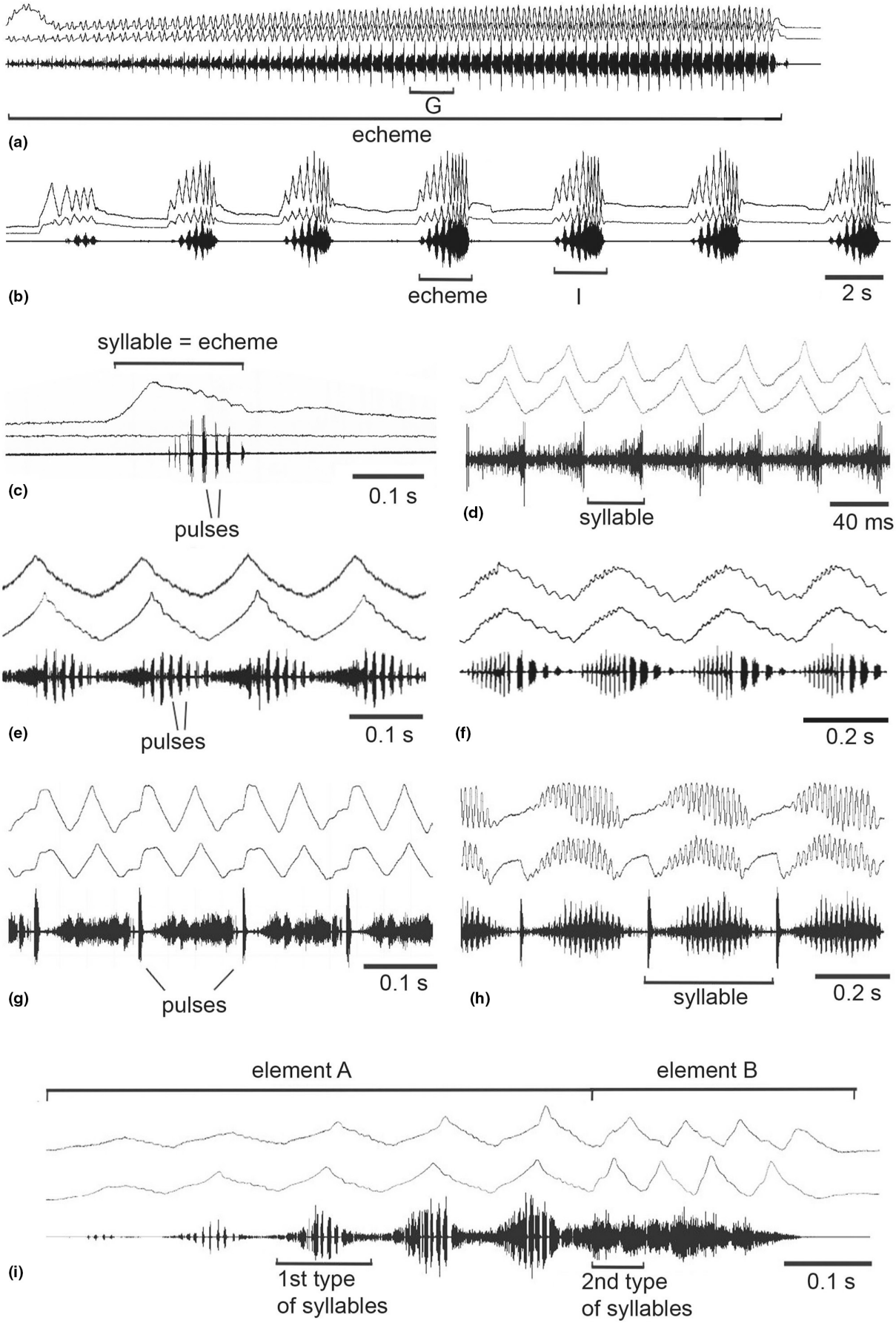
A PCR amplification of the mitochondrial cytochrome C oxidase subunit I (COI) gene was performed with the universal primers jg LCO-HCO (575 bp, Geller et al., 2013). Primers LR5-SP6R (Bruns et al., 1992) were used for the amplification of the nuclear internal transcribed spacers 1 (ITS1) and 2 (ITS2) genes as a part of ITS1-5.8-ITS2 region (the length of ITS1 and ITS2 were 395 bp and 263 bp, respectively); then some ITS2 sequences were obtained with 2A_5-2B_5 primers (Porter & Collins, 1991; Walton et al., 1999). PCR was performed in Veriti® Thermal Cycler. The quality of the PCR products was tested by gel electrophoresis and cleaned using ethanol precipitation. Sequencing was performed from the PCR primers in both directions with the ABI BigDye Terminator v. 1.1 kit on an ABI 3500, according to the protocol of the manufacturer.

The newly collected sequences were edited, contigs were assembled and sequence proofreading performed using the CodonCode Aligner, versions 6.0–7.0, and BioEdit, version 4.5–7.1 (Alzohairy, 2011). For the phylogenetic analysis, 35 sequences for mitochondrial cytochrome B gene (cytB), 67 additional sequences for the COI and 13 sequences for the ITS were obtained from GenBank (Table S1). The sequences generated for this study were deposited in GenBank with accession numbers presented in Table S1. Totally, we used 76 sequences of COI (575 bp), 35 sequences of cytB (579 bp), 20 sequences of ITS1 (395 bp) and 51 sequences of ITS2 (263 bp). We used the member of another subfamily (Oedipodinae), *Locusta migratoria*, as outgroup.

Sequences were aligned with MAFFT and ClustalW within MEGA X software (Kumar et al., 2018). Phylogenetic trees were reconstructed based on Bayesian inference methods (MCMC) using BEAST v1.10.4 (Suchard et al., 2018). We tested substitution models within MEGA software using the Bayesian Information Criterion (BIC, Schwarz, 1978). The model TN93 + G + I was applied for COI, the model GTR + G + I was applied for cytB and the model JC + G was applied for ITS1 and ITS2. Partition into codon positions was used for COI and cytB genes because of different results of the model test for first, second and third codon positions. The substitution rate parameters, the rate heterogeneity model and the base frequencies across codon positions were unlinked.

We used the 'Fixed local clock' model for estimation of the divergence time. We calibrated the molecular clock using splitting events for which time estimates were available from a previous study by Hawlitschek et al. (2022): (1) the split of the lineages comprising *Locusta* and Gomphocerinae at 37.9 ± 1.6 mya, (2) the split between Gomphocerini and Stenobothrini tribes at 13.7 ± 1.6 mya, (3) the split of the lineages comprising *Euchorthippus declivus* and *Euthystira brachyptera*

FIGURE 1 (a–i) Oscillograms of the Gomphocerinae calling song and terminology used in the acoustic analysis. (a) *Chorthippus apricarius* (Kazakhstan), calling song consists of one echeme. (b) *Ch. Dorsatus* (Russia, Moscow region), calling song consists of several echemes. (c) *Notostaurus albicornis* (Ukraine, Crimea), echeme consists of one syllable and sound is produced by movements of one leg. (d) *Omocestus haemorrhoidalis* (Russia, Orenburg region), each syllable consists of one up- and downward leg movement. (e) *Ch. dubius* (Russia, Altai republic). (f) *Stenobothrus eurasius* (Russia, Altai republic). (g) *Ch. apricarius*, part of the song shown at (a). (h) *Ch. mollis* (Russia, Orenburg region). Each syllable consists of several up- and downward movements. (i) *Ch. dorsatus*, part of the song shown at (b); elements a and b contain syllables of different structure. The two upper lines are recordings of hind leg movements and the lower line is the sound recording.



at 12.8 ± 1.6 mya, (4) the split between *Chorthippus* (*Glyptobothrus*) and *Chorthippus* (*Chorthippus*) subgenera at 6.4 ± 0.9 mya, (5) the split between *Omocestus* and *Stenobothrus* genera at 6.1 ± 1.0 mya, and (6) the split of the lineages comprising *Ch. dorsatus* and *Ch. albomarginatus* at 4.0 ± 0.6 mya.

We performed a BEAST analysis running for 20 million generations and sampling every 2000 generations. BEAUti v1.10.4 was used to generate XML and set the analysis. We assessed the convergence of samples using Tracer v.1.7.1 (Rambaut et al., 2018) by checking the stability of the log-likelihood curve and the split frequencies of the runs; we used a 10% burn-in because of the rapid convergence of the runs. TreeAnnotator v1.10.4 was used to compute consensus tree and estimate the posterior probabilities (PP) and 95% confidence interval (CI) of divergence time.

2.3 | Ancestral state reconstruction (ASR)

We have used the implementation of this method in the phytools 0.7–20 package (Revell, 2012) in the R software (version 4.2.1). The parameter 'marginal = FALSE' was accepted for all characters. For each character, we tested every possible model (Equal rates 'ER', Symmetrical 'SYM' and All rates different 'ARD'). The software estimated log-likelihood, evolutionary rates for character changes, standard-errors of the rates and character estimations for tree nodes. We chose models that allow to compute the scaled likelihoods of the character values at the tree root. If several models were appropriate according to these criteria, we compared the Akaike information criterion (AIC) value computed on the basis of the model log-likelihood. The result of the analyses was a phylogenetic tree with circle diagram for each node of the tree for the discrete characters and approximate value for the continuous characters. For the latter characters, we used the logarithmic values. The ASR was made for all characters except for those describing the rate of the leg movements (characters 22–24). For the latter characters, there were missing data for some species, which prevented the reconstructions of the ancestral states.

After reconstruction of the ancestral state of characters, we obtained several values. The transition rates, standard errors of transition rates and approximated character values at each node of the phylogenetic tree were used for all characters. In addition, the CI was used to evaluate the quality of continuous character values. For discrete characters, the algorithm calculated the probability of each character state for each tree node. A success of the ASR depended on the standard errors of the transition rates. If the standard errors were lower than the transition rate value, the ASR was mostly successful. Otherwise, the algorithm could not reliably determine the ancestral character state in most nodes of the tree.

2.4 | Estimation of phylogenetic signal

We used a Pagel's lambda (λ) statistics to measure and test phylogenetic signal. The phylogenetic signal was calculated in the same way for both quantitative and qualitative characters (Pagel, 1999). We used the implementation of this analysis in the phytools 0.7–20 package (Revell, 2012) in the R software (version 3.6.2). The result of the analysis for each character included a λ value that ranges from 0 to 1 and a *p*-value.

We determined the relationships between ASR and phylogenetic signal. Phylogenetic signal that was close to zero was typical for predictable reconstructions of the ancestral states. The character value was the same for the overwhelming majority of the nodes. Further, we will call such characters as 'uninformative' ones. Also, a relatively low ($\lambda < 0.4$) phylogenetic signal being insignificant ($p > .05$) was consistent with chaotic reconstructions, when the standard errors of transition rate were very large. In such cases, the estimate of the probability of the character state was large for several character states. The characters with the low phylogenetic signal and chaotic ASR will be further called as 'phylogenetically incongruent' or 'incongruent' ones. By contrast, the high ($\lambda > 0.4$) and significant ($p < .05$) phylogenetic signal that matched the successful ASR was attributed to 'phylogenetically congruent' or 'congruent' characters.

3 | RESULTS

3.1 | Phylogenetic analysis

A phylogenetic tree was obtained after trimming and multiple alignment of 183 sequences of the four phylogenetic markers (COI, cytB, ITS1 and ITS2) by Bayesian analysis (MCMC method) (Figure 2). The oldest split within Gomphocerinae was dated to 23.69 mya. The first cluster combined the tribes Ramburiellini (*Ramburiella*), Dociostaurini (*Dociostaurus and Notostaurus*) and Arcypterini (*Arcyptera*), but this cluster had a low support (PP = 0.35). Monophyly of *Ramburiella* was also poorly supported (PP = 0.61). Topology within cluster Dociostaurini + *Arcyptera* was strongly supported (PP = 0.89–1). The genus *Dociostaurus* appeared to be paraphyletic. Notably, the genus *Eremippus* comprised a separate cluster, despite having been suggested to

belong to Dociostaurini (Hodjat, 2016; Mistshenko, 1989). According to our results, *Eremippus* genus comprised a monophyletic sister group to the tribes Stenobothrini and Gomphocerini. Thus, the tribe Dociostaurini appeared to be polyphyletic. In the following song analysis, we considered the genus *Arcyptera* and the combined cluster *Arcyptera* + Dociostaurini, but did not consider the genera *Dociostaurus*, *Notostaurus* and *Eremippus*. The tribe Chrysochraontini represented a separate well-supported clade (PP = 1) and included not only the expected genera *Mongolotettix*, *Euthystira*, *Chrysochraon and Podismopsis* but also *Euchorthippus*. The relationship of *Euchorthippus* to this tribe was claimed by Defaut (2012).

Other large clusters contained the species belonging to the two tribes: Stenobothrini (*Stenobothrus*, *Omocestus* and *Myrmeleotettix*) and Gomphocerini (*Gomphocerippus*, *Gomphocerus*, *Stauroderus*, *Aeropedellus*, *Schmidtiacris and Chorthippus*) according to subdivisions of Harz (1975) and Storozhenko (1986). The split between these tribes was dated to 11.25 mya. The species of the genus *Pseudochorthippus* and *Chorthippus pullus* appeared to be the sister group to the tribe Stenobothrini, despite belonging to the tribe Gomphocerini. On the contrary, the species of the genus *Myrmeleotettix*, which are attributed to the tribe Gomphocerini, were nested within Stenobothrini. Thus, we indicate a polyphyletic status of both tribes Stenobothrini and Gomphocerini.

It was possible to distinguish two groups in the tribe Stenobothrini. The first small well-supported group included five species of *Omocestus*, whereas the second larger group included all species of *Stenobothrus*, three species of *Omocestus* and the *Myrmeleotettix* species. It is remarkable that some *Omocestus* species (*O. bolivari*, *O. raymondi* and *O. minutus*) and the *Myrmeleotettix* species did not cluster together within each genus. At the same time, topology within *Stenobothrus* species was poorly supported. For the further song analysis, we distinguished two monophyletic clusters within the tribe Stenobothrini. The first one included five species of *Omocestus*, whereas another cluster included all species of *Stenobothrus*, two species of *Myrmeleotettix* and *O. bolivari* (Figure 2).

Another cluster corresponding to the tribe Gomphocerini could be divided into several groups. Unexpectedly, four species (*Schmidtiacris schmidti*, *Ch. angulatus*, *Mesasippus kozhevnikovi* and *Aeropedellus variegatus*) were clustered basally with a high support (PP = 1). The second group comprised the species of the subgenus *Chorthippus* (*Ch. albomarginatus* group and *Ch. dorsatus* group). The third group included mainly the species of *Glyptobothrus* subgenus, but also the species from the genera *Gomphocerippus*, *Gomphocerus*, *Stauroderus* and *Megaulacobothrus*. In the further analysis, we name this third group as '*Chorthippus* (*Glyptobothrus*) subgenus'. The cluster corresponding to the *Chorthippus biguttulus*-group was found to be poorly supported.

3.2 | Phylogenetic signal and ASR

To estimate phylogenetic signal (Table 2) and to perform an ASR, we used the phylogenetic reconstruction (Figure 2) based on combined molecular data (COI, cytB, ITS1 and ITS2) and the matrices of the character statements (Tables S2–S4).

The results of the ASR and estimation of phylogenetic signal allowed us to divide the characters into three groups: (1) 'phylogenetically congruent' characters, (2) 'uninformative' characters and (3) 'phylogenetically incongruent' characters (Table 3). The 9 'congruent' song characters, which demonstrated the successful ASR, included almost all characters describing the echeme temporal structure (characters 2–6) and only four characters describing the syllable temporal structure. For these characters, phylogenetic signal was found to be high ($\lambda > 0.4$) and significant ($p < .05$) (Table 2). The ancestral character states were unambiguously established for most nodes of the tree. This group included both continuous characters 3–5 and 7 and discrete characters 2, 14, 16 and 19. The continuous characters described the echeme structure (echeme number per song, echeme duration and syllable number per echeme). The discrete 'congruent' characters described mainly the syllable structure.

One of the 'congruent' discrete characters appeared to be syllable homogeneity (character 2, Figure 3a). The reconstruction showed that ancestral song in Gomphocerinae consisted of only one type of syllables. The second type of syllables evolved independently in many different branches of the tree. The presence of two types of syllables is supposed to be ancestral state for the two clusters: *Chorthippus* subgenus and *Arcyptera* genus. Another example of the 'congruent' discrete characters was usage of two legs (character 19, Figure 3b). The ancestor of Gomphocerinae was found to generate the calling song by using only one leg. This character state could be found within some *Dociostaurus* species. Echeme duration (character 4) was found to be one of the 'congruent' continuous characters (Figure 3c). Ancestral echemes were relatively short, and the pathway of song evolution was mainly from short to long echemes. However, the *Chorthippus* subgenus demonstrated an opposite trend, namely, a decrease in echeme duration.

The ASR was also successful for the 'uninformative' discrete characters: echeme homogeneity (character 1), sound-producing mechanisms (character 10), stepwise

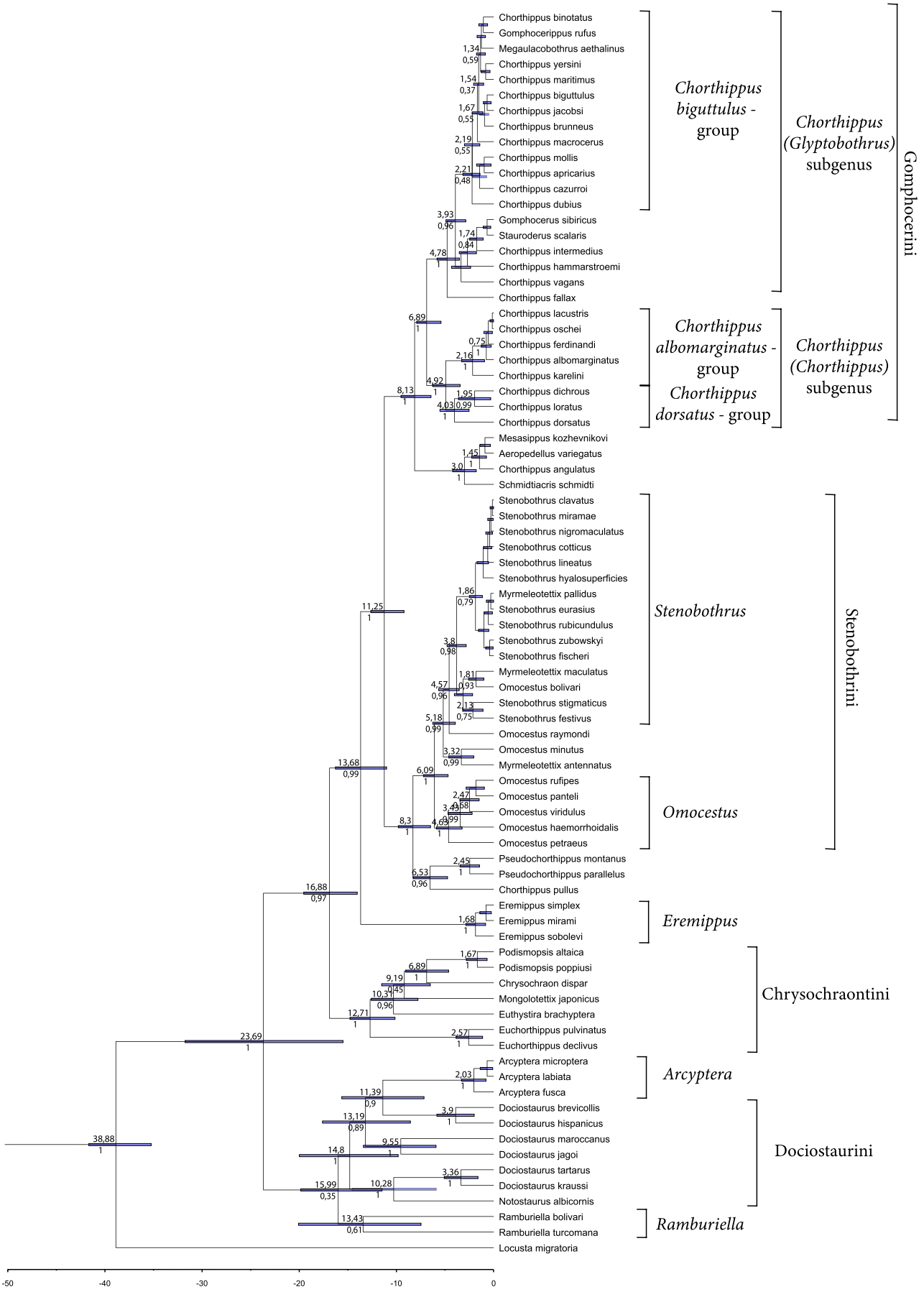


FIGURE 2 Phylogeny of the 79 Gomphocerinae species inferred from MCMC analysis. Phylogeny is based on the sequences of the four markers (COI, 575 bp; cytB, 579 bp; ITS1, 395 bp; ITS2, 263 bp). Numbers above the nodes are the divergence time estimation, numbers below the nodes are posterior probability values. Monophyletic taxons are marked from the right. Scale below the tree indicates the divergence time.

upstroke (character 12), silent syllable part variation (character 15), phase shift variation (character 17). However, each reconstruction was rather predictable and did not provide any new data for the character evolution. There was a uniform character state for most species (more than 90%). Phylogenetic signal was close to zero (Table 2). According to the analysis of these characters, the ancestral calling songs contained echemes of similar structure (character 1), the sound was generated by femoro-tegminal stridulation (character 10) and by gradual upstroke (character 12). In the ancestral calling song, there were no variations in the leg movements within syllable (characters 15 and 17). For the 'uninformative' characters describing the rate of the leg movements (characters 22–24), we could not estimate the ancestral character values because of the method constraints (these characters were only found in one third of the species studied).

The ASR was not successful for the 'incongruent' characters (character 8 – syllable period, character 9 – syllable duration, character 11 – syllable complexity, character 13 – stepwise downstroke, character 18 – phase shift, character 21 – pulses). An estimated variance of evolutionary rates was rather high to compute the ancestral character state in the most of the tree nodes. The phylogenetic signal was either high but insignificant (e.g. character 18) or low (e.g. characters 9 and 11). Notably, we suggest all these characters to be quite important for description of the syllable temporal structure (see Section 4). The reconstruction presented for the character 13 (Figure 3d) shows the inability to establish whether the ancestral song had stepwise or smooth leg movements within the syllable.

TABLE 2 Results of the phylogenetic signal estimation in Gomphocerinae.

Character	Pagel's lambda	*p*-value (based on LR test)
1. Echeme homogeneity	<0.001	1.000
2. Syllable homogeneity	0.471*	0.023
3. Echeme number	0.721*	<0.001
4. Echeme duration	0.435*	<0.001
5. Syllable number	0.579*	<0.001
6. Echeme and syllable equality	0.926*	<0.001
7. Syllable number per echeme	0.637*	<0.001
8. Syllable period	0.216	0.348
9. Syllable duration	0.239*	0.043
10. Sound-producing mechanisms	<0.001	1.000
11. Syllable complexity	0.192*	0.016
12. Stepwise upstroke	<0.001	1.000
13. Stepwise downstroke	0.413	1.000
14. Silent syllable part	0.504*	0.012
15. Silent syllable part variation	<0.001	1.000
16. Leg-movement pattern difference	0.422*	0.044
17. Phase shift variation	<0.001	1.000
18. Phase shift	0.590	0.059
19. Usage of two legs	1.000*	<0.001
20. Variation of usage of two legs	<0.001	1.000
21. Pulses	<0.001	1.000
22. Stepwise upstroke rate	1.000	0.462
23. Stepwise downstroke rate	<0.001	1.000
24. Superimposed movements rate	0.089	0.767

Note: The calling song characters with significant phylogenetic signal are marked by*.

3.3 | Evolution of calling song

We selected the key nodes on the tree of Gomphocerinae (Figure 4) to analyse the results of the ASR for "congruent" characters (Table 4). The results suggested that ancestral song of Gomphocerinae consisted of only one type of syllables (character 2). Two different types of syllables evolved independently in *Arcyptera* genus and *Chorthippus* subgenus. Some species of the tribe Stenobothrini and the subgenus *Glyptobothrus* also independently shifted to generation of the two syllable types. We suggested characters 3 (echeme number) and 4 (echeme duration) to be the main echeme features that should be considered together. Gomphocerinae produced many short echemes ancestrally. There was a common pathway of the song evolution in decreasing of echeme number and increasing of echeme duration. The subgenus *Glyptobothrus* and the tribe Stenobothrini independently evolved the calling songs consisting of one long echeme. At the same time, some species of *Glyptobothrus* (e.g. *Ch. brunneus*) returned to the ancestral song structure. We also found such reversal in the subgenus *Chorthippus*.

Results of reconstructions for the 1st and 2nd nodes (ancestor of Gomphocerinae, ancestor of *Arcyptera* and Dociostaurini) for characters 5–7 (syllable number, echeme and syllable equality, syllable number per echeme) showed some discrepancies (Table 4). According to character 6, the ancestral echeme comprised only one

syllable. However, according to characters 5 and 7, syllable number per echeme varied in the range of 4–8 on average. Taking into account a relatively large confident interval of characters 5 and 7 for the 1st node (CI = 1–46), we rely more on character 6. In some nodes (3, 10–14), the values of character 7 were almost twice as low as those of character 5. The reason for these differences was that character 5 summarized the number of both syllable types, while character 7 considered two types of syllables separately. The difference between the two characters was highest for the subgenus *Chorthippus*, because the presence of the two syllable types was suggested to be an ancestral state for this group. We can observe a general trend towards an increase in the syllable number in the process of evolution: starting with only one syllable within echeme, the syllable number increased to 42 on average in the genus *Omocestus* and to 49 on average in the *Chorthippus biguttulus* group (Table 4). However, an opposite tendency was found in the *Chorthippus* subgenus and some species of *Glyptobothrus* (e.g. *Ch. brunneus*, *Ch. jacobsi*).

We designated characters 14 (Silent syllable part) and 16 (leg-movement pattern difference) as 'congruent' characters on the basis of the estimation of phylogenetic signal. The results of ASR for these characters were similar to the results typical for 'uninformative' characters, when most species showed the same character statement. In most species, sound was generated during both upstroke and downstroke (state '0' of character 14) and two legs were moved with the same pattern (state '0' of character 16). The ASR for character 19 (usage of two legs) shows that an ancestor of Gomphocerinae generated the calling song by stridulation with only one leg. A common ancestor of the tribes Gomhocerini, Stenobothrini and Chrysochraontini, an ancestor of the genus *Arcyptera* and some species of Dociostaurini started to use both legs independently (Table 4).

The analysis of 'congruent' characters enabled us to trace the calling song evolution in Gomphocerinae (Figure 5). The ancestral calling song consisted of numerous short echemes lasting on average 0.9 s. Each echeme comprised only one syllable produced by movements of only one leg. This plesiomorphic song type was preserved in some species of the tribe Dociostaurini. The ancestor of the genus *Arcyptera* (node 3) evolved another type of the song comprising two types of syllables; syllable number increased up to 8 per echeme (character 5). Similarly to the 1st and 2nd nodes, we found a discrepancy of this character with character 6 that showed one syllable per echeme. However, taking into account a smaller confident interval of character 5 for node 3 (CI = 3–18) than for node 1 (CI = 1–46), we rely more on character 5 than on character 6.

A common ancestor of the tribes Chrysochraontini, Stenobothrini and Gomphocerini started to produce echemes consisting of numerous syllables (node 4). The average echeme lasted 1.2 s and consisted of 12 syllables. This ancestral calling song could remind the calling song of modern Chrysochraontini. A comparison of the common ancestor of the tribes Stenobothrini and Gomphocerini (node 6) with node 4 demonstrated two main trends of the calling song evolution: increase in echeme duration (from 1.2 s to 2.2 s) and in syllable number (from 12 to 23). Two main lineages of Gomphocerinae, the tribes Stenobothrini and Gomphocerini, continued this way of evolution. The ancestral song of Stenobothrini (node 7) probably consisted of only one long echeme (3.2 s on average) containing about 37 syllables. Most species of modern Stenobothrini produce similar calling songs. Ancestor of the genus *Omocestus* (node 8) produced echeme lasting 4 s, with the average syllable number equal to 42. The *Stenobothrus* ancestral song (node 9) tended to be shorter (3.5 s) and to contain fewer number of syllables (35).

TABLE 3 The calling song characters in Gomphocerinae grouped on the basis of the results of ancestral state reconstruction and phylogenetic signal estimation.

'Congruent' characters	'Uninformative' characters	'Incongruent' characters
2. Syllable homogeneity	1. Echeme homogeneity	8. Syllable period
3. Echeme number	10. Sound-producing mechanisms	9. Syllable duration
4. Echeme duration	12. Stepwise upstroke	11. Syllable complexity
5. Syllable number	15. Silent syllable part variation	13. Stepwise downstroke
6. Echeme and syllable equality	17. Phase shift variation	18. Phase shift
7. Syllable number per echeme	20. Variation in usage of two legs	21. Pulses
14. Silent syllable part	22. Stepwise upstroke rate	
16. Leg-movement pattern difference	23. Stepwise downstroke rate	
19. Usage of two legs	24. Superimposed movements rate	

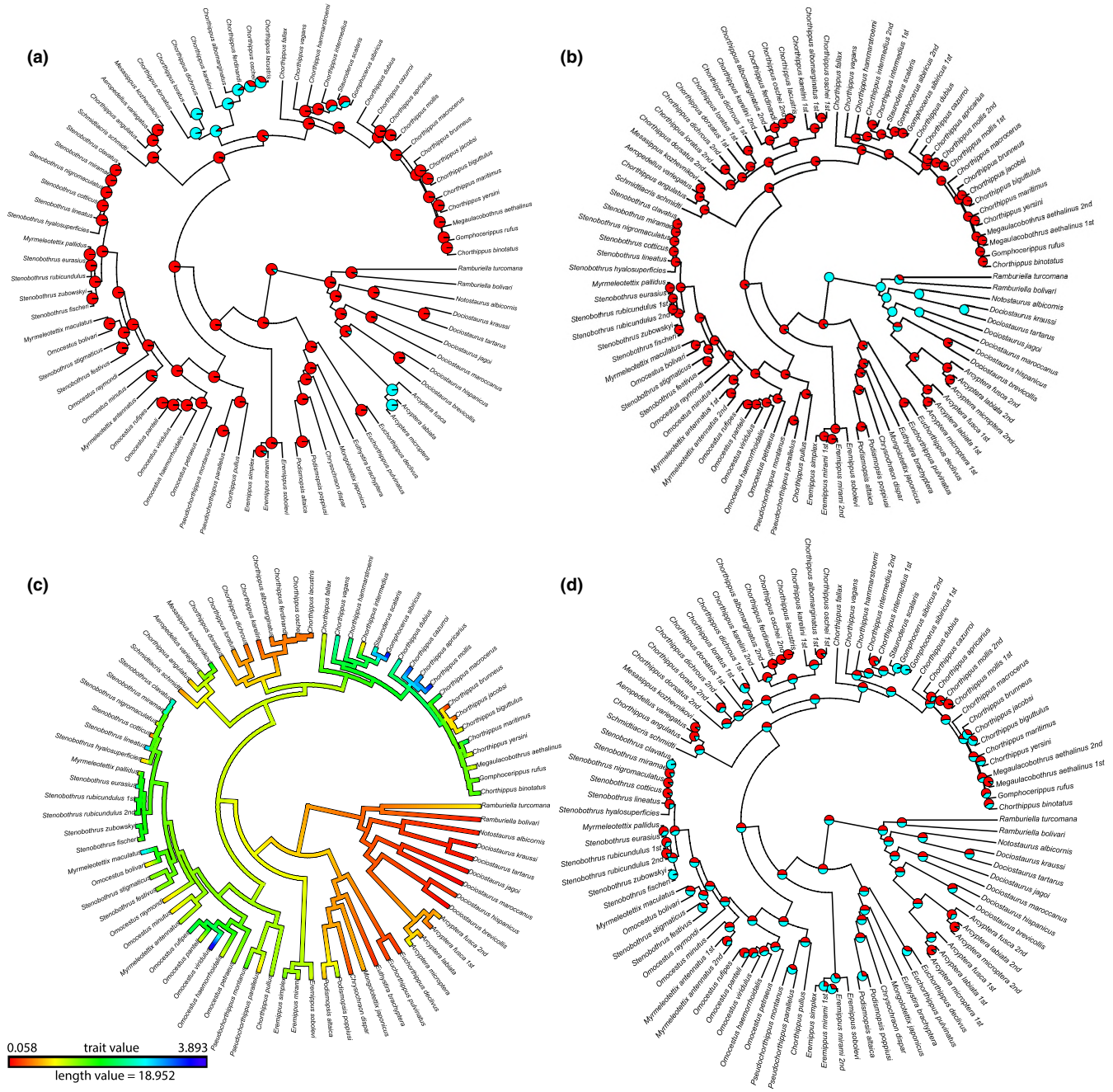


FIGURE 3 (a–d) Ancestral state reconstruction of the characters. The pie charts in the nodes show probability of the different character state for a, b and d. (a) character 2, syllable homogeneity (red colour indicates the presence of one syllable type, blue colour – the presence of two syllable types). (b) character 19, usage of two legs (red colour indicates the usage of both legs, blue colour – the usage of only one leg). (c) character 4, echeme duration (colours of the nodes show the most probable value for the character, that is ancestral state estimate; the legend is shown at logarithmic scale). (d) character 13, stepwise downstroke (red colour indicates the absence of stepwise leg-movements, blue colour – the presence of stepwise leg-movements).

The ancestor of the tribe Gomphocerini (node 10) produced calling song similar to that in the common ancestor of the tribes Stenobothrini and Gomphocerini. This song comprised three echemes, each lasting 2.2 s and consisting of 22 syllables. The two main lineages of the Gomphocerini, the subgenera *Chorthippus* (node 11) and *Glyptobothrus* (node 14), evolved completely different types of calling song. The ancestral *Glyptobothrus* song reminded the Stenobothrini ancestral song containing two echemes of longer duration (about 6.1 s); the number of syllables per echeme was also higher (36). The ancestral *Chorthippus* song demonstrated the opposite trend of evolution. Echeme number increased to 4, echeme duration decreased to about 1.3 s and the number of syllables of both types also decreased to 15. Most of *Chorthippus* species, similarly to *Arcyptera* species, produced two types of syllables, which suggested that ancestral song consisted of two syllable types as well. Ancestral song of the

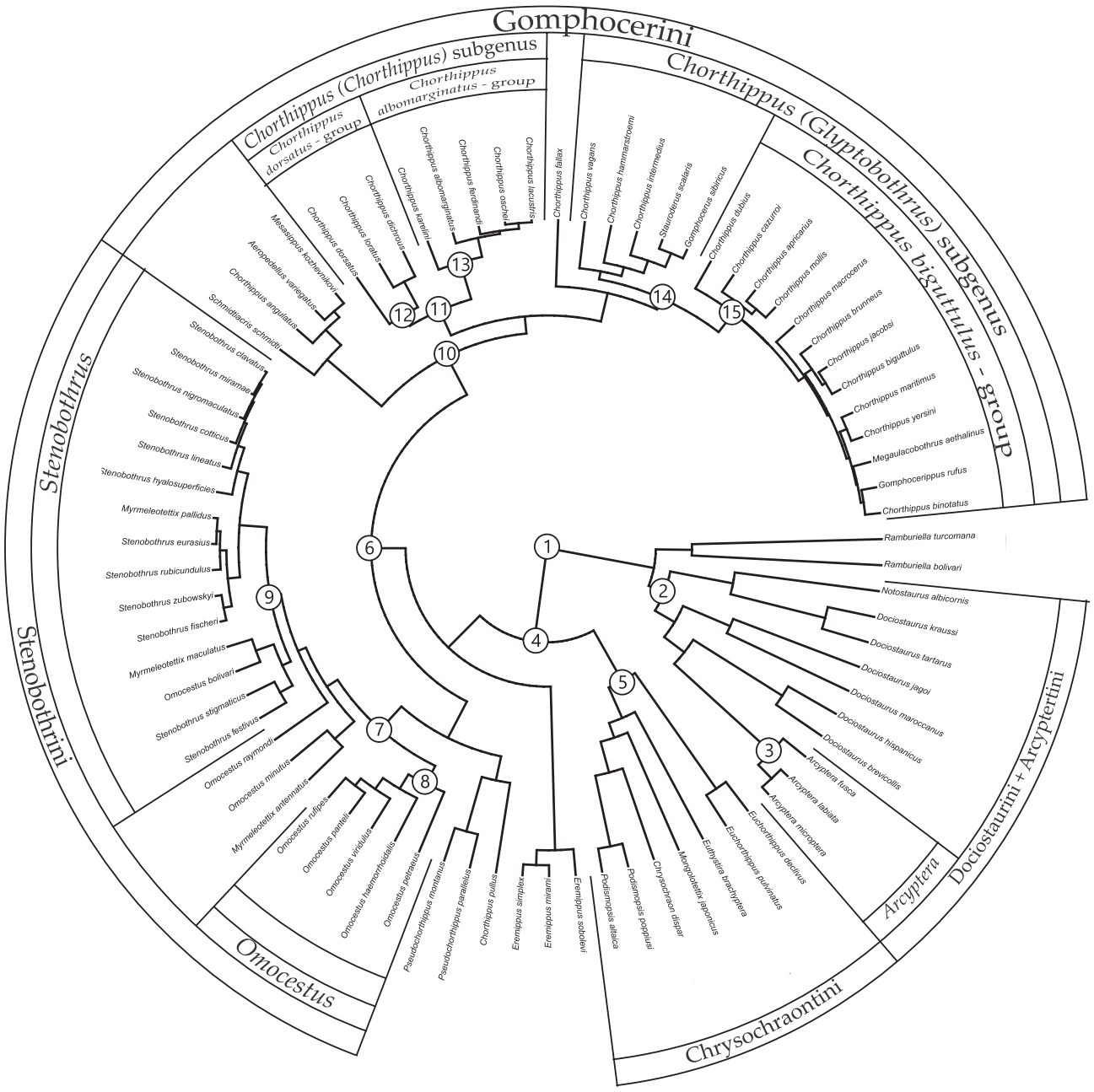


FIGURE 4 Phylogenetic tree of Gomphocerinae with selected nodes and main clusters.

Ch. albomarginatus group (node 13) consisted of about 4 echemes each lasting about 0.7 s and containing about 18 syllables of both types. The ancestor of the *Ch. dorsatus* group (node 12) produced about 5 longer (1.1 s) echemes consisting of 13 syllables of both types.

On the basis of the current analysis, we suggest five main types of calling song in Gomphocerinae. The first type could be the ancestral song type (Figure 5, nodes 1 and 2). This song type consists of many short echemes containing only one syllable produced by the movements of only one leg. The second type (node 3) is unique for the genus *Arcyptera*. The calling song consists of few (3–5) echemes containing relatively low number (<7) of syllables of two types. The third type of calling song is the most common variant (nodes 4–6 and 10). The calling song is composed of few (3–5) echemes; each echeme is characterized by medium (0.9–2.2 s) duration and by medium (10–23) number of syllables. The fourth type evolved independently in the tribe Stenobothrini and the subgenus *Glyptobothrus* (nodes 7–9 and 14). The calling song is one or two long (>3.2 s) echemes consisting of rather high number of syllables (>22). The fifth song type is the common type for the subgenus *Chorthippus* (nodes 11–13). The calling song is composed of few (4–5)

TABLE 4 Ancestral state estimates, 95% confident interval (CI, for continuous 'congruent' characters) and posterior probability (PP, for discrete 'congruent' characters). The characters with uniform states = 0 (character 14 – silent syllable part, average PP = 0.96 and character 16 – leg-movement pattern difference, average PP = 0.99) are not shown.

Node name	Node number	2. Syllable homogeneity (PP)	3. Echeme number (CI)	4. Echeme duration (CI)	5. Syllable number (CI)	6. Echeme and syllable equality (PP)	7. Syllable number per echeme (CI)	19. Usage of two legs (PP)
Gomphocerinae ancestor	1	0 (0.88)	4 (1–17)	0.9 (0–8.7)	8 (1–46)	1 (0.94)	7 (1–51)	1 (1.0)
Arcyptera + Dociostaurini ancestor	2	0 (0.97)	6 (2–14)	0.4 (0–3.3)	4 (1–12)	1 (1.0)	4 (1–14)	1 (1.0)
Arcyptera ancestor	3	1 (0.99)	5 (3–9)	0.8 (0.01–2.3)	8 (3–18)	1 (0.86)	4 (1–23)	0 (0.84)
Chrysachraontini + Stenobothrini + Gomphocerini ancestor	4	0 (0.97)	4 (1–11)	1.2 (0–6.7)	12 (3–49)	0 (0.87)	11 (2–50)	0 (0.86)
Chrysachraontini ancestor	5	0 (0.98)	5 (2–13)	0.9 (0–5.0)	10 (3–36)	0 (0.91)	9 (2–39)	0 (0.9)
Stenobothrini + Gomphocerini ancestor	6	0 (0.99)	3 (1–6)	2.2 (0.2–7.5)	23 (8–69)	0 (0.94)	19 (6–62)	0 (0.92)
Stenobothrini ancestor	7	0 (1.0)	2 (1–3)	3.2 (1.1–7.6)	37 (17–81)	0 (0.99)	28 (10–83)	0 (0.95)
Omocestus ancestor	8	0 (1.0)	1 (1–2)	4.0 (1.4–9.3)	42 (19–95)	0 (0.99)	40 (17–98)	0 (0.98)
Stenobothrus ancestor	9	0 (1.0)	1 (1–2)	3.5 (1.5–7.3)	35 (18–68)	0 (0.99)	32 (15–70)	0 (0.98)
Gomphocerini ancestor	10	0 (0.96)	3 (2–6)	2.2 (0.3–6.7)	22 (8–58)	0 (0.96)	15 (5–44)	0 (0.95)
Chorthippus (*Chorthippus*) subgenus ancestor	11	1 (0.89)	4 (2–8)	1.3 (0.08–3.9)	15 (7–35)	0 (0.98)	6 (3–14)	0 (0.98)
Chorthippus dorsatus – group ancestor	12	1 (0.96)	5 (3–9)	1.1 (0.02–3.46)	13 (6–29)	0 (0.98)	5 (3–11)	0 (0.99)
Chorthippus albomarginatus – group ancestor	13	1 (0.94)	4 (2–6)	0.7 (0–2.2)	18 (9–35)	0 (0.99)	7 (3–14)	0 (0.99)
Chorthippus (*Glyptobothrus*) subgenus ancestor	14	0 (1.0)	2 (1–4)	6.1 (2.9–12)	36 (19–71)	0 (0.99)	27 (13–58)	0 (0.98)
Chorthippus biguttulus – group ancestor	15	0 (1.0)	1 (1–2)	8.1 (4.9–13)	49 (30–80)	0 (1.0)	39 (23–67)	1 (1.00)

and rather short (0.7–1.3 s) echemes consisting of two types of syllables.

4 | DISCUSSION

4.1 | Phylogenetic relationships and taxonomy

Our results mainly support the previous phylogenetic studies on Gomphocerinae. However, some interesting questions arose because a number of species was included for the first time in our analysis. The current study provides the most well-resolved phylogenetic reconstruction of Gomphocerinae, although we focus on the calling song evolution.

Species of four genera (*Ramburiella*, *Notostaurus*, *Dociostaurus* and *Arcyptera*) formed a sister cluster to all other genera within our reconstruction. This result differs from the data of the study of Vedenina and Mugue (2011) where these genera are separated into three clusters. According to Vedenina and Mugue (2011), *R. bolivari* is associated with the species of *Dociostaurus* genus, whereas the current study revealed a sister position of *Ramburiella* to Dociostaurini + *Arcyptera* cluster. The discrepancies could be explained by the usage of additional marker (ITS2) for *R. bolivari* in the current study. A basic position of *R. turcomana* is inconsistent with the results of Contreras and Chapco (2006) and of Nattier et al. (2011). In the latter article, *R. turcomana* is clustered with the species of *Dociostaurus*, whereas the species of *Arcyptera* comprise a basic branch to all Gomphocerinae; however, this relationship is poorly supported.

The sister relationship between the genera *Arcyptera*, *Dociostaurus* and *Notostaurus* shown in this study is consistent with the results of Bugrov et al. (2006, 2012) and Contreras and Chapco (2006). This topology is, however, inconsistent with the data of Nattier et al. (2011) and Vedenina and Mugue (2011). At the same time, the basal nodes receive low support in the reconstructions of the two latter studies. The species of *Eremippus* were previously considered as the sister group to the genera *Dociostaurus* and *Arcyptera*, and different authors included them either in the tribe Dociostaurini (Hodjat, 2016; Mistshenko, 1989) or in the tribe Arcypterini (Otte, 1995). Our reconstructions show that species of *Eremippus* have the sister relationship to the tribes Gomphocerini and Stenobothrini. This was also demonstrated by Bugrov et al. (2012). However, in the latter study the authors did not include any species of Stenobothrini into their analysis; therefore, they suggest an intermediate position of *Eremippus* between Dociostaurini and Gomphocerini (Bugrov et al., 2012).

Most species of Gomphocerinae considered in the current paper belong to the advanced tribes Stenobothrini and Gomphocerini. The tribe Stenobothini includes three genera, *Stenobothrus*, *Omocestus* and *Myrmeleotettix*. This is consistent with the results of previous phylogenetic reconstructions (Contreras & Chapco, 2006; Nattier et al., 2011; Vedenina & Mugue, 2011). Three species of the genus *Myrmeleotettix* do not cluster together. It is not surprising since they differ considerably both by songs and morphology. Only the clubbed antennae make them similar to each other, and they were mainly grouped into one genus on the basis of this feature. However, evolving of the clubbed antennae could occur convergently in those species that perform the strokes with antennae during courtship (Vedenina & Mugue, 2011). The fact that different species of *Myrmeleotettix* demonstrate a different pattern of the antennae stroke (Berger & Gottsberger, 2010; Vedenina et al., 2020) could support our hypothesis about their convergent evolution.

Our results confirm the polyphyly of both genera *Omocestus* and *Stenobothus*. Five species of *Omocestus* comprise one group with high support. The monophyly of this cluster is also found by other authors (Nattier et al., 2011; Vedenina & Mugue, 2011). Another cluster includes many poorly supported nodes where the species of *Stenobothrus*, *Myrmeleotettix* and *Omocestus* are mixed. A close relationship between some species of *Stenobothrus* and *Omocestus* is also shown by Berger (2008) on the basis of the song and morphological analyses. Not only the *Myrmeleotettix* species but also some species of *Stenobothrus* have the antennae tips thickened to different degree, which are used in visual display during courtship (Ostrowski et al., 2009; Tarasova et al., 2021).

The species of the *Pseudochorthippus* genus form a sister group to the tribe Stenobothrini. Previously they were attributed to the genus *Chorthippus* but now they are classified as a separate genus (Defaut, 2012). The topology of the genus is consistent with the data of other authors (Nattier et al., 2011; Vedenina & Mugue, 2011). It is interesting that we revealed a relationship between *Ch. pullus* and species of *Pseudochorthippus*. We expected *Ch. pullus* to stay apart from the species of *Pseudochorthippus* because of quite different songs and morphology. This topology requires future studies.

Most species of the tribe Gomphocerini form three robust clades. One clade is surprising because it combines the species that are expected to be separate. These species are very different in both morphology and song behaviour. However, our data partly coincide with previous results. For example, the genus *Aeropedellus* was shown as a sister group to other Gomphocerini (Contreras & Chapco, 2006; Nattier et al., 2011), whereas *Ch. angulatus* and *M. kozhevnikovi* comprise a sister group to other

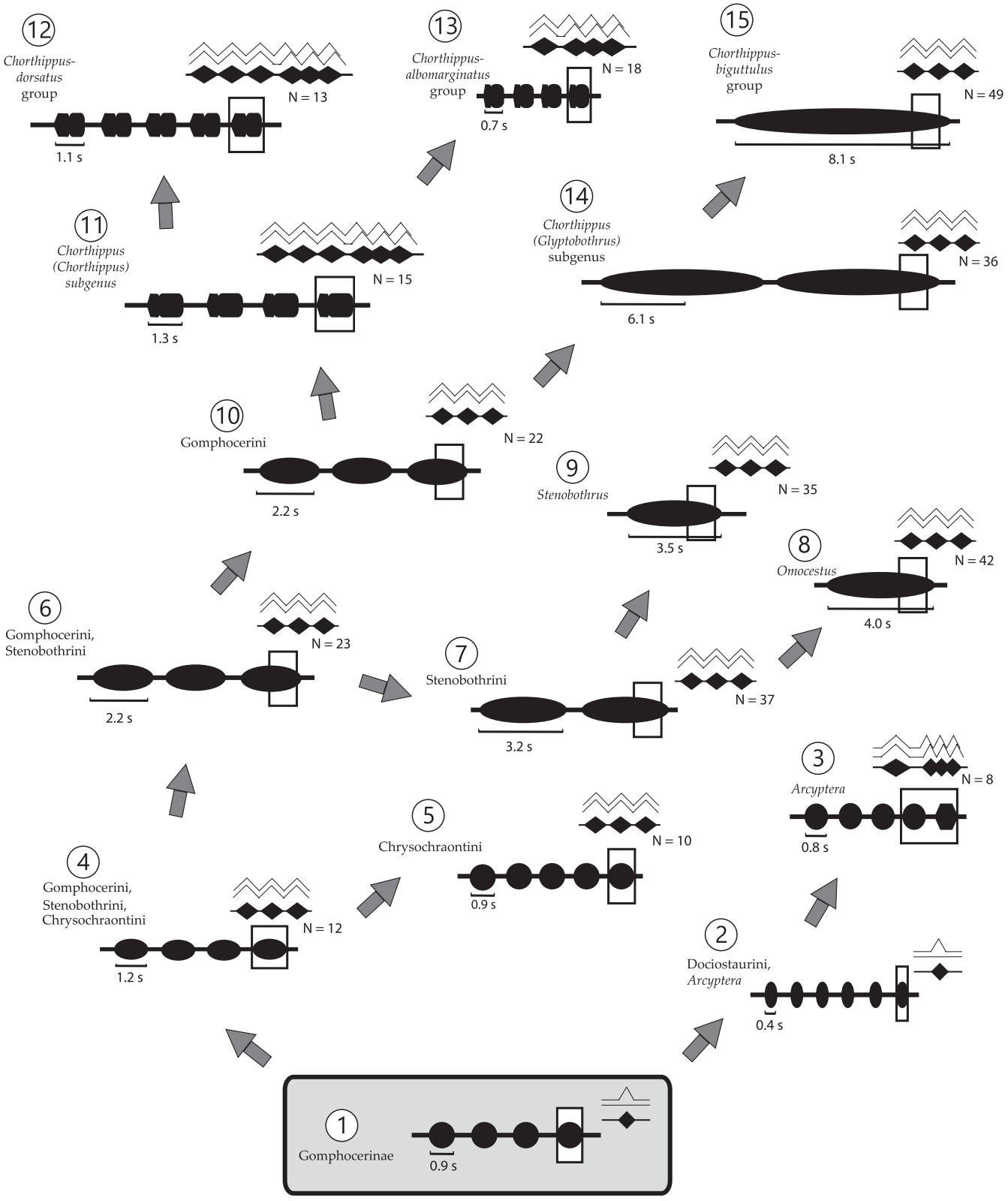


FIGURE 5 Reconstruction of the calling song evolution in Gomphocerinae. Schemes show structure of echemes and syllables; for syllables, the two upper lines are schemes of leg movements and the lower line is the sound scheme. Ancestral state estimates for echeme duration and syllable number are shown without 95% confident intervals; the latter ones are shown in Table 4. The circle numbers correspond to the numbers of the nodes shown in Figure 4 and Table 4.

Gomphocerini (Bugrov et al., 2012). At the same time, *Sch. schmidti* was clustered with the *Glyptobothrus* species in reconstructions of Nattier et al. (2011).

According to our reconstructions, we suggest a monophyly of the *Chorthippus* subgenus that includes two species groups, *Ch. dorsatus* group and *Ch. albomarginatus* group. The species of the two groups are pretty similar in morphology and have some common characters in the calling songs (Figure 5). At the same time, they are extremely different in the courtship songs (Stumpner & von Helversen, 1994; Vedenina & von Helversen, 2009). Notably, these two groups form the separate clades in the reconstructions of Vedenina and Mugue (2011).

The third clade of Gomphocerini combines all species of the *Glyptobothrus* subgenus, in particular, the sibling species of the *Ch. biguttulus* group. However, the species of other genera (*Stauroderus*, *Gomphocerus*, *Gomphocerripus* and *Megaulocobothrus*) are found within this clade. Notably, the species of the *Ch. biguttulus* group do not cluster together, which is pretty consistent with the previous results (Mayer et al., 2010; Nattier et al., 2011; Vedenina & Mugue, 2011). Most nodes within this cluster have a weak support and the interspecific differences are very small. We reveal only one well-supported monophyletic cluster, which combines *G. sibiricus*, *S. scalaris*, *Ch. intermedius* and *Ch. hammarstroemi*. This cluster was also noted by other authors (Bugrov et al., 2006; Nattier et al., 2011; Vedenina & Mugue, 2011).

4.2 | Evolution of complex calling songs in Gomphocerinae

It is suggested by von Helversen and von Helversen (1994) and Vedenina and Mugue (2011) that the most 'primitive' sounds in Gomphocerinae are produced by straight upward and downward movements of the hind legs or by straight upward and stepwise downward movements. A main slow rhythm of 1–10 kHz could originate from the walking or breathing rhythms (Heinrich & Elsner, 1997), whereas rapid vibratory movements of 50–70 Hz during downstroke could derive from flight (Elsner, 1994). Vedenina and Mugue (2011) suggest both song patterns to be plesiomorphic. Nattier et al. (2011) argue that hypothetical ancestral song has no echeme-sequence and only a few syllables per echeme. At the same time, all the authors suggest that an ancestral gomphocerine male produced song by movements of both legs simultaneously or with a slight shift. According to the current analysis, however, each echeme of the ancestral calling song in Gomphocerinae comprised only one syllable produced by movements of only one leg. The complete song consisted of numerous short echemes lasting on average 0.3 s. This plesiomorphic song type was preserved in some species of the tribe Dociostaurini.

Unfortunately, we cannot say when the stepwise downstroke could evolve since no good ASR was obtained for this character (character 13). The number of syllables per echeme in the ancestral song is difficult to be compared between different studies because of different definitions of syllable (see Methods). A notable conclusion of our study is that the ancestral sound was produced by one leg (character 19). Other authors suggest that both legs participated in the ancestral song generation. At the same time, usage of only one leg in sound generation could originate from the non-special movement of one leg. The non-special movements are more likely produced by one leg than by both legs. Neurophysiological basis for stridulation by both legs requires a more complex organization of neurons in thoracic ganglia. It was shown that longitudinal splitting of the meso- and metathoracic ganglia resulted in almost normal stridulation patterns on both sides, but the left–right coordination of the song subunits was impaired (Ronacher, 1989). Thus, the coordination of both pattern generators must depend on commissures within the metathoracic ganglion, that is additional nervous elements are necessary.

Could the numerous short echemes produced by an ancestral gomphocerine male have any adaptive value? If we consider some species of the tribe Dociostaurini, which demonstrate plesiomorphic song pattern (Figure 5), generation of short echemes seems to be rather adaptive. Males actively move, stopping only briefly and generating few short echemes (Savitsky, 2000, 2007). Thus, the male bypasses a large territory and has a chance to meet more females that rarely respond acoustically. Berger (2008) also suggests this searching strategy to be phylogenetically old system in gomphocerine grasshoppers.

A next step of evolution in searching strategy could be the production of longer echemes or longer echeme-sequence, which can be found in many extant species of the tribes Chrysochraontini and Stenobothrini, as well as in some species of the tribe Gomphocerini (Figure 5). A singing male is sitting at one place and a receptive female approaches him without acoustic responses. The female acoustic response cannot be heard by a singing male because of peripheral effects acting on the tympanum during stridulation (Hedwig, 1990). Some species from the tribes Stenobothrini and Gomphocerini evolved another searching strategy: males produce relatively short echemes and listen for acoustic responses of females; both sexes may approach each other, periodically exchanging songs (von Helversen & von Helversen, 1994). In this case, a male can also wander about the terrain, similarly to the species of Dociostaurini.

4.3 | Increasing or decreasing complexity?

It was previously suggested by different authors (Mayer et al., 2010; Vedenina & Mugue, 2011; von Helversen & von Helversen, 1994) that calling song in Gomphocerinae evolved linearly through increased complexity. Nattier et al. (2011), however, observed no clear trend towards increasing complexity and claimed that the calling song evolution in Gomphocerinae involved many parallel transformations and reversals. A study of evolution of calling songs in the Lebinthini crickets showed that both continuous trills and monosyllabic calls (that are suggested to be simpler patterns) were acquired secondarily and multiple times (Tan et al., 2021). Notably, the call architecture in Lebinthini is partly stable across lineages, but finer temporal patterns are relatively labile among closely related species. In the current study, we also did not find any clear trends in evolution of the syllable temporal structure. The ASR results for the characters that characterize the fine temporal patterns within a syllable (characters 11, 13, 18 and 21) are difficult to interpret (Table 3, Figure 3d). This means that temporal patterns of the calling songs could undergo multiple and independent complications and simplifications across the phylogeny. An example of simplification might be related with the usage of different sound-producing mechanisms in Gomphocerinae (character 10). Some species of *Stenobothrus eurasius* group (Tarasova et al., 2021) and *S. rubicundulus* group (Elsner & Wasser, 1995; Vedenina et al., 2012) produce calling songs by wing clapping. The sound generated by wing clapping is a simple trill of pulses repeated at a rate of wing beats during flight. Such pattern is even simpler than the simplest song patterns in other Stenobothrini. Such simplification, however, could be partly provoked by the bifunctionality of some thoracic muscles that can move both the wing and the leg. In *S. rubicundulus*, it was shown that simple changes in muscle coordination can convert the movement patterns typical of legs and wings into one another (Elsner, 1974b; Elsner & Wasser, 1995). On the other hand, wing clapping could appear under sexual selection because the wing beats are incorporated into courtship songs serving as an additional visual signal for a female.

Thus, most characters of the syllable temporal structure in the gomphocerine calling songs appear to be relatively labile and probably under natural or sexual selection. Our data are partly consistent with the results of Nattier et al. (2011), which describe a pattern of the calling song evolution as 'dynamic'. However, the authors did not find a clear trend of evolution for all characters, while our results indicate a general trend for some of them. For example, the ancestral song in Gomphocerinae consisted of only one type of syllables (character 2; Figure 3a), whereas the second type of syllables evolved independently in many different branches of the tree. In the ancestral song, sound was generated by gradual upstroke (character 12) and without variations in the leg movements within syllable (characters 15 and 17). The stepwise upstroke and variations in phase shift or silent syllable part evolved later. Our findings on evolution of the gomphocerine calling song are comparable with the data on the Lebinthini crickets (Tan et al., 2021), which show that the call architecture is partly stable across lineages, but finer temporal patterns are relatively labile among closely related species.

4.4 | Identifying the evolutionary forces shaping calling song in Gomphocerinae

Most mating traits studied in various animals were shown to have a high phylogenetic signal (e.g. Erdtmann & Amézquita, 2009; Price & Lanyon, 2002). By contrast, the calling songs of gomphocerine grasshoppers include only a third of characters that are conservative in their evolution. These characters describe mainly the echeme structure. Of the numerous characters describing the syllable structure, only three show evolutionary stability. A high phylogenetic signal for a given trait implies that the trait can be phylogenetically informative in forming clades. In terms of evolutionary forces, a high phylogenetic signal is expected for a given trait if it has evolved because of non-adaptive changes, for example under the effects of random genetic drift (Gerhardt & Huber, 2002; Panhuis et al., 2001) or pleiotropic effects that follow indirectly from morphological evolution (e.g. Cocroft & Ryan, 1995; Podos, 2001; Seddon, 2005).

At the same time, behavioural experiments conducted on grasshoppers show the importance of some echeme characters in mate choice and sexual selection. For example, females of *Ch. brunneus* respond only to echemes lasting between 0.05 and 0.3 s, which corresponds to the range of the echeme duration (von Helversen & von Helversen, 1994). In *Ch. dorsatus*, the average duration of one echeme element is even well below the durations females prefer (Stumpner & von Helversen, 1992), which indicates that this character is responsive to sexual selection. Notably, our estimate of phylogenetic signal for echeme duration is lower ($\lambda = 0.435$) than for other echeme characters (Table 2), which assumes an interaction of several selection vectors, one of which is sexual selection. Nevertheless, we suggest that the characters with relatively high phylogenetic signal ($\lambda > 0.4$) can be informative in forming clades. Song and Bucheli (2010) show similar discrepancies between the phylogenetic signal value and

proposed evolution rate when comparing the male genitalia and non-genital traits in insects. According to their estimates, male genitalia have similar phylogenetic signal as compared with non-genital characters, despite the fact that male genitalia as a whole are under sexual selection. They explain this by the composite nature of male genitalia: some genital components could be phylogenetically conserved, such as the features that may be functionally constrained, while other characters could be phylogenetically much more labile, perhaps because they are involved in copulation. In our song analysis, echeme is a structural unit of the highest level, which assumes the composite nature of this song unit, similarly to that of male genitalia.

Of the characters describing the syllable structure, the highest phylogenetic signal is shown for usage of two legs (character 19). Sound generated by the movements of only one leg is only typical among members of the tribe Dociostaurini representing the most basal clade. We suggest that as soon as Gomphocerinae started to use both legs in sound generation, this feature remained to be unchanged in the process of song evolution. It is of interest that phylogenetic signal was found to be very low for variation in usage of two legs (character 20). This character implies that both legs are moved in one part of syllable, whereas one leg is only moved in another part of syllable. We expect this character to be subjected to sexual rather than natural selection (see below).

The weak phylogenetic signal was found for many syllable characters, in particular, for syllable period and syllable duration (characters 8 and 9). Syllable period is one of the few song characters that form a so-called acoustic niche, which is a part of an ecological niche in the grasshopper community (Bukhvalova, 2006; Bukhvalova & Zhantiev, 1994; Tishechkin & Bukhvalova, 2009). The species producing signals with similar syllable period are always either allopatric or inhabit different biotopes and thus avoid competition for acoustic communication channels. When females are making a choice, the first thing they must do is reject males of other species. Therefore, employment of a communication system as premating isolating mechanism is an important component of natural selection. Some authors suggest it even to be a component of sexual selection (e.g. Reichert & Ronacher, 2015; Searcy & Andersson, 1986). However, it is a fundamental difference between the question 'is it a conspecific?' that can be answered 'yes' or 'no' and the question 'who is the best out of a group of conspecifics?' that cannot be answered so easy (von Helversen & von Helversen, 1994).

The concept of acoustic niches can be considered as a part of the more general concept about environmental selection on evolution of the signal temporal structure (Gerhardt & Huber, 2002). Noise is an especially relevant environmental condition because it interferes with the capabilities of sensory systems to process relevant signals (Wiley, 2013). Reichert and Ronacher (2015) showed how female preferences for the calling song characteristics in the grasshopper *Ch. biguttulus* are affected by noise. The noise stimuli chosen by the authors covered the range of the frequency spectra typical of heterospecific signals. The results suggest that different signal characteristics can be favoured under different noise conditions, and therefore signal evolution may proceed differently depending on the extent and temporal patterning of environmental noise. Notably that in these experiments, the authors changed the fine temporal details of songs, namely, on- or offset amplitude of syllable, gap duration between pulses or pauses between syllables. For pause durations, there was a significant effect of noise on the strength of preferences. On the contrary, noise did not influence the female responsiveness to syllables with large gaps. An absence or presence of gaps between pulses depends on whether males have both legs or only one leg because of varying phase shift between the movements of two legs. A female was shown to prefer pulses without gaps because she prefers males with both legs (Klappert & Reinhold, 2003; Kriegbaum, 1989; von Helversen & von Helversen, 1997). This result showed that female preferences for pulses without gaps could be the result of sexual selection. This also matches our results on the weak or insignificant phylogenetic signals for song characters 17 (phase shift variation), 18 (phase shift) and 21 (pulses) in our estimations.

At the same time, differences in phase shift or in patterns between two legs are not always reflected in the sound pattern. When a female hears calling song at a distance, she might not evaluate whether the male lost one leg. A question arises why gomphocerine grasshoppers evolved such incredibly complex pattern of the leg movements? To answer this question, we must take into account not only the calling but also the courtship songs. About 60% of Gomphocerinae species considered in the current study show only slight differences between calling and courtship songs. Moreover, the complex courtship songs found in 40% of other species usually contain one element similar to the calling song (Berger, 2008; Tarasova et al., 2021; Vedenina et al., 2020; Vedenina & von Helversen, 2009). When a female is sitting near-by a courting male, she perceives not only acoustic but also visual signals. Such a female might distinguish between synchronous and alternative leg-movements, and a loss of one leg by the male could be critical for perception of the courtship song by the female. Thus, evolution of syllable characters chosen in our analysis of the calling song should be considered in the context of evolution of the courtship song.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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