Bioacoustics
The International Journal of Animal Sound and its Recording, 2008, Vol. 18, pp. 17–34
0952-4622/08 $10

THE EVOLUTION OF CICADA SONGS CONTRASTED WITH THE RELATIONSHIPS INFERRED FROM MITOCHONDRIAL DNA (INSECTA, HEMIPTERA)

PAULO J. FONSECA[11*], ESTER A. SERRÃO[2], FRANCISCO PINA-MARTINS[1], PEDRO SILVA[3], SARA MIRA[2], JOSÉ A. QUARTAU[1], OCTÁVIO S. PAULO[1] AND LEONOR CANCELA[2]

[1]*Centro de Biologia Ambiental/Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, P-1749-016 Lisboa, Portugal*
[2]*CCMAR, CIMAR-Laboratório Associado, F.C.M.A., Universidade do Algarve, Gambelas, P-8005-139 Faro, Portugal*
[3]*Centro de Genética e Biologia Molecular/Departamento de Biologia Vegetal, Faculdade de Ciências da Universidade de Lisboa, P-1749-016 Lisboa, Portugal*

ABSTRACT

The molecular phylogeny of nine Palaearctic species of cicadas (Hemiptera, Cicadoidea) was inferred using two mitochondrial DNA genes, Cytochrome Oxidase I and II. The two main groups detected, namely species within *Tettigetta* and *Tympanistalna*, as well as the two species investigated in the genus *Cicada,* are robustly supported across the analytical methods. The structure of the song syllables, generated during single tymbal cycles of males of the analysed group of species is remarkably consistent in these two phyletic lines. This reflects the morphology and the mechanics of the tymbal. However the higher level song patterns, which depend on the activity of the central nervous system and have evolved to advertise receptive mates, do not seem to be consistent with either the inferred molecular topology or the basic tymbal cycle. The observed similarities between the molecular phylogeny and the basic tymbal cycles seem to reflect the basic conservative nature of the tymbal structure, while the discrepancy between the former and the calling song pattern is probably related to the high plasticity of the pattern generator in the central nervous system and dependent on species-specific selection.

Keywords**:** insect calling songs, sound production, molecular phylogenies, cicadas, cytochrome oxydase

INTRODUCTION

Acoustic communication has evolved in Vertebrates and Arthropods. Among the arthropods, certain insects have extensively exploited this communication channel. Acoustic signals are especially frequent in

[1*]Corresponding author: Email: pjfonseca@fc.ul.pt

the Orthoptera and the cicadas, where they evolved as the basis for mate finding and species recognition. Cicadas exhibit songs that can be very complex in their amplitude patterns (e.g. Popov 1975; Young & Josephson 1983; Fonseca 1991; Sueur et al. 2004) and, especially in tropical species, frequency modulation can be extensively used (Gogala 1995). Thus, cicadas can be viewed as excellent models to study the evolution of acoustic signals. In spite of this, there is still much to be learned on the subject.

In cicadas, only the males produce loud acoustic signals by means of a tymbal mechanism. There are two components in the production of such a sound signal: the first one is the sound producing apparatus itself, and the second one is the control of this apparatus through the nervous system. The first component, the sound producing apparatus, is a stereotyped tymbal mechanism which is located in the first and second abdominal segments (Pringle 1954). The tymbal muscle pulls on a tymbal plate, which then modifies the convex tymbal membrane loading each of a series of sclerotized ribs. These ribs may buckle inward in synchrony, or sequentially, and from one to a series of sound pulses may be produced per muscle contraction (Young & Bennet-Clark 1995; Fonseca & Bennet-Clark 1998).

The elasticity of the tymbal, which contains resilin, assures that its resting state is restored upon relaxation of the tymbal muscle, which may or may not be accompanied by sound. Moreover, the overall shape of the tymbal – and so the sound pulses – may be modified by a tensor muscle acting on the tymbal frame (Fonseca & Hennig 1996). A period of contraction and subsequent relaxation of the tymbal muscles, *i.e.* a single cycle of the sound producing apparatus, generates the sound pulses that constitute the basic element of a song, the syllable.

The second component of the sound producing system is the nervous activity which controls the tymbal and tensor muscles. The coordination of the several elements of the sound producing system can generate diverse sounds by assembling the basic syllables in higher order and more complex song elements.

Our main objective is to examine how the different patterns of cicada calling songs correlate with the molecular phylogenetic relationships among the species investigated. To accomplish this we infer the phylogenetic relationships among a sample of nine species of Palaearctic cicadas using sequences from two fragments of mitochondrial genes, Cytochrome Oxidase I and II, and comparing the topologies obtained with the well known patterns of the songs and the morphological and physiological parameters of the sound producing system. These two genes were chosen because they are widely used for this level of phylogenetic analysis and have already been successfully used to study relationships among cicadas (e.g. Cooley *et al.* 2001; Buckley *et al.* 2001a,b). We are aware that the number of

taxa analyzed in this work is small when compared with the number of cicadas known. However, there were not many more species we could use or from which the physiological data is available.

MATERIAL AND METHODS

Sampling

Nine species of cicadas present in Portugal, belonging to six genera and two families, were selected for this study. The Cicadidae were *Cicada barbara* Boulard 1982, *Cicada orni* Linnaeus 1758 and *Lyristes plebejus* (Scopoli, 1763), while within the Tibicinidae we used *Tibicina garricola* Boulard 1983, *Tympanistalna gastrica* (Stål, 1854), *Tettigetta argentata* (Olivier, 1790), *Tettigetta estrellae* Boulard 1982, *Tettigetta josei* Boulard 1982, and *Tettigetta mariae* Quartau and Boulard 1995. The species *Philaenus spumarius* (L.) of the family Cercopidae, a related group of the Cicadoidea, was selected as the outgroup. We used two sequences, one, produced in our lab and another from the GenBank (Accession Number AY630340)

All specimens were collected in central and southern Portugal in June and July. The type of sample material used varied from freshly collected insects, to insects preserved in ethanol or in a high salt buffer, to dried insects kept in entomological boxes. DNA was generally extracted from the tymbal muscles, but for smaller insects the whole individual was extracted.

DNA extraction, amplification and sequencing

DNA extraction

Whole or parts of insects were ground in microcentrifuge tubes with liquid N, and then incubated at 55° C in extraction buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 µM EDTA, 0.5% SDS, 200 µg/ml proteinase K) with frequent agitation, followed by a purification step with phenol and chloroform:isoamyl alcohol and precipitation with 3 M sodium acetate and 100% ethanol. The pellet was washed with 70% ethanol, resuspended in TE, RNA was digested, and RNAse was removed with chloroform:isoamyl alcohol followed by an overnight precipitation with 100 % ethanol and sodium acetate. DNA quality and quantity in the resuspended pellet were assessed on agarose gels. An alternative DNA extraction method was used for some of the samples: ground in 5% chelex and 15-20 µl proteinase K (20µg/ml), incubated at 55-57° C for 3-4 h, followed by 15-20 min at 100° C. However, the first method yielded DNA of better quality and quantity.

PCR Amplification

Cytochrome oxidase I (COI) and II (COII) from each sample was symmetrically amplified by PCR, with the following primers. COI: C1-J-2195 – TTGATTTTTTGGTCATCCAGAAGT and TL2-N-3014 – TCCAATGCACTAATCTGCCATATTA; COII: C2-J-3400 – ATTGGACATCAATGATATTGA and C2-N-3661 – CCACAAATTTCTGAACATTGACCA (Simon *et al.*, 1994). Amplification conditions were: 1 X buffer II, 1.9 mM MgCl2, 0.2 mM/dNTP, 0.3 μM/primer, 1.25 U of AmpliTaq (Perkin Elmer, Norwalk, CT) and 2 ng of genomic DNA per 25 μL reaction. The PCR parameters were: 94°C for 5 min, 35 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. PCR products were electrophoresed in agarose gels, the target bands were cut under UV light, and the DNA was purified with a gel extraction kit (Qiagen, GmbH, Germany).

Cloning and sequencing

The PCR products were directly ligated into pGEM-T easy vector (Promega, Madison, Wi) and transformed into supercompetent cells (Xl1-Blue MRF', Stratagene, La Jolla, CA). Plasmid DNA from each recombinant colony was extracted using alkaline lysis minipreps. The COI and COII of 1-2 individuals of each species were sequenced in both directions using cycle sequencing with dye-labelled terminators (Perkin Elmer) on an ABI 373 automated sequencer or in ABI 310 (Applied Biosystems). For both COI and COII it was possible to fully sequence the genes in both directions using universal primers in the vector. Sequences were deposited with Genbank (Accession Numbers for COI: EU401964-EU401973; COII: EU401954-EU401963).

Sequence alignment and phylogenetic analyses

DNA sequences were initially aligned using CLUSTAL X v1.8 (Thompson *et al.*, 1997) and the resulting alignment was then inspected and manually changed to eliminate a few frame shifting gaps. Three sets of analyses were carried out, the first with combined sequences from CO I and CO II fragments, and the second with only CO I or CO II sequences.

Phylogenetic analysis was performed using PAUP*4.0.b4a (Swofford 2000). Modeltest 3.0 software (Posada & Crandall 1998) associated with PAUP* was used to select the most appropriate evolutionary model for the different data sets. The most appropriate model was then used to calculate the maximum likelihood phylogenetic tree (Felsenstein 1988). The optimal tree was found by a heuristic

search with tree-bisection-reconnection as the branch-swapping algorithm. Initial trees were obtained via stepwise addition with 100 replicates of random addition sequence. The neighbour-joining tree (Saitou & Nei 1987) for the dataset was also calculated with the previously selected maximum likelihood distance. This calculation involved the following parameters: a shape parameter of the gamma distribution used to set the relative size of four rate categories, the proportion of invariable sites, and the proportion of the two types of transition and transversions (or the transition / transversion ratio in simpler models). Unweighted parsimony analyses were also carried out on PAUP. For unweighted maximum parsimony, the optimal tree was found by a heuristic search with tree-bisection-reconnection as the branch-swapping algorithm. Initial trees were obtained via stepwise addition with 100 replicates of random addition sequence. The g1-statistic was calculated from the frequency distribution of lengths of a thousand random trees and the tree length of the optimal tree compared with this distribution (Hillis & Huelsenbeck 1992). Ensemble indices – consistency index (Kluge & Farris 1969), retention index (Farris 1969) and homoplasy index (Archie 1989) – were calculated to describe the amount of homoplasy. Gaps were treated as a fifth character state for the parsimony analysis or as missing data for other analyses.

In all, three forms of analyses bootstrapping with 1000 pseudoreplicates was performed to evaluate the robustness of the nodes, with the trees obtained in the same way of the original inference one.

To assess if the sequences evolved in a clock-like way, a likelihood ratio test was performed. The log likelihood value of a tree with the same topology and evolutionary model was calculated with and without enforcing a molecular clock. Twice the difference between calculated values was then compared with a χ^2 distribution with n-2 degrees of freedom, where n is the number of sequences used in the analysis (Huelsenbeck & Crandall 1997).

Finally Bayesian analyses were also undertaken using MrBayes v3.0b4 (Ronquist & Huelsenbeck 2003).The posterior probabilities of the phylogenetic trees were estimated by a Metropolis-Coupled, Markov Chain Monte Carlo sampling algorithm (MCMCMC). The Markov Chain Monte Carlo (MCMC) procedure ensures that trees are sampled in proportion to their probability of occurrence under the given model of gene-sequence evolution while the MCMCMC approach ensures that the Markov chain did not become trapped in local optima. The conditions for the Bayesian analysis were previously set up in order that the likelihood scores of the trees would reach stationarity. Using a general-time-reversible model of sequence evolution with a gamma distribution for the among sites rate variation, a total of 1.5×10^6 generations were sampled every 100 generations with a “burn-in”

of the first 7.5% of the trees. Clade credibility values were obtained from a 50% majority-rule consensus tree of the remaining trees. Two runs using different random starting seed were used to assess congruence of the likelihood values (Huelsenbeck *et al.* 2002). The prior model was estimated with MrModeltest v2.2 (Nylander 2004). The Shimodaira-Hasegawa test (Shimodaira & Hasegawa 1999) was implemented, when necessary, to test alternative tree topologies.

Sound recording and analysis

Calling songs were recorded at 19 cms^{-1} with a Uher 4200 report tape recorder through an AKG D202 or a Sennheiser MKE 2 microphone. These audio assemblages have a frequency response within ±3 dB in the range 1-18 kHz. Sound analysis was made with software written by the authors (e.g. Fonseca 1991).

Evaluation of physiological parameters

The tymbal muscle phase and changes in the leading muscle during singing were evaluated with electromyograms (EMG's) of the tymbal muscles obtained from spontaneously singing cicadas. For details on the EMG recordings see Fonseca (1996). The tensor muscle effect on the sound amplitude was evaluated with simultaneous electrical stimulation of the tymbal motoneuron and the tensor nerve. For a description of the method see Fonseca & Hennig (1996).

Morphology

Morphological and anatomical observations of dry and ethanol preserved insect specimens were made using a stereomicroscope (Wild M5A) equipped with a camera lucida that was used to make the drawings. We paid special attention to structures related to the sound producing apparatus such as the structure of the ribbed tymbals with the presence or absence of a coupling bar, the presence of tymbal covers and the appearance and thickness of the abdominal wall.

RESULTS

Sequence alignment and variation

The dataset comprises eleven aligned sequences including two outgroup sequences. All sets of analyses were performed for the COI

and COII dataset and for the combined dataset of COI and COII. Only the results for the concatenated dataset are shown since each dataset give essentially congruent results but with less resolution and robustness. In a total of 1184 nucleotide sites, 659 are constant in all sequences, and 341 are parsimony-informative sites. The fragment of COI has 878 nucleotide sites and COII has 306. The alignments are available from the authors.

For the datasets there was a strong bias towards AT, the average for the combined dataset is A – 30.54% and T – 41.09%. In all cases the sequences passed a Chi-square test of heterogeneity in nucleotide frequencies (for the combined dataset: Chi-square = 15.35, df=30, P>0.98). Uncorrected pair wise distances range between 2.1% to 25.0% for the combined dataset.

Phylogenetic analyses

Maximum parsimony analysis, with gaps treated as missing data or as a fifth character, produced two equally parsimonious trees with length of 1021 and three with size 1147 respectively. The consistency, the retention and the homoplasy indices were respectively 0.704, 0.607, 0.296 for the first analysis (gaps treated as missing data) and 0.730, 0.641, 0.270 for the latter (gaps treated as fifth character). The random generation of trees produced a highly skewed distribution for the two analyses, suggesting phylogenetic informative sequences (g1=-0.766; p<0.01 when gaps are treated as missing data g1=-0.875; p<0.01 when gaps are treated as fifth character).

The selected evolutionary model for the concatenated dataset was the TVM with a proportion of invariable sites and a gamma correction (TVM+I+Γ) with a G-A and C-T transition rate of 22.2452, and a A-C transversion rate of 2.9196, A-T rate of 6.1279, C-G rate of 6.1334 the proportion of invariable sites was 0.3743 and the shape parameter of the gamma distribution was 1.1088. For the combined dataset, the likelihood ratio test between trees with the same evolutionary model but with the molecular clock enforced or not, show statistical significant difference (2δ=19.47, df=9) and the molecular clock hypothesis was rejected at 95% level.

Tree topologies

The dataset produced overall similar topologies that were robust to the different phylogenetic inference methods which were used in the analyses.

Two well-supported clades are shown in all the analyses (Figure 1). The first one is formed by the Tibicinidae species *Tympanistalna*

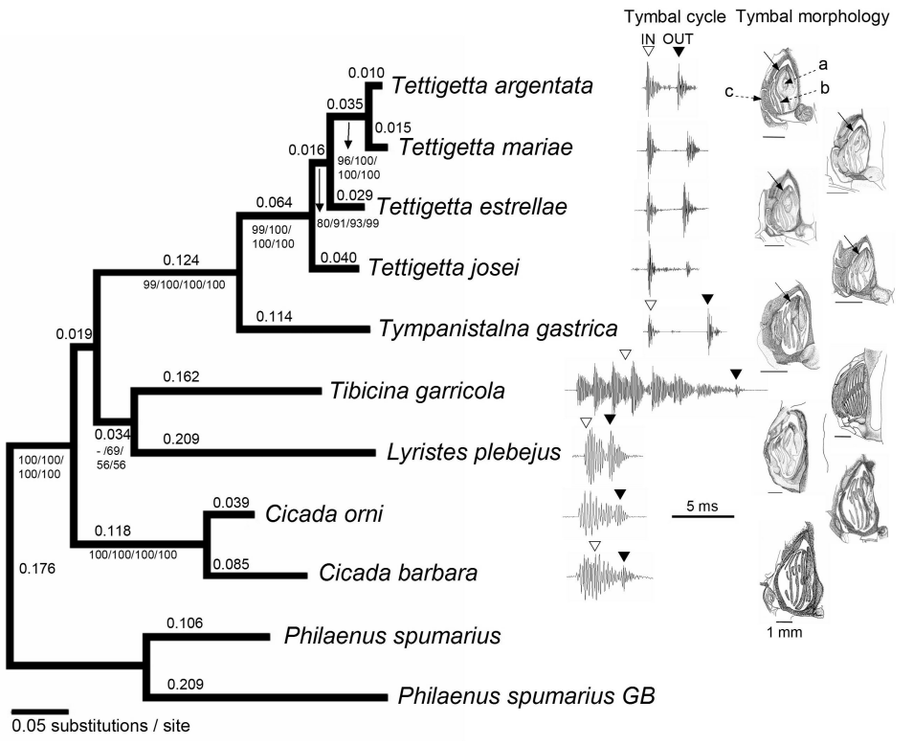

Figure 1. Phylogram obtained by Bayesian inference of 1184 base pairs of the Mitochondrial DNA, Cytochrome Oxidase subunits I and II. Numbers above the branches are inferred number of substitutions per site and the numbers below branches are the bootstrap branch support values obtained from 1000 pseudoreplicates from Maximum Likelihood analysis, Neighbour-Joining trees with the corrected maximum likelihood distance, parsimony and clade credibility values from Bayesian phylogenetic inference, respectively. Adjacent to each terminal node (*i.e.* each species) there is a scheme with the IN/OUT pulses of the Tymbal cycle and the respective Tymbal morphology. The time scale of the pulses is in milliseconds (ms) and the respective scale is shown. The scale of the morphological schemes represents 1 mm. The tymbal morphology is characterised by a) the tymbal plate, b) the longitudinal ribs and c) the tensor sclerite. The presence of a dorsal coupling bar connecting the longitudinal ribs is shown (see solid arrows).

gastrica, *Tettigetta josei*, *Tettigetta estrellae*, *Tettigetta mariae* and *Tettigetta argentata,* all within the tribe Cicadettini. This clade shows a remarkable internal stability across inference techniques and datasets. The second one is formed by only two species, the Cicadidae *Cicada barbara* and *Cicada orni*, both within the tribe Cicadini.

Somewhat unexpectedly, the tibicinid species *Tibicina garricola* never clustered with the remaining Tibicinidae as would be expected

according to traditional taxonomy, in spite of belonging to a different tribe (Tibicinini). Similarly, the third cicadid, *Lyristes plebejus*, within the tribe Lyristini, also never clusters with the two other Cicadidae, always clustering instead with *Tibicina garricola,* although with low robustness values (Figure 1). These two species seem to have an unresolved position in the tree either forming a polytomy with the two other clades (bayesian) or cluster with the other Tibicinidae species (maximum likelihood, neighbour-joining, parsimony) or with the other Cicadidae species (parsimony) or even appear in basal position in relation with the two other clades (parsimony).

A Shimodaira-Hasegawa test showed no statistical significant difference between the optimal tree (Figure 1) and several alternative suboptimal trees with the topology constrained according to different hypothesis namely with a different basal clade (p=0.330; p=0.432) or non-monophyly of the three main ingroup clades (p=0.330; p=0.432; p=1.000).

Sound producing apparatus and song characteristics

The characteristics of the sound producing apparatus and the pattern of the calling songs of the nine species are summarized in Table 1 (for clarity in the text each parameter indicated in Table 1 is shown in bold).

The tymbal mechanism of sound production consists of a ribbed tymbal that is pulled by the tymbal muscle that, upon contraction, leads to the buckling of the tymbal with subsequent sound generation. Sound pulses may be produced during the inward (IN) movement of the tymbal as well as when the tymbal pops out (OUT) due to elastic forces (Figure1). In the *Tettigetta* and *Tympanistalna* the tymbal generates two sound pulses in each **tymbal cycle,** or syllable, one at the inward and the other upon the outward movement of the tymbal. In *Tibicina* there is a series of well delimited pulses generated by the collapse of the same number of ribs during the IN, while the OUT is almost silent. In the Cicadidae the inward buckling is accompanied by two (*Lyristes)* or three (*Cicada*) pulses more or less fused together in a complex wave while the OUT produces one sound pulse.

The number and position of the tymbal ribs is different among species. Unlike the species included in the family Cicadidae, the *Tettigetta* spp. and *Tympanistalna gastrica* exhibit a **tymbal coupling bar** dorsally joining the long ribs of their tymbals (see arrows in Figure1) at their dorsal edges. This coupling bar causes the tymbal to collapse as a whole producing a single IN sound pulse. The other Tibicinidae, *Tibicina garricola*, also has the longitudinal long ribs connected dorsally to a rib-like bar, but here the ribs are spaced out dorsally (cf. Figure1). Moreover, while the three Cicadidae, *Lyristes*

TABLE 1

Selected song, physiological and morphological parameters of the cicadas analysed in this study. The representatives of the two families (Tibicinidae and Cicadidae) are separated, and the species are clustered by genera and presented in the same order as in Figure 1. Identical values for the parameters analysed are shown in identical grey shading. A question mark (?) indicates lack of data.

		Morphological parameters			Song parameters			Physiological parameters		
Family	**Species**	**Tymbal coupling bar**	**Tymbal covers**	**Thickness of abdominal wall**	**Tymbal cycle**	**Frequency peak of calling song (kHz)**	**Gross temporal pattern of calling song**	**Tymbal muscle phase**	**Changes in the leading muscle**	**Tensor muscle effect on sound pulses**
Tibicinidae	***Tettigetta argentata***	Yes	No	Thick	1 IN / 1 OUT	13-15	Simple phrases	0.1	No	Strong amplitude increase
	Tettigetta mariae	Yes	No	Thick	1 IN / 1 OUT	13-14	Simple phrases	?	?	Strong amplitude increase
	Tettigetta estrellae	Yes	No	Thick	1 IN / 1 OUT	11-14	Complex phrases	< 0.1	?	Strong amplitude increase
	Tettigetta josei	Yes	No	Thick	1 IN / 1 OUT	15-17	Complex phrases	0.03 (close to synchrony)	No	Strong amplitude increase
	Tympanistalna gastrica	Yes	No	Thin and translucent	1 IN / 1 OUT	10-13	Complex phrases	0.15	No	Strong amplitude decrease
	Tibicina garricola	Yes, but ribs well separated dorsally	No	Thick	3-7 IN / 1 quiet OUT	8-10	Continuous song	0.3-0.5	Yes	Not clear
Cicadidae	***Lyristes plebejus***	No	Yes	Thick	2 fused pulses IN / 1 OUT	5-6	Complex phrases	0.4 -0.5	?	?
	Cicada orni	No	Yes	Thin and translucent	3 fused pulses IN / 1 OUT	4-5	Simple discontinuous song	0.5	Yes	Not clear
	Cicada barbara	No	Yes	Thin and translucent	3 fused pulses IN / 1 OUT	5-7	Continuous song	0.5	Yes	Not clear

plebejus, *Cicada orni* and *Cicada barbara*, possess **tymbal covers**, *i.e.* integument expansions that project anteriorly and cover in part (*Cicada*) or completely (*Lyristes*) the tymbals, these structures are not present in the other species. **The thickness of the abdominal wall** also varies. It is thick in the majority of the cicadas, but in *Tympanistalna gastrica* and in the two species of the genus *Cicada* the wall of the abdomen is thin and translucent.

The vibrations induced in the tymbal by the pull of the muscle, which correspond to the resonant properties of this structure backed by an internal air chamber, are then radiated to the air by a number of structures. These include the main generator, the tymbal, that usually contributes the spectral peak to the song, but sound is conveyed also through the tympana. The abdominal wall may also play an important role, especially if it is thin. The species studied can be grouped according to the **frequency peaks of their calling songs**. The *Tettigetta* species generate sounds with higher frequency components (10-17 KHz), followed by *Tympanistalna gastrica* (10-13 kHz), and by *Tibicina garricola* (8-10 KHz). In contrast, the Cicadidae produce lower pitched sounds 4-7 KHz).

The nervous system dictates which tymbal is activated first and the phase between the paired tymbal muscles. **Changes in the leading muscle** were not seen in the *Tettigetta* and *Tympanistalna* but were observed in *Tibicina* and in the Cicadidae (*Lyristes* and *Cicada* spp.). A similar pattern was seen in the **tymbal muscle phase**. The *Tettigetta* species and *Tympanistalna* show smaller phases between the activation of the tymbal muscles (from almost synchrony in *Tettigetta josei* to about 0.15 in *Tympanistalna gastrica*) while in the other species the tymbals alternate with phases ranging from 0.3 to 0.5.

Finally, **the tensor muscle effect on the sound pulses** can be different in the several species. While in *Tettigetta* its contraction strongly increased the sound amplitude, in *Tympanistalna gastrica* the opposite effect was obtained with a marked decrease of the sound amplitude. The effect was not clear in *Tibicina* and the two *Cicada* species, and it was not assessed in *Lyristes plebejus*.

The songs can be classified according to their gross temporal pattern. In continuous songs observed in *Cicada barbara* and *Tibicina garricola* the syllables are simply repeated in a continuous sequence for many seconds to several minutes. In contrast, in *Cicada orni*, which exhibits a simple discontinuous song, this sequence is interrupted by regular pauses. In other species the sound sequence is organized in characteristic phrases, *i.e.* long sound units composed of a certain sequence of groups of syllables and pauses, which are repeated over time. *Tettigetta argentata* and *Tettigetta mariae* have simple phrases with groups of similar syllables along the phrase. In contrast, in the complex phrases of *Tettigetta estrellae*, *Tettigetta josei*, *Tympanistalna*

gastrica and *Lyristes plebejus* there are sequences of different groups of syllables along the phrase (see Fonseca 1991 and Sueur *et al*. 2004 for a detailed description of the songs).

The observed **Gross temporal pattern of the calling songs**, which measures the contribution of the second component of the sound producing system, shows a remarkably different pattern from the one inferred by the phylogenetic results. Simple and complex phrases appear in both groups, and continuous or discontinuous songs are produced by species in the several groups.

DISCUSSION

Although the topologies produced by different inference methods were not always identical, there was no strong support for conflicting arrangements. One possible reason for this unstable situation could be related to the asymmetry of the species sample by using single species from both of the genera *Tibicina* and *Lyristes*. A more balanced and larger taxonomic coverage and other genes might help in solving the current ambiguities. However, the well supported clades of the phylogenetic analyses are in agreement with the traditional classifications based mostly on morphological characters (Webb 1979; Boulard 1982,1987). Inside the well-supported Tibicinidae clade (Figure 1) the relations among the species are identical across the different inference methods. In relation to the Cicadidae species, this set of results support what others already have shown with morphology and genetics: that *Cicada barbara* and *Cicada orni* are closely related and well defined species (Quartau 1988; Pinto *et al*. 1998; Quartau *et al*. 2001).

The phylogeny obtained with this dataset, in spite of some ambiguities, was used as a framework for the comparisons of the sound apparatus and acoustic patterns of the several species.

The calling songs of cicadas exhibit high variability, ranging from continuous to discontinuous songs with different degrees of amplitude modulation, to songs with a complex pattern repeated over time (e.g. Popov 1975; Young & Josephson 1983; Fonseca 1991; Sueur *et al*. 2004). Our main objective was to find out how exactly the molecular phylogeny of the cicadas correlates with the observed pattern of the songs.

The first component of the sound producing system, the sound producing apparatus itself, is responsible for the basic sound elements produced during one tymbal cycle. These are similar in the *Tettigetta spp*. and *Tympanistalna gastrica*, which generate two loud pulses, one during the inward buckling of the tymbal (IN pulse), and another resulting from its outward movement (OUT pulse), a tymbal cycle pattern shared with many *Cicadetta* species (e.g. Popov 1975; Gogala

et al. 1996). These species cluster in a well-supported phylogenetic clade. The Cicadidae group show a different tymbal cycle, with a complex IN pulse resulting from two (*Lyristes*) or three (*Cicada*) partially fused pulses followed by one loud OUT pulse (Popov 1975; Fonseca 1991), a pattern also seen in other genera of this group like the North-American *Tibicen* (e.g. Hennig *et al.* 1994). In the phylogram the two *Cicada* species are grouped together, while *Lyristes plebejus* has an unresolved status. *Tibicina garricola* shows a distinct syllable producing up to seven pulses during the inward tymbal movement, while the OUT pulse is almost silent, a pattern similar to other *Tibicina* (e.g. Sueur & Aubin 2003) and to the American *Magicicada* (Moore & Sawyer 1966) and *Okanagana* species (Stölting *et al.* 2004). This species also has an unresolved relationship with the well-defined clades of the phylogenetic trees.

All the species producing 1 IN –1 OUT pulse have a coupling bar located dorsally to the longitudinal tymbal ribs, coming close to the tymbal plate. This bar, probably responsible for the buckling of the tymbal as a whole (Fonseca & Bennet-Clark 1998), is not present in any of the other species except *Tibicina garricola.* Consequently, this organization of the tymbal and the sound produced (syllable) is exclusive of the robust clade that assembles *Tettigetta* and *Tympanistalna*. In *Tibicina*, although all the ribs are dorsally linked by a sclerotized rib-like structure, the arrangement of the ribs and the tymbal is very different. Here the long ribs are well separated in all their length and are not apposed as in *Tettigetta* and *Tympanistalna*, and the resulting IN produces not a single but instead several pulses, one per each rib collapsed.

Despite the similarities of the basic sound production found in the group of species with a coupling bar, the effect of the tensor muscle, responsible for the amplitude modulation of the songs, introduces another source of variability. Its effect is opposite in the *Tettigetta spp.* and in *Tympanistalna gastrica* (Fonseca & Hennig 1996). In the former it increases the sound amplitude while in the latter the contraction of the tensor muscle generates a decrease. This opposite effect seems to corroborate the phylogenetic results, as these two genera seem to be well-defined sister taxa. Moreover, while the abdominal wall is very thin in *Tympanistalna gastrica*, playing a significant role in sound radiation (Fonseca & Popov 1994), in *Tettigetta* it is much thicker and therefore does not work as a radiator.

In the *Tympanistalna* and *Tettigetta* clade, not only do the tymbal buckle as a whole, but the left-right tymbal cycle shows only a small phase difference (< 0.2 of a cycle), while in the other four species the tymbals almost alternate (phase differences about 0.5). It is especially noteworthy that *Tibicina garricola*, which is traditionally classified with the Tibicinidae, appears outside the group including

other traditional members of this group in this phylogenetic analysis. *Tibicina* also shows a tymbal cycle phase difference inconsistent with the other members of the traditional classification. The same pattern can be observed in the activation of the muscles, since changes in the leading muscle during the calling song were never observed in *Tympanistalna* and *Tettigetta*, while they have been found in the *Cicada* and *Tibicina* (Fonseca 1996). Finally, all three Cicadidae species display tymbal covers, which no other species show, suggesting that this character could have evolved in the lineage leading to this group.

The phylogenetic relations seem also to be reflected in the spectral peak of the calling songs, which is greatly dependent on the mechanics of the tymbal (Fonseca & Popov 1994) and, according to Bennet-Clark & Young (1994), also on body size. The calling songs with higher frequency peaks are associated with the *Tettigetta* / *Tympanistalna* clade, also the smaller species in our sample, while the Cicadidae species (*Cicada* and *Lyristes*) exhibit lower frequencies, but here the much larger *L. plebejus* has a frequency peak similar to *Cicada spp*. *Tibicina garricola,* a species with a body length similar to *Cicada* has an intermediate spectral peak, *i.e.* higher then *Cicada* and *Lyristes* but lower than *Tettigetta* and *Tympanistalna*, as do other members of the genus *Tibicina* (Sueur & Aubin 2003) and the possibly related American species of *Okanagana* (*e.g.* Stölting *et al.* 2004).

While the syllable depends on the structure and thus the mechanics of the tymbal, the second component, the role of the nervous system which generates the calling song pattern by controlling the sound producing apparatus, is a major determinant of the species specificity of the songs. This is obtained as a result of the activity of a very plastic nervous system that assembles the bilateral tymbal cycles into complex and diversified temporal sequences. At this level there are less biomechanical constraints to the evolution of the songs, more in line with the constraints of the vocal system of vertebrates (e.g. frogs, Ryan & Rand 2003).

In contrast with the close phylogenetic relations shown by the species with similar tymbal cycles, as expected, this second level in the assemblage of the songs does not seem to agree with the phylogenetic trees. Similar types of songs can be found in distant positions and, conversely, sister species can have rather different song patterns. Another example of such plasticity in a closely related group can be found in New Zealand *Maoricicada* species (Buckley *et al.* 2006). Nevertheless, examples exist of close related species (*e.g. Tibicina spp*., Sueur & Aubin 2003; Sueur *et al.* 2007) that exhibit similar calling songs even when sympatric. This, however, only reinforces the plasticity of the nervous system and behaviour, since in those cases other cues should be used by females, at least at close range, to discriminate males of their own species.

The same was found in other groups where characteristic songs are considered premating signals mediating reproductive isolation. For instance, In the *Drosophila willistoni* species complex Gleason & Ritchie (1998) found no correlation between song and genetic divergence, and phylogenies derived from song patterns did not reflect molecular phylogenies. Likewise, the phylogenies of frogs in the *Physalaemus pustulosus* species group obtained from molecular sequences, allozymes and morphological characters were significantly different from the tree derived from characters of the advertisement calls (Cannatella *et al.* 1998).

This diversity in the patterns exhibited by calling songs is likely the result of selection on these congregating signals where related species overlap in time and space. A good example can be seen in the sympatric sister species of *Cicada*. While *Cicada barbara* produces a continuous signal, *Cicada orni* has a simple discontinuous song. Similarly, *Tettigetta mariae* has a simple discontinuous song, while *Tettigetta estrellae* shows a complex pattern composed of short elements, and *Tettigetta josei* has a complex pattern of long phrases (Fonseca 1991). Thus, as often seen, closely related sympatric species exhibit considerable diversity in their signals (e.g. Popov *et al.* 1974; Ritchie & Gleason 1995; Gerhardt & Huber 2002). Conversely, *Lyristes plebejus* exhibits a complex pattern of the phrases of its song, along with the complexity of the calling songs of some of its phylogenetically unrelated *Tettigetta* species (cf. table 1).

The diversity of the calling songs seems to be very homoplasious (Gleason & Ritchie 1998), while the basic song characteristics which depend on the structural elements, such as the tymbal are more conservative and robust during evolution and consequently seem to have a closer correspondence to the phylogeny of genera and higher groups.

This set of results illustrates the complex evolution of the cicada sounds in relation to molecular phylogeny, clearly with two components. One component, more basic, is strongly related with a more conservative evolutionary pattern, while the other, at higher level, is closely related with the intrinsic neural plasticity and has been probably selected under different regimes that may have been acting in the mating signals, namely adaptation to the environment for improved signalling to the receiver, avoidance of heterospecific encounters and sexual selection, among other selective forces. The complex pattern shown in this study is expected to turn into a more robust one with enlarged datasets and better knowledge of the phylogenies of the cicadas and of the mechanisms involved in their song production. A much larger taxa dataset may reveal additional relationships in the evolution of cicadas and their song signals.

ACKNOWLEDGMENTS

This work was carried out with support from the Centro de Biologia Ambiental, CCMAR, and a grant by FCT, Portugal and FEDER (POCTI/BSE/48365/2002). We thank two anonymous referees for their comments.

REFERENCES

Archie, J.W. (1989). Homoplasy excess ratios – new indexes for measuring levels of homoplasy in phylogenetic systematics and critique of the consistency index. *Syst. Zool.*, **38**, 253-269.

Bennet-Clark, H. & Young, D. (1994). The scaling of song frequency in cicadas. *J. Exp. Biol.*, **191**, 291-294.

Boulard, M. (1982). Les cigales du Portugal, contribution à leur étude (Homoptera, Cicadidae). *Annls. Soc. Ent. Fr. (NS*), **18**, 181-198.

Boulard, M. (1987). Taxonomie et nomenclature superieures des Cicadoidea, histoire, problèmes et solutions. *École Pratique des Hautes Études, Travaux du Laboratoide d'Entomologie, Biologie et Evolution des Insectes Hemipteroidea*, Paris. **1**, 1-89

Buckley, T.R., Cordeiro, M., Marshall, D.C. & Simon,C. (2006). Differentiating between hypothesis of lineage sorting and introgression in New Zealand alpine cicadas (Maoricicada Dugdale). *Syst. Biol.*, **55** (3), 411-425.

Buckley, T.R., Simon,C., & Chambers, G.K. (2001a). Phylogeography of the New Zealand cicada Maoricicada campbelli based on mitochondrial DNA sequences: ancient clades associated with cenozoic environmental change. *Evolution*, **55**, 1395-1407.

Buckley, T.R., Simon,C., Shimodaira, H., & Chambers, G.K. (2001b). Evaluating hypotheses on the origin and evolution of the New Zealand alpine cicadas (Maoricicada) using multiple-comparison tests of tree topology. *Mol. Biol and Evol.*, **18**, 223-234.

Cannatella, D.C., Hillis, D.M., Chippindale, P.T., Weigt, L., Rand, A.S., & Ryan, M.J. (1998). Phylogeny of frogs of the Physalaemus pustulosus species group, with an examination of data incongruence. *Syst. Biol.*, **47**, 311-335.

Cooley, J.R., Simon, C., Marshall, D.C., Slon, K., & Ehrhardt, C. (2001). Allochronic speciation, secondary contact, and reproductive character displacement in periodical cicadas (Hemiptera: Magicicada spp.): genetic, morphological, and behavioural evidence. *Mol. Ecol.*, **10**, 661-671.

Farris, J.S. (1969). A successive approximations approach to character weighting. *Syst. Zool.*, **18**, 374-385.

Felsenstein, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Annu. Rev. Genet.*, **22**, 521-565.

Fonseca, P.J. (1991). Characteristics of the acoustic signals in nine species of cicadas (Homoptera, Cicadidae). *Bioacoustics*, **3**, 173-182.

Fonseca, P.J. (1996). Sound production in cicadas: timbal muscle activity during calling song and protest song. *Bioacoustics*, **7**, 13-31.

Fonseca, P.J. & Bennet-Clark, H.C. (1998). Asymmetry of tymbal action and structure in a cicada: a possible role in the production of complex songs. *J. Exp. Biol.*, **201**, 717-730.

Fonseca, P.J. & Hennig, R. (1996). Phasic action of the tensor muscle modulates the calling song in cicadas. *J. Exp. Biol.*, **199**, 1535-1544.

Fonseca, P.J. & Popov, A.V. (1994). Sound radiation in a cicada: the role of different structures. *J. Comp. Physiol.*, **175**, 349-361.

Gerhardt, H.C. & Huber, H. (2002). *Acoustic Communication in Insects and Anurans, Common Problems and Diverse Solutions*. Chicago, London: The University of Chicago Press.

Gleason, J.M. & Ritchie, M.G. (1998). Evolution of courtship song and reproductive isolation in the Drosophila willistoni species complex: Do sexual signals diverge the most quickly? *Evolution*, **52**, 1493-1500.

Gogala, M. (1995). Songs of four cicada species from Thailand. *Bioacoustics*, **6**, 101-116.

Gogala, M., Popov, A.V., & Ribariè, D. (1996). Bioacoustics of singing cicadas of the western palearctic: Cicadetta tibialis (Panzer) (Cicadoidea: Tibicinidae). *Acta Entomologica Slovenica*, **4**, 46-62.

Hennig, R.M., Weber, T., Moore, T.E., Huber, F., Kleindienst, H.U., & Popov, A.V. (1994). Function of the Tensor Muscle in the Cicada Tibicen Linnei. *J. Exp. Biol.*, **187**, 33-44.

Hillis, D.M. & Huelsenbeck, J.P. (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *J. Hered.*, **83**, 189-195.

Huelsenbeck, J.P. & Crandall, K.A. (1997). Phylogeny estimation and hypothesis testing using maximum likelihood. *Annu. Rev. Ecol. Syst.*, **28**, 437-466.

Huelsenbeck, J.P., Larget, B., Miller, R.E., & Ronquist, F. (2002). Potential applications and pitfalls of Bayesian inference of phylogeny. *Syst. Biol.*, **51**, 673-688.

Kluge, A.G. & Farris, J.S. (1969). Quantitative phyletics and the evolution of anurans. *Syst. Zool.*, **18**, 1-32.

Moore, T.E. & Sawyer, R.T. (1966). Mechanism of Cicada Timbal Action (Insecta – Homoptera - Cicadidae). *Am. Zool.*, **6**, 509.

Nylander, J.A.A. (2004). *MrModeltest v2*. Program distributed by the author. Uppsala University: Evolutionary Biology Centre.

Pinto, G.A., Quartau, J.A., Morgan, J.C., & Hemingway, J. (1998). Preliminary data on the sequencing of a fragment of cytochrome B gene of mitochondrial DNA in Cicada orni L. (Homoptera: Cicadidae) in Portugal. *Bol. Soc. Port. Ent.*, **188**, 57-66.

Popov, A.V. (1975). The structure of the tymbals and the characteristics of the sound signals in singing cicadas (Homoptera, Cicadidae) in the southern regions of the USSR. *Entomol. Obozr.*, **54**, 258-291.

Popov, A.V., Shuvalov, V.F., Svetlogorskaya, I.D., & Markovich, A.M. (1974). Acoustic behaviour and auditory system in insects. In *Mechanoreception* (Ed. by J. Schwartzkopff), pp. 281-306. Abh. Rheinisch-Westfäl.Akad. Wiss.

Posada, D. & Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Pringle, J.W.S. (1954). A physiological analysis of cicada song. *J. Exp. Biol.*, **31**, 525-560.

Quartau, J.A. (1988). A numerical taxonomic analysis of interspecific morphological differences in two closely related species of Cicada (Homoptera, Cicadidae) in Portugal. *Great Basin Naturalist Memoirs*, **12**, 171-181.

Quartau, J.A., Ribeiro, M., Simões, P.C. & Coelho, M.M. (2001) Genetic divergence among populations of two closely related species of Cicada Linnaeus (Hemiptera: Cicadoidea) in Portugal. *Insect Syst. Evol*, **32**, 99-106.

Ritchie, M.G. & Gleason, J.M. (1995). Rapid Evolution of Courtship Song Pattern in Drosophila-Willistoni Sibling Species. *J. Evol. Biol.*, **8**, 463-479.

Ronquist, F. & Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572-1574.

Ryan, M.J. & Rand, A.S. (2003). Sexual selection in female perceptual space: How female tungara frogs perceive and respond to complex population variation in acoustic mating signals. *Evolution*, **57**, 2608-2618.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406-425.

Shimodaira, H. & Hasegawa, M. (1999). Multiple Comparisons of Log-Likelihoods with Applications to Phylogenetic Inference. *Mol. Biol. Evol.*, **16**, 1114-1116.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Ent. Soc. Am.*, **87**, 651-701.

Stölting, H., Moore, T.E., & Lakes-Harlan, R. (2004). Acoustic communication in Okanagana rimosa (Say) (Homoptera : Cicadidae). *Zoology*, **107**, 243-257.

Sueur, J. & Aubin, T. (2003). Specificity of cicada calling songs in the genus *Tibicina* (Hemiptera : Cicadidae). *Syst. Entomol.*, **28**, 481-492.

Sueur, J., Puissant, S., Simoes, P.C., Seabra, S., Boulard, M., & Quartau, J.A. (2004). Cicadas from Portugal: revised list of species with eco-ethological data (Hemiptera: Cicadidae). *Insect Syst. Evol.*, **35**, 177-187.

Sueur, J., Vanderpool, D., Simon, C., Ouvrard, D. & Bourgoin, T. (2007). Molecular phylogeny of the genus Tibicina (Hemiptera: Cicadidae): rapid radiation and acoustic behaviour. *Biol. J. Linn. Soc.*, **91**, 611-626.

Swofford, D.L. (2000). *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods).* v. 4.0. Sunderland, MA: Sinauer Associates.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., & Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, **25**, 4876-4882.

Webb, M.D. (1979). Revision of Rambur's Homoptera species from the types in the British Museum. *Annls. Soc. Ent. Fr.* (NS), **15**, 227-240.

Young, D. & Bennet-Clark, H.C. (1995). The role of the tymbal in cicada sound production. *J. Exp. Biol.*, **198**, 1001-1019.

Young, D. & Josephson, R.K. (1983). Mechanisms of Sound-Production and Muscle-Contraction Kinetics in Cicadas. *J. Comp. Physiol.*, **152**, 183-195.

Received 28 June 2007, revised 18 October 2007 and accepted 30 October 2007.