

Vibratory Communication and its Relevance to Reproductive Isolation in two Sympatric Stink Bug Species (Hemiptera: Pentatomidae: Pentatominae)

Raul A. Laumann¹ · Andrej Čokl² ·
Maria Carolina Blassioli-Moraes¹ · Miguel Borges¹

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Abstract Communication is in phytophagous stink bugs of the subfamily Pentatominae related to mating behavior that among others includes location and recognition of the partner during calling and courting. Differences in temporal and frequency parameters of vibratory signals contributes to species reproductive isolation. *Chinavia impicticornis* and *C. ubica* are two green Neotropical stink bugs that live and mate on the same host plants. We tested the hypothesis that differences in temporal and spectral characteristics of both species vibratory signals enable their recognition to that extent that it interrupts further interspecific communication and copulation. To confirm or reject this hypothesis we monitored both species mating behaviour and recorded their vibratory songs on the non-resonant loudspeaker membranes and on the plant. The level of interspecific vibratory communication was tested also by playback experiments. Reproductive behavior and vibratory communication show similar patterns in both *Chinavia* species. Differences observed in temporal and spectral characteristics of female and male signals enable species discrimination by PCA analyses. Insects that respond to heterospecific vibratory signals do not step forward to behaviors leading to copulation. Results suggest that species isolation takes place in both investigated *Chinavia* species at an early stage of mating behavior reducing reproductive interference and the probability of heterospecific mating.

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✉ Raul A. Laumann
raul.laumann@embrapa.br

¹ Semiochemicals Laboratory, Embrapa Genetic Resources and Biotechnology, Avda W5 Norte (Final), Brasília, DF 71070-917, Brazil

² Department of Organisms and Ecosystems, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

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Introduction

Communication plays a central role in animal behavior and evolution. Signals transmit information that among many tasks reduces uncertainty and modulates sexual behavior (Scott-Philips 2008; Seyfarth et al. 2010). Insects communicate with signals of different modalities transmitted in air, solids and water. Chemical and acoustic signals predominantly transmit information through the air (Greenfield 2002) and on plants most insect communicate with the substrate-borne component of their vibratory emissions (Cocroft and Rodrigues 2005). In plant-dwelling stink bugs of the subfamily Pentatominae vibratory signals are produced principally by abdomen vibration, they are related to reproductive behavior, modulating location, recognition and choice of mates and attraction and/or rivalry (Čokl 2008; Čokl et al. 2014). Vibratory signals are part of a communication repertoire that starts when males release sex pheromone to attract females to the same plant (Borges et al. 1987; Aldrich 1988; Blassioli-Moraes et al. 2008).

Prezygotic reproductive isolation among populations is one of the principal forces in patterns of speciation (Kirkpatrick and Ravigne 2002). In this context differences in courtship behavior and signals characteristics, the phenotypic architecture of behavioral isolation, among closely related species are relevant to preventing hybridization (Ryan and Rand 1993; Mendelson and Shaw 2012). Notwithstanding reproductive interference may take place when signals characteristics and/or receivers preferences of sympatric species overlap conducting to heterospecific interactions with direct or indirect fitness costs (Gröning and Hochkirch 2008).

In Pentatominae vibratory signals differences in temporal and spectral characteristics are related to the level of species specificity (Čokl and Virant-Doberlet 2003; Blassioli-Moraes et al. 2005; Čokl 2008; Žunič et al. 2011). This is especially relevant to sympatric species that live, feed and mate at the same time and at the same place. To our knowledge the only investigation among Pentatominae of the reproductive barrier carried by vibratory signals among sympatric species was conducted on *Nezara antennata* Scott, 1874 and *N. viridula* (Linnaeus 1758) (Kon et al. 1988). The presence of heterospecific mating between individuals of populations in the sympatric area of the Wakayama Prefecture in Japan indicates that isolation barriers based on species specificity of vibratory, chemical, tactile and optical signals do not exclusively prevent reproductive interference at pre-copulatory levels (Kon et al. 1994). In the specific case of sympatric *Nezara* species, Kon et al. (1993) confirmed that hybridization is prevented by the complete post-mating barrier.

Chinavia impicticornis (Stål, 1872) and *C. ubica* (Rolston, 1983) are widely distributed in Brazil (Schwertner and Grazia 2007), being found in both natural and agricultural habitats on the same host-plants (Panizzi et al. 2000; Schwertner and Grazia 2007) and, like other stink bugs, frequently show aggregate spatial distribution (Higuchi 1992; Roggia 2009; Tillman et al. 2009). The high possibility of reproductive interference is based on observation that both species populations have the same spatial-temporal distribution in central Brazil and that several couples were detected along the insect life (Silva et al. 2015).

Chemical signals (pheromones) in closely related stink bugs species have similar composition with variations in minor components or in specific blends between species (Blassioli-Moraes et al. 2008). The sex pheromone of *C. impicticornis* appears to be formed by only one component, *trans*-(*Z*)-(4*S*)-bisabolene epoxide (BE); on the other hand, the sex pheromone blend of *C. ubica* is composed of *trans*-(*Z*)-(4*S*)-BE and their isomer, *cis*-(*Z*)-(4*S*)-BE, in an approximately 10:90 ratio (Blassioli-Moraes et al. 2012). At long range, these differences in sex pheromone composition specifically attract females of each species (Blassioli-Moraes et al. 2012). These compounds are also components of *Chinavia hilaris* (Say, 1832) (McBrien et al. 2001) and *Nezara viridula* sex pheromone (Baker et al. 1987; Aldrich et al. 1987).

Because of the similar sex pheromone composition and ecological characteristics species specific vibratory signals could play a decisive role in reproductive isolation in both sympatric *Chinavia* species. Vibratory communication and the song repertoire of *C. impicticornis* were first described by Blassioli-Moraes et al. (2005). The aim of the presents work was to determine the role of vibratory communication signals in species isolation in closely related sympatric species *C. impicticornis* and *C. ubica*. To achieve this goal, we compared temporal and spectral parameters of vibratory signals of both species, recorded on non-resonating loudspeaker membrane and on plants, comparing differences and correlated them with specific behaviour during calling and courting. With playback experiments we tested the level of interspecific vibratory communication. We hypothesize that *C. impicticornis* and *C. ubica* produce vibratory signals that significantly contribute to reproductive isolation by their species specific temporal and spectral characteristics.

Material and Methods

All experiments were conducted at EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil) in a sound insulated room on a shock-proof table to decrease environmental noise. Observations and recordings were conducted between 08:00 and 18:30 when most of the mating activities were detected in preliminary observations.

Insects and Plants

Adults of *C. impicticornis* and *C. ubica* originated from colonies maintained in the Semiochemicals Laboratory of Embrapa Genetic Resources and Biotechnology, Brasília, Distrito Federal, Brazil. Voucher specimens were deposited in the insect collection at Embrapa Genetic Resources and Biotechnology (Brasília, DF - Brazil). Rearing procedures followed those described by Blassioli-Moraes et al. (2012). In brief, the insects were reared in 8 l transparent plastic containers and fed with a standard diet composed of green bean pods (*Phaseolus vulgaris* L.), dry soybean seeds (*Glycine max* L), raw peanuts (*Arachis hypogaea* L.), sunflower seeds (*Helianthus annuus* L.) and water. A 15 cm² plastic mesh (~ 40 mesh) was placed against the inner wall of each container as an oviposition substrate and shelter for the bugs. The containers were covered with voile screen and kept at 26 ± 1 °C temperature, 65 ± 10 % RH under a 14 L:10D photoperiod (light, 06:00 to 20:00 h). Food supply was renewed three times per week. Egg masses were collected daily and incubated in 9 cm ID plastic Petri dishes

until hatch. After nymphs moulted into second instars, they were transferred to plastic containers and reared following the procedures described above. To prevent interactions between the sexes, males were separated from females after their imaginal moult and cuticular hardening (ca. 24 h after moulting). Sexually mature adults, ≥ 8 d after the final moult (Blassioli-Moraes et al. 2012), were used for all bioassays.

Soybean plants were grown in sterilized soil in plastic pots 20 cm high \times 15 cm diameter and used in the V3 stage (Fehr et al. 1971). At this phenological stage the soybean plants are characterized by two unifoliolate ($\sim 7 \times 7$ cm) and two trifoliolate ($\sim 6 \times 8$ cm) leaves and a 15 to 25 cm high stem (Laumann et al. 2013).

Recording Vibratory Signals

Vibratory signals were recorded from *C. impicticornis* and *C. ubica* stinkbugs communicating either on a non-resonant substrate (loudspeaker membrane) or on the host plant. The experimental set-up for recordings on the loudspeaker membrane was similar to that described by Blassioli-Moraes et al. (2005). A couple was placed in an arena constructed on the membrane of a 10 cm diameter low-midrange loudspeaker (40–6000 Hz frequency response, 8 Ω impedance; RadioShack, Taiwan). An acrylic box (9 cm diameter \times 4 cm high) was placed over the edge of the loudspeaker, without contacting its membrane, to prevent insects from walking away from the membrane surface. After the first emitted vibratory signal the box was removed. The vibratory signals emitted as vibrations of the loudspeaker membrane or soybean plant were recorded using a portable digital laser vibrometer (Polytec, model PDV 100, Waldbronn, Germany). The laser beam was oriented perpendicularly to the recording point from a distance of ca. 20 cm. Recording points were located at one of the laterals of the loudspeaker membrane (~ 3 cm from the external edge) or on the middle part of the plant stem (~ 10 cm above the soil). To obtain better reflection of the laser beam a small piece (0.5 cm²) of a reflective tape was attached to the loudspeaker membrane or the plant stem. Signals were digitised with a sound card (24-bit, 96-kHz, 100-dB signal-to-noise ratio, Sound Blaster Extigy, Creative Laboratories Inc., Milpitas, CA), and stored on a computer using the software Cool Edit Pro 2.0 (Syntrillium Software 2001 – Fort Wayne, Indiana, USA). Signal emissions were monitored with headphones during all times of insect observation.

Behavioral Observations

The same test protocol was used for experiments on the loudspeaker membrane and on the plant. Experiments on the loudspeaker membrane were conducted with 64 *C. impicticornis* and 54 *C. ubica* couples placed in the arena. The behavior and vibratory signal emissions were monitored and recorded until copulation. If insects did not start mating behavior within 20 min the couple was removed and the trial was classified as failed. Behavior and communication with vibratory signals on soybean plants, was tested with 20 pairs of each *Chinavia* species under the same experimental protocol as described for those performed on the loudspeaker membrane. On plants, a male and a female were placed on opposite leaves of the soybean plant and mating with vibratory communication signals was recorded until copulation. The trial was classified as a failure if the insects displayed no mating behavior within 20 min or if a male or a female left the plant.

Terminology and Data Analysis

Sequences of courtship and copulation behaviors were described using behavior categories previously determined for other pentatomids (Harris and Todd 1980; Borges et al. 1987; Zahn et al. 2008) (Table 1). Pulse was defined as a unitary homogeneous parcel of vibration of finite duration, pulse trains as repeatable and temporally distinct groups of pulses and a song as a sequence of pulses and/or pulse trains with a distinct beginning and end (Figs. 2 and 3) (Broughton 1963). Signal duration was measured between its onset and its end where its amplitude reached the noise level. Repetition time was defined as the time between onsets of two sequential signals. Frequency characteristics were described by frequency spectra (fast Fourier transform (FFT) size 32,768, FFT overlap 75 %, smoothing window Blackman-Harris and display range 60 dB) and sonograms (FFT size 8192, FFT overlap 99 %, smoothing window Blackman-Harris display range 80 dB). Frequency characteristics were described by the dominant and harmonic peaks and by frequency modulation described as downward or upward-orientated frequency sweeps. Songs were classified in the sequence of their emission (Čokl et al. 2001). All spectral and temporal characteristics were determined using the software Sound Forge 6.0 (Sonic Foundry <http://www.sonicfoundry.com>).

Heterospecific Couples Experiments in Arenas

To check how the different behavioral steps/vibratory communication could benefit reproductive isolation in these sympatric species, 25 heterospecific couples were

Table 1 Behavioral categories performed by *Chinavia impicticornis* and *Chinavia ubica* in a closed arena and described in the ethogram

-Behavioural category code	Description of behaviour
nrb	No reproductive behavior (insects that did not show any behavior)
FapM	Female approaches the male
MapF	Male approaches the female
FS-1a or 1b	First vibratory signal of female type a or b (for description see text)
MS-1	First vibratory signal of male (for description see text)
MS-2	Second vibratory signal of male (for description see text)
An	Antennation
FrejM	Female rejects the male
MrejF	Male rejects the female
Mp	Male pivoting, male moves in semi-circle around abdomen of female during antennation
MbF	Male beats female: Male puts head behind the female abdominal tip and beats her abdomen until she adopts the copulatory position
Fcoppos	Female adopts copulatory position. Female elevates the tip of the abdomen and stays immobile
Mcoppos	Male adopts copulatory position. Male goes down from the female and turns 180°; the insects are oriented end to end in copulatory position
Copulation	Pairs copulate
No copulation	No copulation, insects separate and walk away from one another

formed and evaluated under the same experimental procedures described above for conspecific couples on loudspeaker membranes.

Playback Experiments

Two types of playback experiments were conducted on the soybean plants. Stimulation programs were synthesized by Cool Edit Pro software using pre-recorded loudspeaker signals from 3 to 5 different individuals of each species. Sequences of pulses or pulses trains of each individual were mixed as required to build up each type of stimulation program. The stimulation vibrational signals were applied on the middle area of the central leaflet of a trifoliate leaf of a soybean plant by a entomological pin (Bioquip Insect Pins N° 2, Bioquip, Rancho Dominguez, CA, U.S.A.) fixed to the top of a vibration exciter (Mini-shaker Type 4810, Bruel & Kjaer, Naerum, Denmark). Horizontally positioned vibration exciter was mechanically isolated from the substrate by iron support coated with polyurethane foam.

Insects were placed individually (one male or one female of each species) on one of the opposite trifoliate leaf and stimulated with different stimulation programs. In the first experiment on the plants, males and females of each species were stimulated with four different stimulation programs that contained two types of female or male songs pre-recorded from the bugs singing on a loudspeaker membrane (see above). Each stimulation program lasted three minutes with one type of song spaced by 1 min of silence. The stimulation programs were played back during 20 min, using the loop play function of the Sound Forge software. The intensity of stimulation was adjusted to the level of the insects' emissions recorded on the loudspeaker. Ten males and ten females of each species were tested with each stimulation program (conspecific and heterospecific male and female signals). Within each test behavior of insects was monitored simultaneously with recording their vibratory emissions to correlate the number of insects that responded to stimulation.

In the second experiment males were stimulated by song programs composed of two types of con- and heterospecific female songs in a sequence of 3 min for each species spaced with 1 min of silence. The males of each species were stimulated with four different song programs. The first started with FS-1a of *C. ubica* followed by FS-1a of *C. impicticornis* and the second by FS-1a signals pre-recorded from females in reverse species sequence. The third program started with *C. ubica* FS-1b followed by the sequence of FS-1b signals of *C. impicticornis*. The fourth program contained the latter sequences in the reverse species order with *C. impicticornis* FS-1b signals in front. Specificity of male responses was determined by the number of male responses to each stimulation test sequence together with analysis of the impact of different stimulation signals on male vibratory response characteristics. For each type of stimulation program, a total of 10 males of each species were tested. The level of response was determined by the ratio of the number of pulses emitted by the tested male to the number of playback pulses.

Statistical Methods

Data from all recorded courtship sequences were used to create a first-order Markovian behavioral transition matrix of total frequency of transitions (i.e. moving from one

behavioral step to the next). The repetition of a single behavior (self-transition) was not included in the records to avoid the possible influence in the relative weight of transitions between behaviors. Transition probabilities were calculated from the observed frequency of a transition between two events divided by the total number of occurrences of the first event (Haccou and Meelis 1992). The expected values of the matrix cells were obtained by multiplying the total values of each column and row of the respective cell and dividing by the grand total of the matrix (total number of transitions observed) and the statistical significance of the individual transitions was evaluated by χ^2 test with significance adjusted by Bonferroni error correction. The results are shown graphically in an ethogram.

Signal parameters (pulse train duration, pulse train repetition time, pulse duration, pulse repetition time and dominant frequency) were compared using a linear mixed model, with species as fixed effects and individuals as the random variable, to consider the repeated measures of pulses or pulses trains made in each individual. The level of significance for the fixed variance was obtained per convergence of Markov chain Monte Carlo test. To compare the number of pulses by pulse train mixed generalized linear models were used; the model considered species as fixed effects and individuals as the random variable and included Poisson error distribution. All analyses were performed in R software, version 3.0.1, using the libraries lme4, languageR and zipfR.

To analyse if FS-1 or MS pulse train signals could be differentiated between species and type (in the case of FS-1a and FS-1b) by their temporal and spectral components, principal component analyses (PCAs) were performed using the correlation matrix as input and mean values of 5 to 10 individuals of each species or signal type. MS2 signals were not included in analyses because of the irregular pattern of emission. PCA analyses were performed using the software PAST version 2.17 (Hammer et al. 2001, <http://folk.uio.no/ohammer/past>).

For all analyses considering temporal and spectral parameters of signals only insects that maintained each type of signal emission for more than 1 min and signals not superimposed with those of the partners were considered.

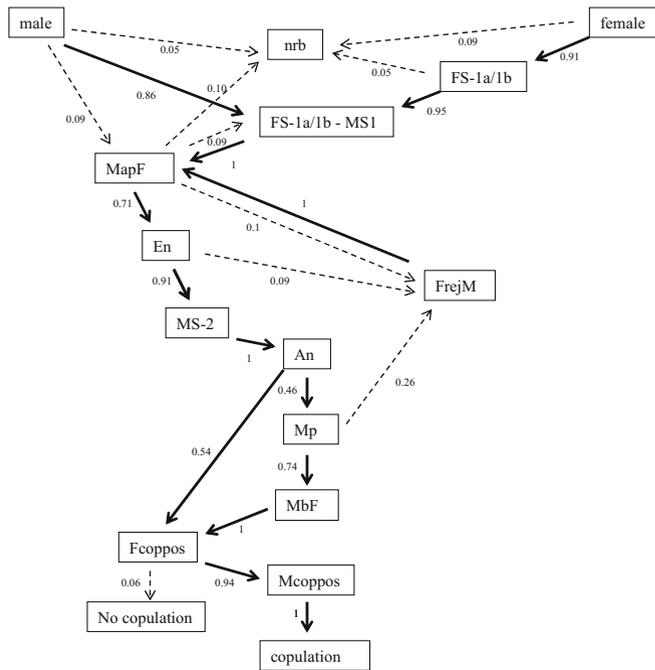
Mean proportion of responses of insects of each species and sex were calculated from parameters of logistic regression using signals as a factor and binomial responses (+ or -) as the response variable. Additionally, to study if the responses to heterospecific signals are similar to those observed in conspecific interactions, odds ratios (chance of response to heterospecific signal in relation to conspecific) and their confidence intervals (95 %) were calculated. The ratio of the number pulses emitted by each insect to the number of pulses of the played-back signals of the stimulation programs was analysed using beta regression considering species and first signal in the sequence as factors.

Results

Reproductive Behavior and Vibratory Signals Emission

In the loudspeaker arena conspecific couples of both species show stereotyped transition sequences of behavior (Fig. 1), with similar behavioral categories and transition sequences. Copulation was recorded in 52 of 64 (81.25 %) *C. impicticornis* and in 41 of 54 (76 %) *C. ubica* couples.

Chinavia impicticornis



Chinavia ubica

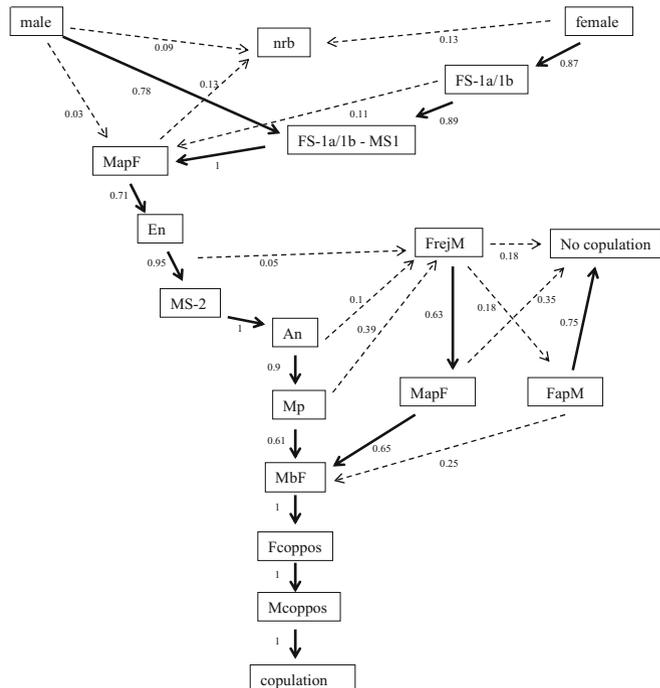


Fig. 1 Sequence of courtship and mating behavior of *Chinavia impicticornis* and *Chinavia ubica* couples. Values at left or below lines represent the probability of transitions between behaviors. Solid-line arrows indicate the significant transitions ($P < 0.05$) and dashed-line arrows the non-significant transitions ($P > 0.05$). Boxes represent behavioral categories. FS-1a = female vibratory signal 1a, FS-1b = female vibratory signal 1b, MS-1 = male vibratory signal 1, MS-2 = male vibratory signal 2. Codes for behavioral categories are listed in Table 1

One female and two different male vibratory songs were identified during different behavioral steps of mating behavior in *C. impicticornis* and *C. ubica*. The behavioral context of the song repertoire did not differ in the two species: females started vibratory communication with the female song FS-1 that triggered male response with the first song (MS-1) that silenced the female by transition to the second male song (MS-2) as typical emission leading to copulation (Fig. 1).

Most of the females observed in closed arenas initiated the reproductive sequence by spontaneous emission of FS-1 signals (91 %, 58/64 in *C. impicticornis* and 87 %, 46/54 in *C. ubica*). FS-1 triggered the emission of MS-1 (86 % of *C. impicticornis* and 78 % of *C. ubica*) forming the duet that, in sequence, initiated directional movement of the male towards the female's position (Fig. 1). Antennation phase characterized by male emission of the MS-2 started when the male reached the female at the distance that enabled physical contact between mates, in many cases (40 of 52 couples (76.92 %) in *C. impicticornis* and 28 of 41 (68.3 %) in *C. ubica*) the emission of these signals was maintained during pivoting and kicking steps until copulation. During these steps female rejection of a male occurred in most cases (Fig. 1). If the male was not rejected mates adopted the copulatory position and copulation started. The most evident difference between species in the courtship phase of reproductive behavior was the higher tendency of *C. impicticornis* females to adopt the copulatory position directly after antennation (Fig. 1). Similar behaviors were observed in couples mating on plants.

Signal Characteristics

Signals recorded from the loudspeaker membrane or plants showed similar characteristics. We show data on the temporal and spectral parameters of signals recorded on a plant with comparative data on parameters of signals recorded on the loudspeaker membrane presented in on-line resources as electronic supplementary material (Tables ES1 and ES2).

The FS-1 of both *Chinavia* species is composed of readily repeated pulse trains of two types (FS-1a and FS-1b) (Fig. 2). Both types may appear in the same sequence with FS-1b usually following FS-1a. The basic pattern of both FS-1 song types does not differ between species. Highly uniform pulses, pulse train duration and repetition time are characteristics of FS-1a. This signal is composed of a sequence of pulse trains with a higher number of pulses per pulse train, longer duration and a lower repetition rate in *C. impicticornis* compared with *C. ubica* (Table 2). The dominant frequency of plant recorded FS-1a signals ranges around 100 Hz, with lower values in *C. impicticornis* than in *C. ubica* (Table 2).

The second type of the female song (FS-1b) (Fig. 2) develops from FS-1a and shows in both species a characteristic pulse train pattern composed of a longer pulse followed by one (*C. impicticornis*) or from one to a few (*C. ubica*) short pulses, so FS-1b appears to develop from fusion of initial pulses of the FS-1a pulse train (Table 2). Similar

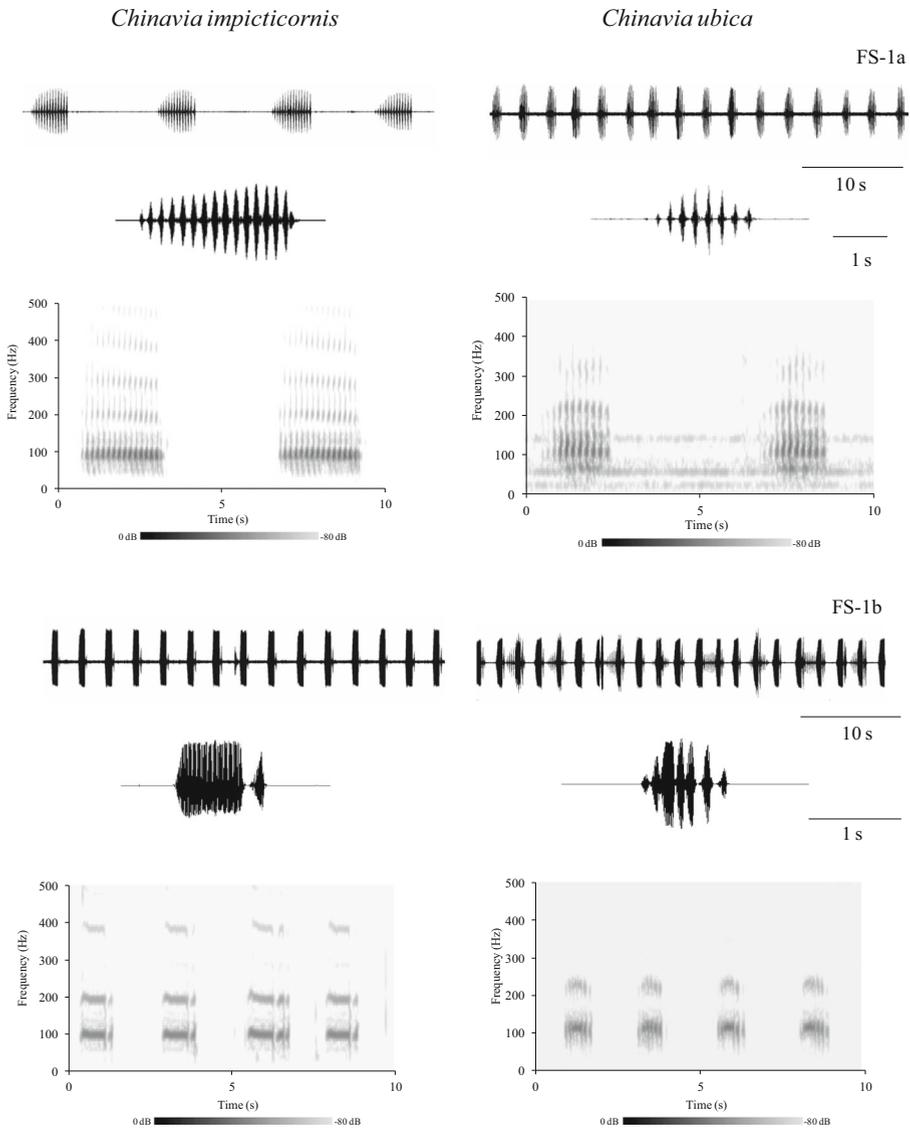


Fig. 2 Typical oscillograms and frequency spectra of *Chinavia impicticornis* and *Chinavia ubica* female songs. An oscillogram of a long sequence of pulse trains (upper), a pulse train showing in details the pulses components (middle) and a frequency spectrum of two to four consecutive pulses trains (inferior). In *Chinavia ubica* FS-1b it is possible to observe some pulses of MS-1 of lower amplitude

differences, as those found in FS-1a, in time parameters and dominant frequency were found when compared *C. impicticornis* with *C. ubica* FS-1b (Table 2).

Males of both species respond to FS-1a and FS-1b by the emission of the first male song (MS-1) pulse trains. In characteristic female-male duets MS-1 pulse trains alternate in a regular 1:(1–3) fashion (Fig. 3). In *C. impicticornis* prolonged MS-1 pulse trains may overlap the following FS-1 signal. In *C. ubica* duration of MS-1 pulse trains is always constrained to the interval between two FS-1 signals. In both species we have

Table 2 Means (\pm SD) of temporal and spectral parameters of *Chinavia impicticornis* and *Chinavia ubica* plant recorded female songs

		<i>Chinavia impicticornis</i>	<i>Chinavia ubica</i>	Statistics	
FS-1a	Pulse train	Duration (ms)	3769.84 \pm 436.07 (N = 6, n = 57)	2359.13 \pm 596.75 (N = 10, n = 97)	$t_{154} = 4.70$ $P < 0.001$
		Repetition time (ms)	10,902.16 \pm 1737.99 (N = 6, n = 57)	4906.20 \pm 1547.36 (N = 10, n = 97)	$t_{154} = 5.52$ $P < 0.001$
		DF (Hz)	92.54 \pm 2.44 (N = 6, n = 57)	108.43 \pm 2.91 (N = 10, n = 97)	$t_{154} = 15.78$ $P < 0.001$
		N° Pulses/ pulse train	15.46 \pm 1.77 (N = 6, n = 57)	12.06 \pm 2.84 (N = 10, n = 97)	$Z_{154} = 2.96$ $P = 0.003$
		Duration (ms)	215.73 \pm 34.85 (N = 6, n = 150)	167.29 \pm 43.97 (N = 10, n = 148)	$t_{298} = 4.27$ $P = 0.002$
FS-1b	Pulse train	Repetition time (ms)	232.53 \pm 29.63 (N = 6, n = 150)	196.17 \pm 54.02 (N = 10, n = 148)	$t_{298} = 4.90$ $P = 0.004$
		Duration (ms)	1068.20 \pm 182.81 (N = 9, n = 105)	1274.94 \pm 225.70 (N = 5, n = 52)	$t_{157} = 3.08$ $P = 0.001$
		Repetition time (ms)	3088.15 \pm 352.48 (N = 9, n = 105)	2836.33 \pm 770.56 (N = 5, n = 52)	$t_{157} = 1.80$ $P = 0.07$
		DF (Hz)	81.46 \pm 2.86 (N = 9, n = 105)	110.46 \pm 2.62 (N = 5, n = 52)	$t_{157} = 20.45$ $P < 0.001$
		N° Pulses/ pulse train	2.54 \pm 0.99 (N = 9, n = 105)	3.21 \pm 1.11 (N = 5, n = 52)	$Z_{157} = 12.40$ $P < 0.001$
First Pulse	Duration (ms)	707.84 \pm 243.55 (N = 4, n = 44)	841.39 \pm 132.63 (N = 3, n = 33)	$t_{77} = 1.00$ $P = 0.25$	
	Repetition time (ms)	241.11 \pm 63.16 (N = 4, n = 44)	898.30 \pm 136.65 (N = 3, n = 33)	$t_{77} = 21.09$ $P < 0.001$	

References: N = number of individuals, n = number of pulse trains or pulses. Mean values between species were compared using mixed linear models and GLMM for number of pulses/ pulse train. DF = dominant frequency

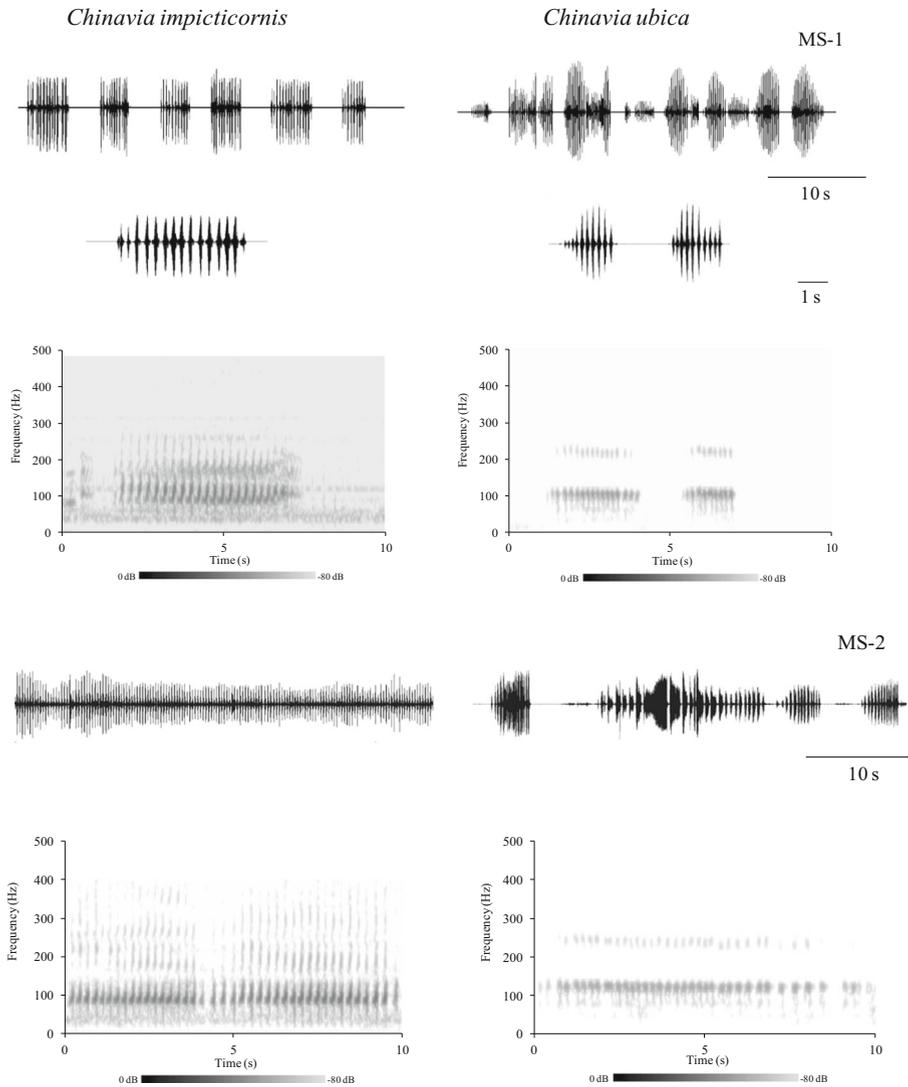


Fig. 3 Typical oscillograms and frequency spectra of *Chinavia impicticornis* and *Chinavia ubica* male songs. An oscillogram of a long sequence of pulse trains (MS-1) or pulses (MS-2) (upper), a pulse train showing in details the pulses components (middle, only for MS-1) and a frequency spectrum of one (*C. impicticornis*) and two consecutive pulse trains (*C. ubica*) and several individual pulses of MS-2 (inferior). In *C. ubica* MS-1 it is possible to observe some pulses of FS-1 of lower amplitude

recorded emission of MS-1 signals also after the female stopped singing and in the silent period after play-back stimulation (see playback experiments). MS-1 pulse trains of both species have a similar time pattern but differ mainly in the pulse train duration and number of pulses per pulse train (Table 3).

Like in FS-1 the dominant frequency of *C. impicticornis* MS-1 is lower compared with that one of *C. ubica* and duration and repetition time values are longer in pulses of

Table 3 Means (\pm SD) of temporal and spectral parameters of *Chinavia impicticornis* and *Chinavia tubica* plant recorded male songs

		<i>Chinavia impicticornis</i>	<i>Chinavia tubica</i>	
MS-1				
Pulse Train	Duration (ms)	4966.07 \pm 785.24 (N = 8, n = 45)	2274.39 \pm 739.15 (N = 8, n = 67)	$t_{112} = 10.12$ $P < 0.001$
	DF (Hz)	92.13 \pm 5.30 (N = 8, n = 45)	108.01 \pm 7.90 (N = 8, n = 67)	$t_{112} = 5.95$ $P < 0.001$
	Pulses/ pulse train	19.53 \pm 3.15 (N = 8, n = 45)	10.82 \pm 2.90 (N = 8, n = 67)	$Z_{112} = 6.95$ $P < 0.001$
Pulse	Duration (ms)	211.87 \pm 30.69 (N = 4, n = 173)	177.70 \pm 25.67 (N = 4, n = 214)	$t_{387} = 4.93$ $P = 0.004$
	Repetition time (ms)	245.49 \pm 48.68 (N = 4, n = 173)	206.69 \pm 27.40 (N = 4, n = 214)	$t_{387} = 2.86$ $P = 0.02$
MS-2				
Pulses	Duration (ms)	211.51 \pm 23.19 (N = 4, n = 120)	405.46 \pm 25.39 (N = 4, n = 149)	$t_{269} = 2.04$ $P = 0.005$
	Repetition time (ms)	265.13 \pm 36.46 (N = 4, n = 120)	581.07 \pm 47.78 (N = 4, n = 149)	$t_{269} = 3.01$ $P = 0.012$
	DF (Hz)	100.50 \pm 3.00 (N = 3, n = 3)	116.00 \pm 11.46 (N = 4, n = 4)	$F_{1,6} = 6841$ $P = 0.04$

References: N = number of individuals, n = number of pulse trains or pulses. Mean values between species were compared using mixed linear models and GLMM for number of pulses/pulse train. DF = dominant frequency. DF of MS-2 was estimated as mean value of a sequence of 20 to 30 individual pulses of each individual and mean values of species were compared using ANOVA test

C. impicticornis compared with *C. ubica* (Table 3). Mainly in the pulse train duration and number of pulses per pulse train (Table 3).

Prior to copulation males change MS-1 into the MS-2 song that silences the female. The well-structured MS-1 pulse train song pattern changes in *C. impicticornis* to a minute-long sequence of readily repeated pulses of unique duration and velocity that cyclically varies along the sequence (Fig. 3, Table 3). A similar pattern is characteristic also for *C. ubica* males MS-2 pulses of that differ mainly by their longer duration, repetition time and lower velocity (Fig. 3, Table 3) that does not show regular variation as shown in *C. impicticornis*. The individual minimal and maximal dominant frequency values of 10 s long sequences were 97 and 104 Hz in *C. impicticornis* and 104 and 135 Hz in *C. ubica*.

Results of PCA analyses for FS-1 pulse train signals showed a clear separation between species and type (1a or 1b) signals. The first component of PCA that explains 66.99 % of total variability was defined principally by temporal parameters of signals and the second component (24.86 % of variability) was defined by dominant frequency (Fig. 4). These results confirm that *C. ubica* FS-1 signals differ from those of the sympatric *C. impicticornis* species by their temporal and spectral (dominant frequency) components and that FS-1a type differs from FS-1b by different pulse duration, repetition time and number of pulses per pulse train (Fig. 4). PCA analyses of the parameters of MS-1 pulse train signals show clear separation between individuals of both species. As for female signals, the first component of PCA that explains most of the observed variability (84.73 % of total variability) was defined principally by temporal parameters of signals and the second component (10.28 % of variability) was defined by the dominant frequency. Differentiation between species is principally obvious in different pulse train temporal parameters being higher in *C. impicticornis* males (Fig. 4).

Heterospecific Couples Vibrational Communication and Behavior

In heterospecific couples most females of both species (60 %, 15/25) spontaneously emitted FS-1. Males did not show regular responses to these signals: only 43 % (7/15) of *C. impicticornis* and 53 % (8/15) of *C. ubica* males responded with MS-1 signals in duet with heterospecific female FS-1 emission (Fig. 5). These duets lasted for a short time from few seconds to one or two minutes. Fifty percent of *C. ubica* female x *C. impicticornis* male and 37 % of *C. ubica* male x *C. impicticornis* female heterospecific couples showed no further reproductive behavior after the duet phase. Those males that preceded mating behaviour walked in the direction of the females and antennated them. Most of the females rejected copulation although males expressed copulatory position (Fig. 5). No heterospecific copulation was observed.

The proportion of responses to played-back conspecific and heterospecific signals were similar for females and males of each species when stimulated with signals of the other gender (Fig. 6). Odd ratio analyses showed no difference in the proportion of male and female responses to heterospecific signals in relation to conspecific signals (Table 4). The sequence of signals played-back does not show any significant effect on proportions of pulses related to number of pulses in playback signals (beta regression $z = -1.15$ $P = 0.25$ and $z = 1.11$ $P = 0.27$ for *C. ubica* males responding to FS1a and

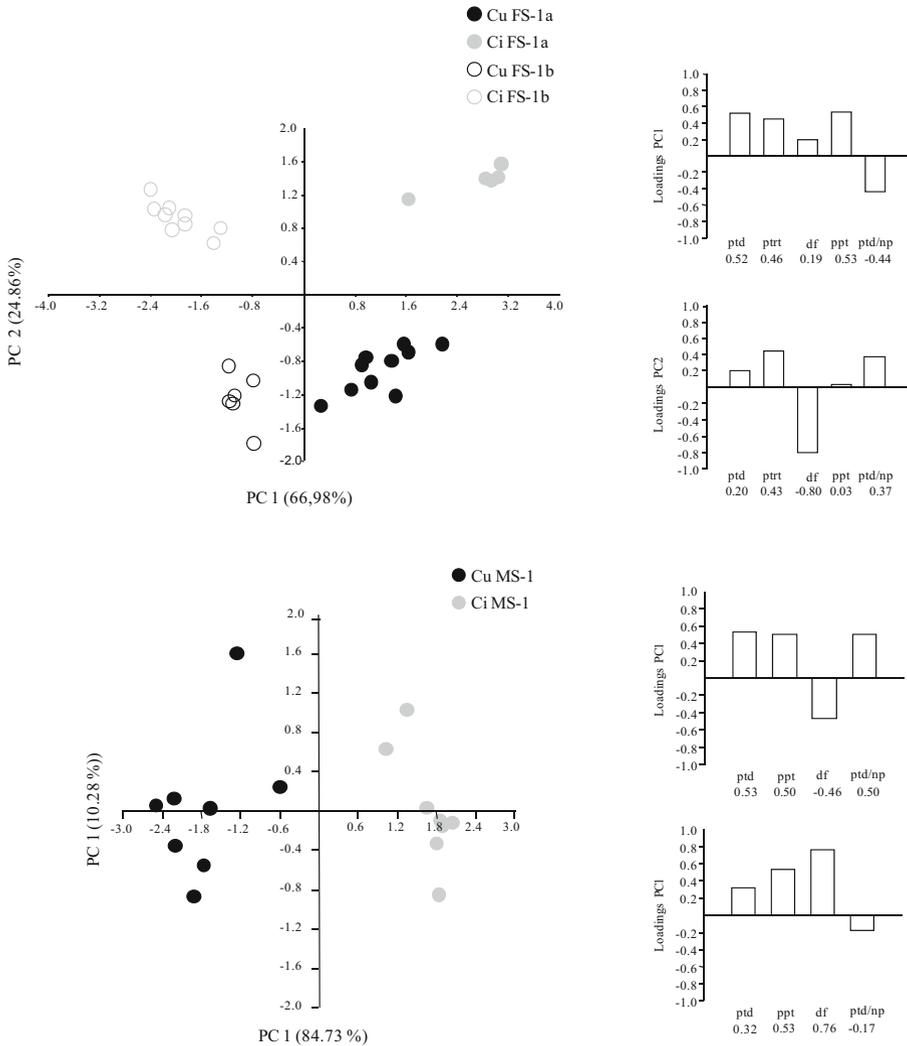
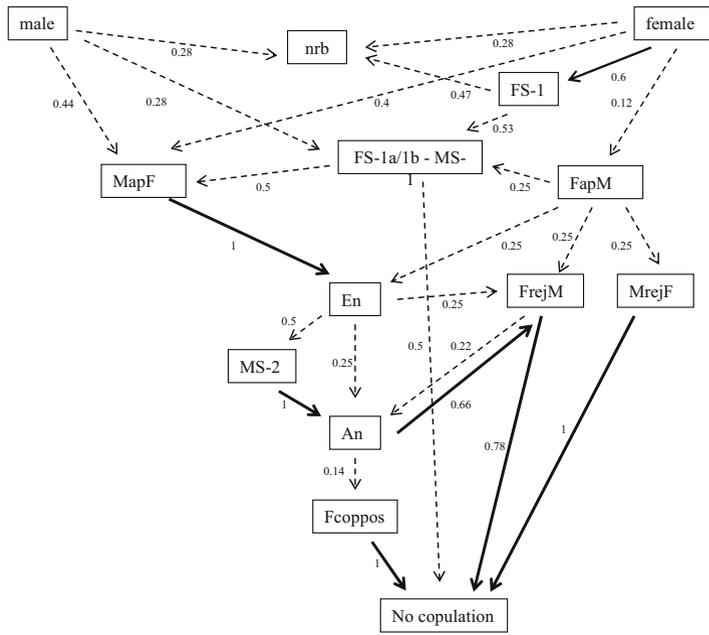


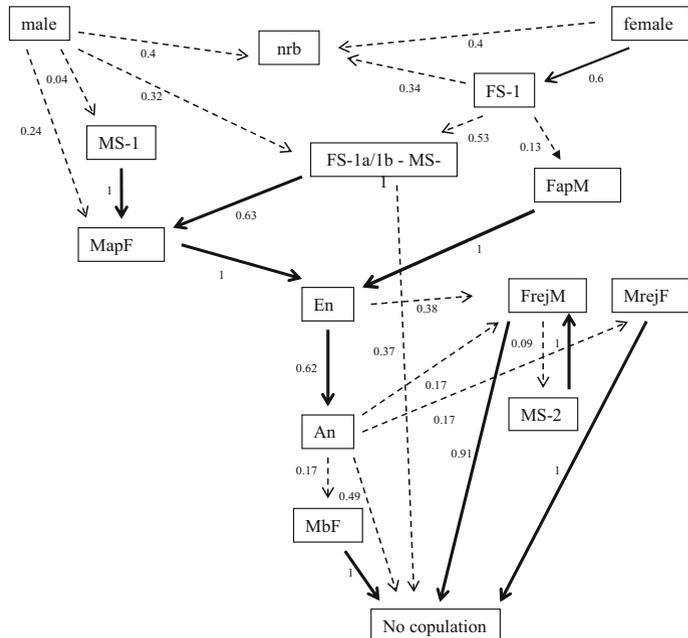
Fig. 4 PCAs for the pulse trains of the female (FS-1a and FS-1b, upper figure) and male (MS-1, lower figure) songs of *Chinavia impicticornis* (Ci) and *Chinavia ubica* (Cu). PCAs were performed using the correlation matrix of different variables measured from 5 to 10 individuals of each species/song. Figures at left show the loading of each variable for the first and second component, numbers between brackets show the % of total variation explained by each component. Ptd: pulse train duration, ptrt: pulse train repetition time, df: dominant frequency of the pulse train, ppt: number of pulses/pulse train

F1b respectively and $z = 0.93$ $P = 0.35$ and $z = -0.38$ $P = 0.71$ for *C. impicticornis* males responding to FS1a and F1b respectively). In contrast males of both species responded with higher proportions of pulses related to number of pulses in playback signals when conspecific female signals were played-back, with the only exception of *C. ubica* males when were stimulated with FS1a (beta regression $z = 0.41$ $P = 0.68$ and $z = 3.60$ $P < 0.001$ for *C. ubica* males responding to FS1a and F1b respectively and $z = -2.89$ $P = 0.004$ and $z = -2.70$ $P < 0.007$ for *C. impicticornis* males responding to FS1a and F1b respectively) (Fig. 7).

C. ubica females x *C. impicticornis* males



C. ubica males x *C. impicticornis* females



◀ **Fig. 5** Sequence of courtship and mating behavior of *Chinavia impicticornis* and *Chinavia ubica* heterospecific couples. Values at left or below lines represent the probability of transitions between behaviours. Solid-line arrows indicate the significant transitions ($P < 0.05$) and dashed-line arrows the non-significant transitions ($P > 0.05$). Boxes represent behavioural categories. FS-1a = female vibratory signal 1a, FS-1b = female vibratory signal 1b, MS-1 = male vibratory signal 1, MS-2 = male vibratory signal 2. Codes for behavioural categories are listed in Table 1

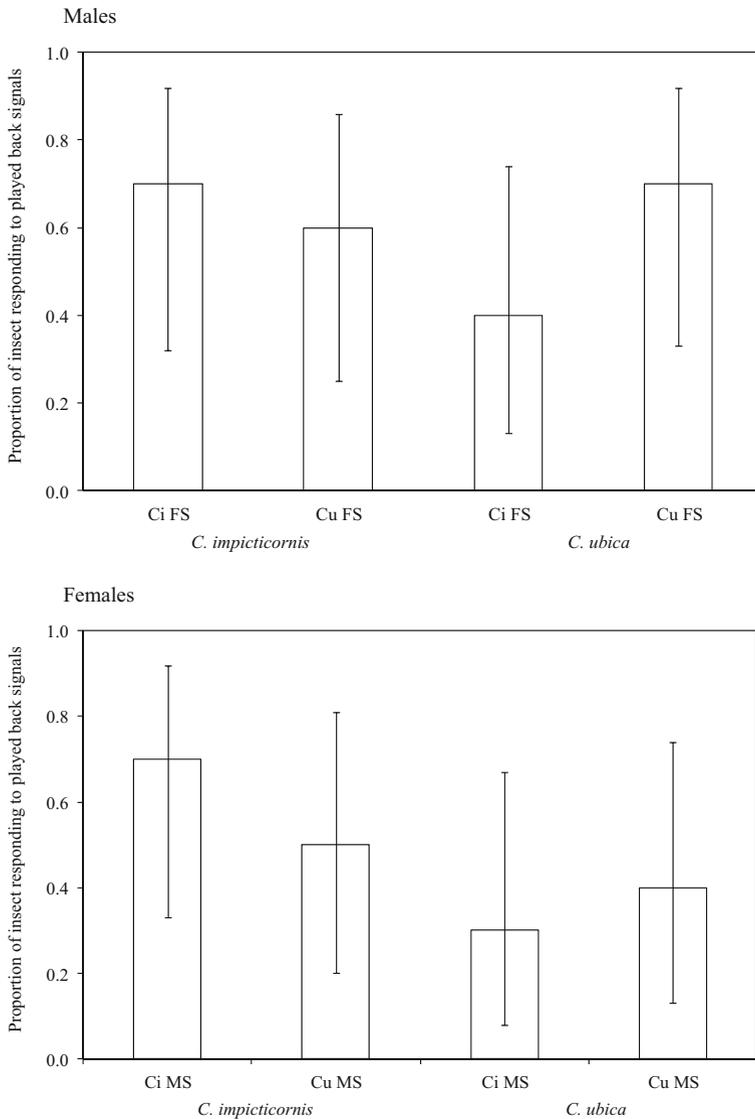


Fig. 6 Mean proportion of responses of male and females of *Chinavia impicticornis* and *Chinavia ubica* when stimulated by conspecific and heterospecific songs. Proportion of responses were calculated from back transform estimated parameter from a logistic regression and lines indicates 95 % CI calculated using the estimated parameter and standard deviation from logistic regression

Table 4 Probability of *Chinavia ubica* and *Chinavia impicticornis* females and males responding to heterospecific signals in playback experiments

Species/sex	Odds ratio	CI (95 %)	Significance
<i>C. ubica</i> females	0.64	0.10–4.10	$Z = 0.47 P = 0.64$
<i>C. ubica</i> males	0.28	0.04–1.83	$Z = 1.33 P = 0.18$
<i>C. impicticornis</i> females	0.43	0.07–2.67	$Z = 0.91 P = 0.37$
<i>C. impicticornis</i> males	0.64	0.10–4.10	$Z = 0.47 P = 0.64$

Discussion

Reproductive behavior and vibratory communication showed similar trends in the two *Chinavia* species studied. The sequence of mating behavioral steps (displays) was highly stereotyped in both species and the repertoire of vibratory communication songs was composed of signals with similar temporal and spectral characteristics. Notwithstanding, some differences observed in temporal and spectral characteristics of female and male signals enable species discrimination in multivariate analyses. Insects that responded to heterospecific vibratory signals did not step forward to behavioral steps

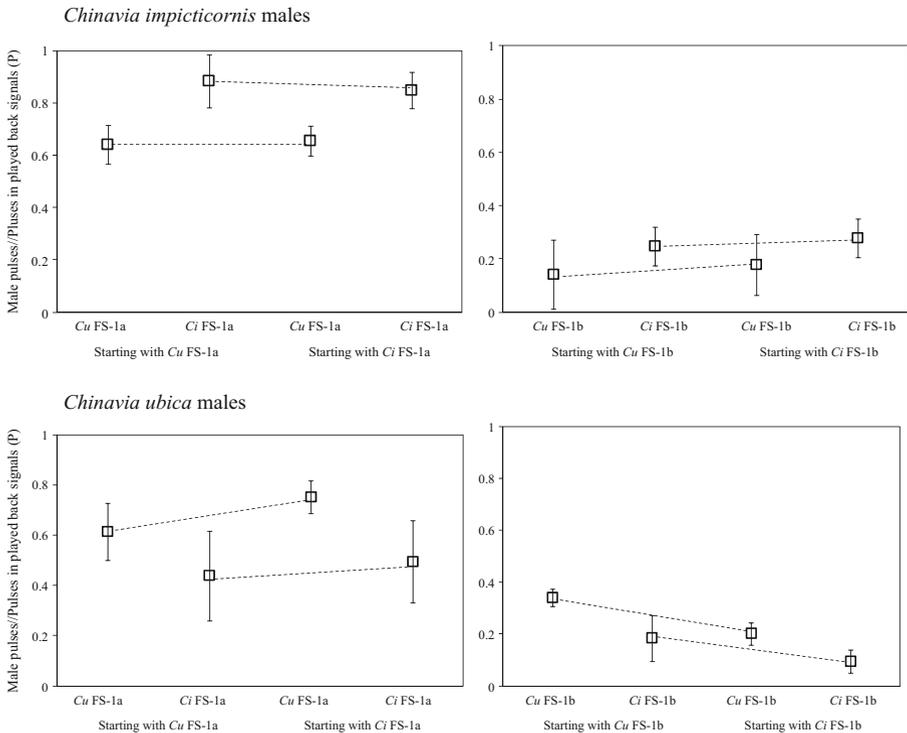


Fig. 7 Ratio of number of pulses of MS of *Chinavia impicticornis* and *Chinavia ubica* to number of pulses of played-back signals (FS1a and FS1b) in different sequences. See text for details of statistical tests

leading to copulation. Interspecific communication remained in all cases at the initial (calling) level and no heterospecific copulations were observed.

The repertoire of signals and their association with specific behaviors of both *Chinavia* species is similar to those reported for other Pentatomine stink bugs (Čokl and Virant-Doberlet 2003; Čokl 2008; Silva et al. 2012; Blassioli-Moraes et al. 2005, 2014) and the sequence of reproductive displays at close range is also similar to that reported previously for phytophagous and predatory stink bugs (Borges et al. 1987; Kon et al. 1988; Wang and Millar 1997; Zahn et al. 2008; Žunič et al. 2008; Silva et al. 2012; Laumann et al. 2013; Blassioli-Moraes et al. 2014).

C. impicticornis vibratory songs recorded with the laser vibrometer on loudspeaker membrane and on plants have similar characteristics to those analysed and described as FS-1 and FS-2 from records obtained directly from loudspeaker membrane by Blassioli-Moraes et al. (2005). Similar temporal and spectral characteristics of songs in the same behavioral context permit consideration that these signals are two types of the female calling song. FS-1a shows a well-structured pattern of temporal parameters with highly uniform pulses and pulse train duration and stable repetition time as typical for Pentatominae calling songs (Čokl and Virant-Doberlet 2003; Čokl 2008). On the other hand, FS-1b signals show higher variation in their temporal characteristics and are related with male signals in a duet, suggesting transition to courtship displays.

Species specific information based predominantly on temporal and frequency characteristics of *C. impicticornis* and *C. ubica* vibratory signals has also been shown in many other stink bug species (Blassioli-Moraes et al. 2005; Čokl 2008; de Groot et al. 2010; Žunič et al. 2011), as well as in other insect groups that communicate either with plant-borne signals, like planthoppers (Claridge et al. 1985, 1988) and treehoppers (Rodríguez et al. 2004, Rodríguez et al. 2006; Rodríguez and Cocroft 2006) or even in animals that use air bone sounds as bush-crickets (Dobler et al. 1994) and katydids (Forrest et al. 2006) or vertebrates as the frog *Xenopus levis* (Daudin, 1802) (Vignal and Kelley 2007).

In particular, for Pentatominae, de Groot et al. (2010) and Žunič et al. (2011) showed that *Nezara viridula* males recognize and respond to the female calling song based principally on pulse train duration and interval. Calling songs of *N. viridula* populations from different geographical origins (continents) have different time characteristics (Čokl et al. 2000; Miklas et al. 2003) and males respond preferentially to female songs of their own population (Miklas et al. 2003). Results of heterospecific duetting in *C. impicticornis* and *C. ubica* suggest that time parameters could be the responsible for the interruption of the initially started vibrational communication and prevents female male duetting, a prerequisite for successful copulation.

Although frequency characteristics of vibratory signals show differences between *Chinavia* species they are in the range of until now investigated pentatomine stink bugs, dominant frequencies around 100 Hz (Čokl et al. 2014). However, stink bugs show some variability of frequency characteristics as observed in signals from different geographically isolated populations (Čokl et al. 2000), in signals with different frequency modulation (McBrien and Millar 2003; Blassioli-Moraes et al. 2005) or frequency sweeps produced as response to environmental (Polajnar and Čokl 2008) or to biotic noise (Čokl et al. 2015). Frequency differences of female calling song signals in a broad range around the dominant frequency did not modulate significantly male responses in *N. viridula* (Žunič et al. 2011). Detailed playback experiments with signals of different time and frequency characteristics are needed in *Chinavia* species to determine their relevance in song recognition.

The stink bugs' vibratory signal repertoire shows similar components (calling, courtship and rivalry signals), with variation between species related to signal temporal and spectral compositions (Blassioli-Moraes et al. 2005; Čokl and Virant-Doberlet 2003; Laumann et al. 2013; Shestakov 2015). This suggests that from a basic signal species specific characteristics could be acquired by gradual changes in signal structure. The same pattern has been described in other insects as, for example, treehoppers (Cocroft et al. 2010), planthoppers (Den Bieman 1986) and crickets (Mendelson and Shaw 2002). These changes in signal systems could be related to biases in the signal emission, in the sensory and cognitive system or even in the behavior associated with the signalling (Endler 1993).

We can conclude that heterospecific mating between investigated *Chinavia* species stops at an earlier stage compared with sympatric *N. viridula* and *N. antennata* (Kon et al. 1988, 1993, 1994). Despite very different vibratory communication signals in the two *Nezara* species, the authors found inter-specific copulation, and this phenomenon was related to the straight range of sympatric distribution of the two *Nezara* species. Kon et al. (1988) stated that this could happen because in allopatric species sexual selection could lead to fast divergence of sexual signals independently of species recognition systems, a fact also observed in allopatric populations of the planthopper *Nilaparvata bakeri* (Muir) (Claridge and Morgan 1993). For the *Chinavia* species studied here, that are sympatric in almost their geographic distribution (Panizzi et al. 2000; Schwertner and Grazia 2007), differences on signals parameters efficiently prevent hybridization. It will be interesting in the future to study the signals and behavioral characteristics of allopatric populations, found in northern and southern limits of geographic distribution (Schwertner and Grazia 2007), of these species to have a complete picture about signals evolution and species recognition systems in stink bugs.

The prezygotic isolation observed in very close *Chinavia* species appear to be based on specificity of pheromone blends (Blassioli-Moraes et al. 2012) and vibratory signals (data presented here) and agree with the hypotheses of phenotypic architecture influence on behavioral reproductive isolation (Mendelson and Shaw 2012). As the ultimate consequence of signals differences is preventing hybridization and gene flow between species, sexual communication could have a central role in speciation (West-Eberhard 1983; Mendelson and Shaw 2012). To test this hypothesis in stink bugs a more detailed study including several species, their known phylogeny and their repertory of signals need to be conducted in the future.

For a full knowledge of the function of species-specific courtship and mating signals and their contribution to communication through a private intraspecific channel one needs to relate the structure and characteristics of signals with the mate preferences (Ritchie 1996, 2007; Ritchie et al. 1999; Mendelson and Shaw 2012). In this way, further experiments are needed to identify and evaluate the decisive role of duration and/or repetition time of signals pulse and pulse train characteristics in discrimination and preferences of conspecific signals by the sympatric *Chinavia* species.

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