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Integrating multiple criteria for the characterization of *Psammotettix* populations in European cereal fields

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Abstract

The wheat dwarf disease is among the most damaging diseases in cereals. Its aetiological agent is the *Wheat dwarf virus* (WDV), which is exclusively transmitted from plant to plant by leafhoppers from the genus *Psammotettix* (Hemiptera, Cicadellidae). The parameters linked to the WDV/*Psammotettix* pathosystem are still poorly understood. We studied *Psammotettix* individuals collected in wheat and barley fields in France and, as a comparison, from grassland at agroecological interface in West Slovenia. Species identity of males and females has been determined using multiple criteria. In the first step, the characterization of the collected individuals included recordings of vibrational signals used in mating behaviour and morphometric analyses. In addition, a 442 nt sequence of the mitochondrial cytochrome oxydase I (COI) gene was obtained for some individuals and compared to COI sequences of the *Psammotettix* leafhoppers available in public databases. In the cereal fields in France, *Psammotettix alienus* was the most numerous species; however, it sometimes occurred together with *Psammotettix confinis*, while in the grasslands in Slovenia, the third syntopic species in *Psammotettix* community was *Psammotettix helvolus*. The temporal parameters of the *P. alienus* male calling song that were measured in this study were very similar to those measured in a previous study. The local biotic and/or abiotic parameters most likely influence the life history of *Psammotettix* leafhoppers, and the proportion of viruliferous individuals collected in cereal fields was 14.9%, while leafhoppers collected in Slovenia were virus-free. Taken together, results show that more detailed information on population structure of *Psammotettix* leafhoppers is crucial for providing an insight into the epidemiology of wheat dwarf disease.

Keywords: *Psammotettix alienus*, *Wheat dwarf virus*, morphometric measurements, Cytochrome oxydase I, vibrational signals

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Introduction

Leafhoppers, hemipteran insects from the family Cicadellidae, are among the most important vectors of viruses (Hogenhaut *et al.*, 2008) and phytoplasmas (Weintraub & Beanland, 2008) that cause plant diseases. Cereals, worldwide one of the most important crops for both human and animal food, can be infected by different pathogens of which the

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Wheat dwarf virus (WDV, a well-described viral species belonging into the genus *Mastrevirus* of the family *Geminiviridae* (ICTV Report, 2012)) is the causal agent of dwarfing, mottling and yellowing symptoms in cultivated cereals (Vacke, 1972). The wheat dwarf disease (WDD), described for the first time in the 1960s in a wheat (*Triticum aestivum* L.) field located in the western part of the former Czechoslovak Socialist Republic (Vacke, 1961), is among the most important sanitary issues in wheat and barley. WDV is exclusively transmitted from plant to plant by leafhoppers from the genus *Psammotettix* (Hemiptera, Cicadellidae, Deltocephalinae) – holartic insects commonly found in cereal fields and in grassland (Raatikainen & Vasarainen, 1973; Lindblad & Areno, 2002). So far, it has been demonstrated that only two out of the numerous *Psammotettix* species have the capability to efficiently transmit WDV. *Psammotettix alienus* (Dahlbom) has been reported to be the main WDV vector (Zhang *et al*., 2010) and *Psammotettix provincialis* (Ribaut) has been described in a single report to be able to transmit WDV (Ekzayez *et al*., 2011). However, the WDV/*Psammotettix* pathosystem is still poorly documented in the literature (reviewed in Abt & Jacquot, 2015) and, in particular, leafhopper species used in WDV studies are often not properly described. Due to the lack of genetic resistance sources against WDD and the absence of antiviral molecules, currently the main protection strategy used against this disease is based on the use of chemicals against leafhopper vectors, and consequently, an observation of *Psammotettix* individuals in a cereal field commonly leads to insecticide sprays. However, it is likely that following the general observation of *Psammotettix* leafhoppers in the cereal fields, our current insufficient understanding about the structure of *Psammotettix* communities and about the efficiency of different *Psammotettix* species in WDV transmission, may often lead to unwarranted insecticide treatments. To prevent excessive use of chemicals, it is important that field technicians are able to accurately determine *Psammotettix* species in order to ascertain whether WDV vectors are actually present in cultivated areas. Such knowledge would be advantageous in adapting pest management strategies in order to minimize the use of insecticide treatments against WDD in cultivated cereal crops.

There are different alternative methods to describe the general and the specific characteristics of organisms, from basic morphological observations to molecular typing. The choice of the most appropriate approach(es) depends on several factors : (i) the aims of the study (biodiversity, ecology, pest management); (ii) the number of samples to be identified, (iii) the acceptable delay between the collection and accurate identification; and (iv) the current knowledge on the studied organism itself. Description of the diversity of organisms is at present in the centre of interest not only for systematists, but also for ecologists, population biologists, pathologists, ethologists as well as for medical and agricultural entomologists (Bortolus, 2008; Schlick-Steiner *et al*., 2010). While morphological characteristics can be easily used to distinguish members of the genus *Psammotettix* from other leafhoppers (Vilbaste, 1982; Della Giustina, 1989), species identification in this genus is challenging even for trained experts. Indeed, assignment of *Psammotettix* nymphs and females to species based on morphological characters is currently not possible. *Psammotettix* species are identified primarily on male genital morphology (Biedermann & Niedringhaus, 2009); however, due to high variability of aedeagus morphological characters, delimitation of *Psammotettix* species is often not reliable (Tishechkin, 1999). To improve the accuracy of species identification of *Psammotettix* individuals, morphometric parameters can be combined with other approaches, such as vibrational signals used in sexual communication or the polymorphism of the cytochrome oxydase I (COI) (Bluemel *et al*., 2014). According to our knowledge and data available in public databases, vibrational signals emitted by *Psammotettix* leafhoppers during sexual communication were described only in few publications (Tishechkin, 1999, 2000, 2014; Derlink *et al*., 2016). Moreover, phylogenetic analysis of DNA barcode has been so far applied to *Psammotettix* only twice and with a limited number of individuals and species sampled in Canada, Japan and Korea (Kamitani, 2011; Gwiazdowski *et al*., 2015) and did not include a single *P. alienus* specimen. Consequently, *Psammotettix* individuals used in published studies (e.g. on wheat dwarf pathosystem) are poorly described by authors and, due to the complex taxonomy of *Psammotettix* species and their assignment to species is often questionable. This could lead to contradictory information and conflicting results, especially for those linked to the role of the *Psammotettix* species in the epidemiology of WDD.

In the present study, we provide the information on the structure of *Psammotettix* populations in the cereal fields and agroecological interface (Alexander *et al*., 2014), as well as on life history and viruliferous status of field-collected individuals. To reliably assign the *Psammotettix* leafhoppers to species, we used multiple criteria (iterative approach sensu Yeates *et al*., 2011), including ecology, vibrational communication data, morphometric measurements (body and aedeagus) and molecular analyses (i.e. COI sequences). Integrating several sources of taxonomic information (biological, morphological, molecular, behavioural and ecological data) into the description processes applied to unassigned individuals should improve the quality of taxonomic data, and such approach is generally considered the most reliable method for delimiting species (Schlick-Steiner *et al*., 2010).

Materials and methods

Plants and insects

In all experiments, the spring wheat *cv. Sunstar* (Posadas & Henry, 2002) and the barley *cv. Express* (Sadeghi *et al*., 2000) were used as host plants for both viruses and insects. Seeds were sown in plastic tubes containing vermiculite. Plants were grown in a temperature-controlled chamber at 24°C with a light/dark period of 16/8 h. At the two-leaf stage, the young plants were transferred in the growth chamber (24°C during the day/20°C during the night, 40% RH and day/night periods of 16/8 h) and used in the experiments.

Adult leafhoppers from the genus *Psammotettix* were collected using a sweep net in French cereal fields in June and September 2013. Sampling was done at different locations in the South (15 fields, 2–49 insects/field) and in the North (19 fields, 11–50 insects/field) of France (table 1). Insects were directly observed in the sweep net after its use for 1 min at different locations in the fields. Immediately after the collection of leafhoppers in the field, each insect was individually transferred on two-leaf stage cereal plantlets (one wheat and one barley) and covered by a microperforated cellophane bag (fig. 1). Such individual rearing systems were transferred to the temperature-controlled chamber (40% RH; day/night periods of 16/8 h; 24 and 20°C for day and night, respectively) where the field-collected leafhoppers (F_0 individuals) were maintained on their host plants until they died.

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Table 1. Leafhoppers collected in France and Slovenia during field surveys carried out in 2013 and 2014.

					Collected insects						
								Gravid[4]			
Country	Date of collection	Region	Field location	GPS coordinates	Male	Female	Ratio[3]	Nb	P	Progenies[1]	WDV[2]
France	June 2013	Aude	Moux	N 43°11.291/E 2°38.643	0	4	/	2	0.5	3 (2–4)	NT
			Aigues vives	N 43°13.563/E 2°38.123	1	5	0.2	4	0.8	7.5 (5–10)	NT
			Marseillette	N 43°12.155/E 2°32.132	24	20	1.2	18	0.9	15 (1–65)	NT
			Carcassonne	N 43°12.056/E 2°18.889	3	7	0.42	6	0.85	16 (1–34)	NT
			Villasavary	N 43°13.834/E 2°03.103	0	3	/	2	0.66	1	NT
			Payra sur l'hers	N 43°15.672/E 1°52.774	1	1	1	1	1	0	NT
			Lagarde	N 43°20.656/E 1°41.258	6	7	0.85	2	0.28	5.5 (2–9)	NT
		Haute-	Auragne	N 43°24.275/E 1°29.875	12	4	3	1	0.25	3	NT
		Garonne	Villenouvelle	N 43°26.667/E 1°40.780	23	26	0.88	10	0.38	3.75 (1–12)	NT
		Herault	Montferrier le L.	N 43°40.182/E 3°51.400	11	20	0.55	17	0.85	11.66 (2–26)	NT
			St Gély du Fesc	N 43°42.390/E 3°48.497	3	9	0.33	9	1	4.57 (1–15)	NT
			Les Matelles	N 43°42.390/E 3°48.497	1	3	0.33	0	0	0	NT
			Assas	N 43°42.870/E 3°53.406	12	27	0.44	15	0.55	5 (1–24)	NT
			St Mathieu de T.	N 43°46.889/E 3°52.223	4	10	0.4	5	0.5	14 (1–33)	NT
			Valflaunes	N 43°48.140/E 3°52.444	11	7	1.57	4	0.57	4 (1–6)	NT
	Sept. 2013	Haute	Villiers le sec	N 48°04.618/E 4°54.484	4	7	0.57	1	0.14	4	0
		Marne	Villiers en lieu	N 48°39.095/E 4°54.484	19	31	0.61	23	0.74	9.52 (2–22)	0
			Vaux sur Blaise	N 48°23.510/E 4°58.439	20	30	0.66	14	0.46	6.07 (1–15)	2
			Vraincourt	N 48°13.839/E 5°07.121	21	29	0.72	6	0.21	3.33 (1–8)	2
			Orges	N 48°04.488/E 4°56.191	19	31	0.61	18	0.58	8.22 (1–19)	0
		Aube	Fontaines	N 48°13.053/E 4°42.804	20	28	0.71	19	0.68	8.52 (2–18)	0
			Villenereuill	N 48°12.140/E 4°05.960	21	28	0.75	16	0.57	6.75 (1–20)	8.2
			St Marlin de B.	N 48°25.932/E 3°40.288	4	11	0.36	5	0.45	5.8 (2–10)	0
			Bucey en othe	N 48°15.306/E 3°52.533	18	32	0.56	17	0.53	5.29 (1–16)	20
		Yonne	Evry	N 48°16.138/E 3°15.063	19	31	0.61	20	0.64	5.15 (1–15)	4
			Sepeaux	N 47°55.877/E 3°13.591	19	31	0.61	11	0.35	6.27 (1–11)	10.4
			Looze	N 47°59.177/E 3°27.338	20	29	0.69	17	0.59	6.76 (1–16)	14.3
			Chitry	N 47°45.948/E 3°42.674	21	29	0.72	15	0.52	6.13 (1–18)	16.3
		Loiret	Dammarie en P.	N 47°35.040/E 2°53.832	17	27	0.63	5	0.18	6.4 (1–17)	0
		Cher	Vailly sur sauldre	N 47°27.541/E 2°39.352	20	30	0.66	16	0.53	5.68 (1–19)	10
			Brecy	N 47°07.541/E 2°35.260	21	29	0.72	13	0.45	4.69 (2–11)	26.5
			St Caprais	N 46°58.336/E 2°17.269	20	30	0.66	14	0.47	7.07 (3–15)	34
			Annoix	N 46°57.748/E 2°32.859	16	16	1	1	0.06	6	41.9
		Indre	La chapelle St L.	N 47°03.922/E 1°47.655	20	30	0.66	3	0.1	4.33 (2–10)	14.3
Slovenia	Aug. 2014	Ravnica	Ravnica (field #1)	N 5°08.322/E 3°99.272	24	14	1.71	0	0	/	0
			Ravnica (field #2)	N 5°09.102/E 4°00.593	17	24	0.71	4	0.16	9 (5–14)	0
				Total :	492	700	0.70	334	0.48	7.29 (2–50)	

NT, not tested.
[1]The mean size of progenies produced by gravid females is indicated for each sampling location. When more than a single progeny was obtained, the range of the number of individuals present in progenies is reported between brackets.
[2]Viral detection (PCR assay) carried out using total nucleic acids extracted from individual leafhoppers (proportion of viruliferous individuals).
[3]The presented sex ratio corresponds to number of males/number of females collected in the field.
[4]The number (Nb) and the proportion (P) of females able to lay egg(s)/to produce a progeny under individual rearing conditions used to maintain leafhoppers alive in the laboratory from their collection to their death.

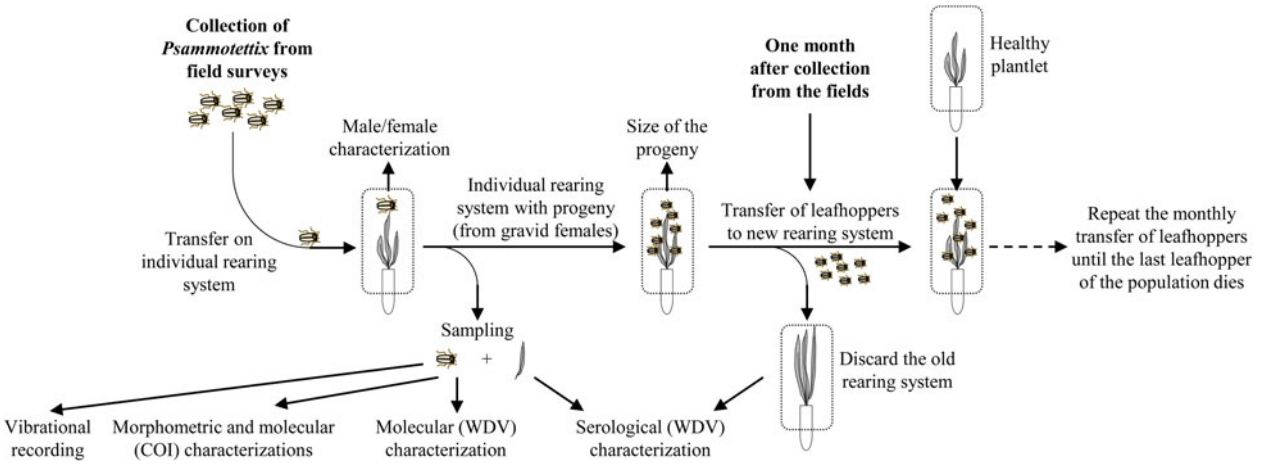

Fig. 1. Schematic representation of the process applied to the field-collected leafhoppers for the characterization of morphometric, molecular, serological and biological parameters.

Once in the laboratory facilities, the sex of collected insects was determined according to morphological differences at the apical part of the abdomen (Biedermann & Niedringhaus, 2009) and sex ratios were determined (table 1). The sanitary status (viruliferous for WDV) of the field-collected leafhoppers and their ability to transmit WDV was determined using appropriate molecular (polymerase chain reaction, PCR) or serological (enzyme-linked immunosorbent assay, ELISA) tools (see below). From the 15th to the 30th day after field collection, individual rearing systems with a female were daily monitored for the presence of eggs and larvae. The size of the F_1 progeny produced by each gravid female was recorded (table 1). After the 30th day, progenies (pool of leafhoppers, mainly larvae) were transferred to new individual rearing systems (progeny of one female per rearing system) to maintain the leafhopper stock as lineages. Such transfer of insects from old to new rearing systems (long-term maintenance of lineages) was repeated each month until the last insect of the lineage died (fig. 1). Dead leafhoppers were individually conserved in 96% ethanol and stored at −20°C until further analysis.

Adult leafhoppers (F_0) were also collected from grassland at agroecological interface in West Slovenia in August 2014 (table 1). These insects were individually transferred to plantlets and characterized (sex, gravid status and viruliferous status) as described above. However, the progenies produced by Slovenian gravid females were not introduced in the long-term maintenance procedure. Data associated with the characteristics of the leafhopper populations were statistically analysed with XLSTAT® software (Addinsoft, Paris, France) for MS Excel.

Detection of WDV in plant tissues using ELISA

The presence of WDV in wheat and barley plants used in individual rearing systems was determined using ELISA (Clark & Adams, 1977). Polyclonal antiserum raised against WDV (DSMZ, Germany) was used in double antibody sandwich ELISA procedures. The serological reagent (rabbit immunoglobulin IgG) was diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH = 9.6) according to the recommendations of the providers. Wells of microtitre plates (NUNC, Maxisorp) were coated 3 h at 37°C with 100 µl of diluted polyclonal antiserum. Between each step of the ELISA protocol, plates were washed three times with PBS buffer (137 mM NaCl, 8 mM Na_2HPO_4, $12H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH = 7.4) supplemented with 0.05% (v/v) Tween 20 (PBS-T buffer). Leaf samples (±0.5 g) were ground in the presence of PBS-T buffer (0.5 ml) supplemented with 2% (w/v) polyvinylpyrrolidone 40T (grinding buffer). Hundred microliters of the plant sap were then incubated in the coated wells overnight at 4°C. Then, 100 µl of a secondary antibody (rabbit IgG–alkaline phosphatase conjugated), diluted according to manufacturer's recommendations in grinding buffer supplemented with 0.2% (w/v) ovalbumine (conjugate buffer), were added in wells. After 3 h incubation at 37°C, plate wells were washed and filled with 100 µl of p-nitrophenyl-phosphate (1 mg ml^{-1}) diluted in substrate buffer (1 N, diethanolamine, pH = 9.8). After incubation at room temperature in the dark for 30 min to 2 h, the absorbance at 405 nm was recorded for each well using a microplate reader (Multiskan FC, Thermo Fisher Scientific, Waltham, Massachusetts, USA). A positive detection of WDV in tested sample was considered when the OD_{405} value is twofold greater than the OD_{405} value obtained for healthy control samples.

Recording of vibrational signals

To record *Psammotettix* vibrational signals, we used an experimental set-up described earlier (Derlink *et al.*, 2016). Briefly, a cv. *Sunstar* wheat plantlet growing in a plastic tube was positioned into the circular opening of a custom-made tripod. The hole was covered with paper surrounding the plant, and the plant above the paper platform was covered with a transparent plastic vial to prevent leafhoppers from escaping. Vibrational signals were registered with a laser vibrometer (PDV 100, Polytec GmbH, Waldbronn, Germany) from a small piece of reflective tape placed on the stem below the platform in order to increase reflectance. Signals were stored in a computer using a Sound Blaster Audigy 2 ZS sound card (Creative Labs, Milpitas, California, USA) and Cool Edit pro 2.0 software (Syntillium Software, Phoenix, Arizona, USA).

To record vibrational signals, a single leafhopper was placed on a plantlet and recordings lasted between 15 and

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60 min. To induce signalling, females were stimulated with pre-recorded male calling signals of *P. alienus*, *Psammotettix confinis* and *Psammotettix helvolus* taken from our library of recordings, since males of these species were found at our collecting sites. Species identity of *P. confinis* males was determined according to aedeagus morphology (see below), while preliminary determination of *P. helvolus* males was initially done according to gross body morphology and hyaline fore wings (Biedermann & Niedringhaus, 2009). Plant stem was vibrated with the conical tip of a 5 cm metal rod (4 mm in diameter) screwed firmly into a head of a vibration exciter (Minishaker type 4810, Büel & Kjaer, Naerum, Denmark) driven from the computer via the above mentioned sound card and the Cool Edit pro 2.0 program. The amplitude of stimulation was adjusted to the level of naturally emitted vibrational signals registered at the point of the recording. Recorded vibrational signals were analysed using Raven 1.4 (Cornel Laboratory of Ornithology, Ithaca, New York, USA) and Cool Edit pro 2.0 software (Syntrillium Software, Phoenix, Arizona, USA) at the sampling rate of 48 KHz and 16-bit resolution. To describe species- and sex-specific characteristics of *Psammotettix* vibrational songs, we determined basic temporal characteristics, i.e. pulse train duration and pulse train repetition time (Derlink *et al*., 2016). Pulse was defined as a unitary homogenous parcel of sound of finite duration (Broughton, 1963), pulse train was defined as a sound consisting of a group of pulses with a characteristic sequence or form, while we defined song as a bout of repeated pulse trains (Derlink *et al*., 2016).

Signals of more than 300 individuals have been recorded; however, the complete analysis of recorded signals has been carried out for 96 leafhoppers (58 F_0 individuals (46 males and 12 females) collected during field surveys, 36 virgin F_1 individuals (25 males and 11 females) produced by females gravid at collection time, and two F_2 individuals from mating experiments carried out under controlled condition in the laboratory using male and female from F_1 populations (Derlink *et al*., 2016)) (tables 2 and 3). The representative vibrational signals are provided as Supplementary material.

Non-destructive DNA extraction procedure applied to leafhoppers

Total nucleic acids were extracted from leafhoppers using a non-destructive procedure. Insects, stored in 96% ethanol, were rehydrated for 2 h in 70% ethanol, individually transferred on wells of a sterile microtitration plate and dried at room temperature. Each well was filled with 150 µl of TNES buffer (50 mM Tris, pH = 7.5, 400 mM NaCl, 20 mM EDTA and 0.5% SDS (w/v)) supplemented with 2 µl of proteinase K (20 mg ml^{-1}). The plate was incubated overnight at 55°C. After a short centrifugation step (1800 *g* for 2 min at 20°C), the 152 µl fractions were transferred in a clean deep-wells microtitration plate. The insect body, left at the bottom of the well of the first plate, was washed twice for 2 h in deionized water then stored at −20°C in the presence of 200 µl of 96% ethanol until it was used in morphometric measurement procedures (see below). Forty-five microlitres of cold 5 M NaCl were added to each well containing the 152 µl fractions and the mixture was gently homogenized. The plate was then centrifuged at 5000 *g* for 10 min at 4°C. The supernatants were transferred in a new deep-wells plate and 500 µl of cold absolute ethanol were added to each well. After 20 min at −80°C, the plate was centrifuged (5000 *g* at 4°C) for 10 min. The supernatants were discarded and wells were washed with 250 µl of 70% ethanol and dried at 30°C using the Mivac Duo concentrator (GeneVac Ltd., Ipswich, UK). Lastly, dried pellets were resuspended in 50 µl sterile water and stored at −20°C until used.

Table 2. Characterization of *Psammotettix* sp. calling songs.

	N	*n*	Pulse train duration (s)	*N*	*n*	Pulse train repetition time (s)
P. alienus females	19	250	0.45 ± 0.06	19	205	1.31 ± 0.10
P. alienus males	53	641	0.07 ± 0.01	53	517	1.12 ± 0.45
P. confinis males	5	106	0.04 ± 0.01	5	100	0.41 ± 0.09
P. helvolus males	14	293	0.14 ± 0.01	14	257	0.54 ± 0.09
P. helvolus females	3	17	7.58 ± 1.98	2	11	36.12
S-70	1	27	0.34	1	23	4.69
51B	1	30	1.84	1	20	3.30

N, number of individuals analysed; *n*, total number of signals analysed.
Means with standard deviations are shown.

Amplification, sequencing and molecular analysis

The presence of WDV in the total nucleic acids extracts from leafhoppers was checked using a PCR procedure. A 935 nt-long region of the WDV genome, corresponding to the 3′end of the coat protein gene, the short inter-genic region and the 3′end of the replicase genes (Rep/RepA), was amplified by PCR using 1.5 U of the GoTaq® flexi polymerase (Promega, Madison, Wisconsin, USA), GoTaq green flexi buffer 1×, 200 nMol of both the forward primer WFb (5′-^{809}CC ACTGACATCTTTACGATGC^{829}-3′, number according to GenBank accession No: AJ311031) and the reverse primer WRb (5′-^{1744}GGAAAGACTTCCTGGGCAAG^{1725}-3′, number according to GenBank accession No: AJ311031), 150 µM of dNTP, 3 mM $MgCl_2$, 2 µl of nucleic acids extracted from a leafhopper and adjusted with RNAse/DNAse-free water to a final volume of 50 µl. The mixture was heated for 5 min at 94°C. Then, the reactions were cycled in a Biometra thermal cycler (Biometra, Goettingen, Germany) for 35 cycles at 94°C for 30 s, 60°C for 1 min and 72°C for 1 min. The run ended with an incubation step at 72°C for 10 min. Simultaneously, the mitochondrial COI gene from *Psammotettix* individuals was amplified by PCR using the LCO1490 and HCO2198 primer pair (Folmer *et al*., 1994). PCR reactions were carried out in the presence of 2 µl of total leafhopper DNA extract, 1.5 U of the GoTaq® flexi polymerase (Promega), GoTaq green flexi buffer 1×, 200 nM of each primer, 150 µM of dNTP and 3 mM $MgCl_2$. The mixture was submitted to 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 90 s. A final extension step was performed at 72°C for 10 min. The PCR products were analysed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and observed under UV illumination. The nucleotide sequences of the 708 nt-long (COI) PCR products were produced by Eurofins MWG Operon (Germany). Sequence data were analysed using Geneious software version 10.1.2 (Biomatters Ltd.).

A phylogenetic tree for the COI gene was built from nucleotide sequences of 70 leafhoppers (18 females and 52 males, table 3) collected in the fields and 26 COI sequences from different *Psammotettix* species retrieved from GenBank (Supplementary table S1). Phylogenetic trees, constructed

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Table 3. Origin of studied leafhoppers and type of available data.

		Origin					Description[1]						Origin					Description[1]			
Name	Sex	Country	Region	Field location	Type	Coll. date	Bod	Aed	Mol	Vib	Name	Sex	Country	Region	Field location	Type	Coll. date	Bod	Aed	Mol	Vib
69Aa	M	Fr.	Aube	Fontaines	F_2	September 2013	Y	N	N	Y	284	M	Fr.	Yonne	Evry	F_0	September 2013	Y	Y	Y	Y
189	M	Fr.	Aube	Villenereuill	F_0	September 2013	Y	Y	Y	Y	286	M	Fr.	Yonne	Evry	F_0	September 2013	Y	N	N	Y
207	M	Fr.	Aube	Villenereuill	F_0	September 2013	Y	Y	Y	Y	299	M	Fr.	Yonne	Evry	F_0	September 2013	Y	Y	Y	Y
209	M	Fr.	Aube	Villenereuill	F_0	September 2013	Y	Y	N	Y	308	M	Fr.	Yonne	Sepeaux	F_0	September 2013	Y	Y	Y	Y
212	M	Fr.	Aube	Villenereuill	F_0	September 2013	Y	Y	Y	Y	319	M	Fr.	Yonne	Sepeaux	F_0	September 2013	Y	Y	Y	Y
217	M	Fr.	Aube	S^t Martin de B.	F_0	September 2013	N	Y	Y	Y	326	M	Fr.	Yonne	Sepeaux	F_0	September 2013	Y	Y	Y	Y
261	M	Fr.	Aube	Bucey en othe	F_0	September 2013	Y	Y	Y	Y	360	M	Fr.	Yonne	Looze	F_0	September 2013	Y	N	N	Y
20E	F	Fr.	Aude	Marseillette	F_1	June 2013	Y	/	N	Y	370	M	Fr.	Yonne	Looze	F_0	September 2013	Y	Y	Y	Y
30A	F	Fr.	Aude	Carcassone	F_1	June 2013	Y	/	N	Y	389	M	Fr.	Yonne	Chitry	F_0	September 2013	Y	Y	Y	Y
30B	F	Fr.	Aude	Carcassone	F_1	June 2013	Y	/	N	Y	400	M	Fr.	Yonne	Chitry	F_0	September 2013	Y	Y	Y	Y
30G	F	Fr.	Aude	Carcassone	F_1	June 2013	Y	/	N	Y	347	F	Fr.	Yonne	Looze	F_0	September 2013	Y	/	N	Y
34Gc	F	Fr.	Aude	Carcassone	F_2	June 2013	Y	/	N	Y	394	F	Fr.	Yonne	Chitry	F_0	September 2013	Y	/	Y	Y
33A	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	Y	Y	A	M	Slo.	Ravnica	Ravnica	F_0	August 2014	N	Y	Y	Y
15B	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	Y	Y	S-01	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
34G	F	Fr.	Aude	Carcassone	F_1	June 2013	Y	/	N	Y	S-02	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	Y
11A	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-03	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	Y
12K	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-07	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
15C	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-09	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	Y
15H	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-11	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	Y
20A	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-12	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	N
27A	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-13	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	N
28A	M	Fr.	Aude	Marseillette	F_1	June 2013	N	Y	N	Y	S-15	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
30E	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-16	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	N
30I	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-17	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	N
31B	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-20	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	Y
31C	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-22	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
31E	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-24	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
34C	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-32	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
35A	M	Fr.	Aude	Carcassone	F_1	June 2013	N	Y	N	Y	S-34	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
524	M	Fr.	Cher	S^t Caprais	F_0	September 2013	N	Y	Y	Y	S-41	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	Y
51B	F	Fr.	Haute Garonne	Auragne	F_1	June 2013	Y	/	N	Y	S-45	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	Y
65A	M	Fr.	Haute Garonne	Villenouvelle	F_1	June 2013	N	Y	Y	Y	S-46	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
34	M	Fr.	Haute Marne	Villiers en lieu	F_0	September 2013	Y	Y	Y	Y	S-48	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
117	M	Fr.	Haute Marne	Orges	F_0	September 2013	Y	Y	Y	Y	S-50	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	N	Y
108	M	Fr.	Haute Marne	Vraincourt	F_0	September 2013	Y	Y	Y	Y	S-56	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
134	M	Fr.	Haute Marne	Vraincourt	F_0	September 2013	Y	Y	Y	Y	S-57	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
49	F	Fr.	Haute Marne	Vaux sur B.	F_0	September 2013	Y	/	Y	Y	S-58	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	Y
57	F	Fr.	Haute Marne	Vaux sur B.	F_0	September 2013	Y	/	Y	Y	S-59	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
15	F	Fr.	Haute Marne	Villiers en lieu	F_0	September 2013	Y	/	Y	Y	S-60	M	Slo.	Ravnica	Ravnica	F_0	August 2014	N	Y	Y	Y
104	F	Fr.	Haute Marne	Vraincourt	F_0	September 2013	Y	/	Y	Y	S-61	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	Y
104G	F	Fr.	Hérault	S^t Gely du Fesc	F_1	June 2013	Y	/	N	Y	S-62	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	N	Y
104H	F	Fr.	Hérault	S^t Gely du Fesc	F_1	June 2013	Y	/	N	Y	S-63	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	N	Y
169A	F	Fr.	Hérault	Assas	F_1	June 2013	Y	/	N	Y	S-64	M	Slo.	Ravnica	Ravnica	F_0	August 2014	N	Y	Y	Y
170B	F	Fr.	Hérault	Assas	F_1	June 2013	Y	/	Y	Y	S-65	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
170C	F	Fr.	Hérault	Assas	F_1	June 2013	Y	/	N	Y	S-66	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	N
190A	M	Fr.	Hérault	S^t Mathieu de T.	F_1	June 2013	N	Y	Y	Y	S-67	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	N
188G	M	Fr.	Hérault	S^t Mathieu de T.	F_1	June 2013	N	Y	N	Y	S-68	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	N
93A	M	Fr.	Hérault	Montferrier sur lez	F_1	June 2013	N	Y	N	Y	S-69	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	Y
97D	M	Fr.	Hérault	Montferrier sur lez	F_1	June 2013	N	Y	N	Y	S-70	F	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	/	Y	Y
97H	M	Fr.	Hérault	Montferrier sur lez	F_1	June 2013	N	Y	N	Y	S-71	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y

Table 3. (*Cont.*)

Name	Sex	Origin					Description[1]				Origin					Description[1]			
		Country	Region	Field location	Type	Coll. date	Bod	Aed	Mol	Vib	Name	Sex	Country	Region	Field location	Type	Coll. date	Bod	Aed	Mol	Vib
190C	M	Fr.	Hérault	S[t] Mathieu de T.	F_1	June 2013	N	Y	N	Y	S-73	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
190E	M	Fr.	Hérault	S[t] Mathieu de T.	F_1	June 2013	N	Y	N	Y	S-74	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
190F	M	Fr.	Hérault	S[t] Mathieu de T.	F_1	June 2013	N	Y	N	Y	S-75	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	Y	Y	Y
267	M	Fr.	Yonne	Evry	F_0	September 2013	Y	Y	Y	Y	S-76	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	Y	Y
268	M	Fr.	Yonne	Evry	F_0	September 2013	Y	Y	Y	Y	S-78	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	N	Y
271	M	Fr.	Yonne	Evry	F_0	September 2013	Y	Y	Y	Y	S-79	M	Slo.	Ravnica	Ravnica	F_0	August 2014	Y	N	N	Y

[1]Each individual was characterized (Y for 'yes') or not (N for 'no') using vibrational data (Vib), morphometric measurement procedures (Bod: measure of parts of leafhopper's body; Aed: measure of the male's *aedeagus*) and molecular approach (Mol: COI sequencing procedure). M, male; F, female; Fr., France; Slo., Slovenia. F_0, F_1 and F_2 correspond to field-collected insects, progenies obtained from gravid females collected during field surveys and progenies resulting from mating obtained during experiments (Derlink *et al.*, 2016), respectively.

using the MrBayes method (Huelsenbeck & Ronquist, 2001) with the HKY85 nucleotide substitution model with the following MCMC settings (chain length: 1.100.000, heated chain: 4, heated chain temp: 0.2, subsampling frequency: 200, burn-in length: 100.000 and random seed: 13.825) and using the PHYML (Guindon *et al.*, 2010) and the Neighbour-Joining methods with the Tamura and Nei (Tamura & Nei, 1993) implemented into Geneious software version 10.1.2 (Biomatters), were obtained from aligned COI sequences. *Macrosteles* COI sequence (GenBank accession number: EU981892.1) was used as outgroup to root the tree.

Morphometric measurements of leafhoppers

Individuals used for morphometric measurements were stored in 96% ethanol. Leafhoppers were placed under the trinoculare stereomicroscope HVZ-S0645T0 (Huvitz) connected to the HC-30MU digital camera (Huvitz). Digitalized pictures of 82 individuals (30 females and 52 males) (table 3) were taken. We measured various body size characters (fig. 2A) using soft basic version of Panasis© software (Micro skopWorld). Male leafhoppers were dried for 2 min at room temperature and afterwards the soft tissue was dissolved by immersion for 5 min in hot 1.8 M KOH. The remaining sclerotized body was washed in deionized water, dried at room temperature and transferred to a drop of glycerol. The last segment of the abdomen was separated from the body and the aedeagus dissection was carried out under the above mentioned trinocular stereomicroscope. Digitalized pictures of aedeagus from 68 males (table 3) were obtained using the LSM700 microscope (Zeiss) and ZEN© software (Zeiss). Morphometric measurements of various aedeagus size characters (fig. 2B) were done by using ImageJ software (Rasband, 2006).

We compared male and female body characters (table 4). A linear discriminant analysis (LDA) was performed based on groups according to the acoustic profile. The ranges of obtained values of morphometric characters associated with acoustic groups are shown in the Supplementary figs S1 and S2 for males and females, respectively. All morphometric analyses were done by R version 3.2.1 (R Development Core Team, 2010).

Finally, general aedeagus morphology has been compared with published drawings (Tishechkin, 1999; Biedermann & Niedringhaus, 2009). Throughout the paper, we follow Wilson *et al.* (2015) in considering *P. alienus* (Dahlbom) the valid name for taxon often referred to in the literature at *Psammotettix striatus* (Linnaeus).

Results

Characteristics of field-collected leafhoppers

From the 36 fields visited in 2013 in France and in 2014 in Slovenia, 1192 leafhoppers (492 males and 700 females; sex ratio$_{(m/f)} = 0.7$) have been collected (table 1), immediately placed in individual rearing systems and transferred to the laboratory facilities for analyses. Under our rearing conditions, 96, 234 and four individuals out of the females collected in June 2013 (South of France), September 2013 (North of France) and August 2014 (West of Slovenia), respectively, produced 2212 larvae illustrating the proportions (i.e. 63, 46 and 11%, respectively) of gravid females at the time of collection. However, the proportion of gravid females was heterogeneous between sampled fields (ranging from 0 to 100, 6 to 74 and 0 to

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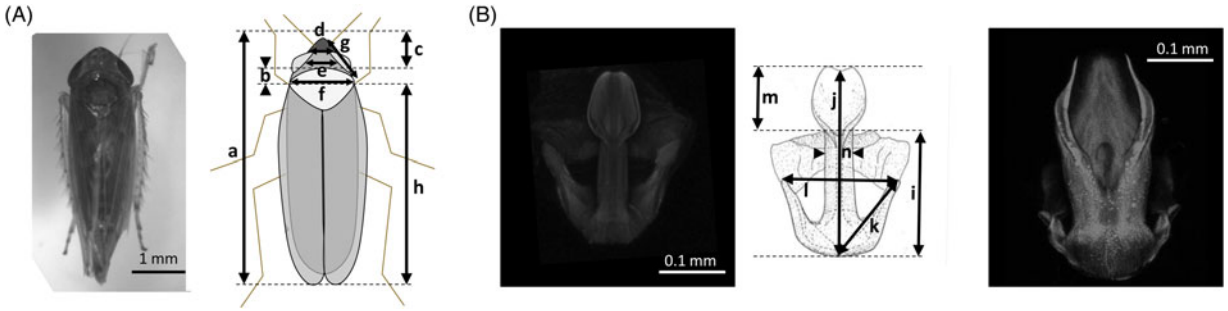

Fig. 2. Morphological characterization of *Psammotettix* body (A) and male *aedeagus* (B). Different parts of the body were measured (A, *a–h*). *Psammotettix* male genitalia (B) were dissected in the presence of potassium hydroxide, observed using the LSM700 microscope and measured (B, *i–n*) with tools included in the ZEN© software (Zeiss). Pictures in B illustrate *aedeagus* from leafhoppers #34 (*Psammotettix alienus*, left) and #524 (*Psammotettix confinis*, right).

16% in fields sampled in June 2013, September 2013 and August 2014, respectively, table 1). Sizes of the progenies produced by F_0 gravid females were highly variable between the collected individuals. Some F_1 progenies included only single individuals while other populations reached up to 65 insects (table 1). Moreover, some F_0 females (e.g. 24/96 for females collected in June 2013) were able to lay eggs on their host plant, highlighting their gravid status, but for an undetermined reason the larvae died few days after hatching.

To get information about the sanitary status of the collected insects and their ability to transmit WDV to host plant, PCR assays targeting the WDV sequence were performed on the total nucleic acids extracted from 818 F_0 individuals sampled in September 2013 and from 79 F_0 individuals sampled in August 2014. Moreover, ELISA procedure was applied to all the plants used for rearing leafhoppers in the laboratory facilities. The proportion of viruliferous leafhoppers was 14.9% for the insects collected in September 2013 (France) with an important between-field variation (table 1). Based on PCR diagnostic, the leafhoppers collected in Slovenia were WDV-free. ELISA results showed that all viruliferous leafhoppers were able to successfully inoculate plants with WDV (not illustrated).

WDV is described to be transmitted in a persistent non-propagative manner by its leafhopper vector (Zhang *et al.*, 2010). This means that once acquired during feeding on infected plants, viral particles are not lost at moulting and persist in the vector for its whole lifespan. To test the possible effect of the viruliferous status on the biology (e.g. size of progenies) of leafhoppers, the progenies of 35 females have been introduced in a long-term maintenance based on monthly transfers of adults and larvae of a progeny on a new individual rearing system (fig. 1 and table 5). This process was applied on each progeny until the last insect of the population died (from 46 days to more than a year). Mean duration of a lineage maintenance was 170.74 days (SD ± 114.58). Three lineages were able to produce generations of larvae during a period of 470 days. No obvious correlation between the size of the progeny initially produced by the gravid F_0 females, which varied in the range 2–33 F_1 individuals, and the duration of the long-term maintenance was observed. However, the number of larvae produced during the maintenance procedure is linearly and positively correlated ($N = 35$; $r^2 = 0.706$) with the length of the maintenance period. According to the sanitary status of the F_0 females, 28 out of the 35 lineages were virus-free while the other lineages were associated with the presence of WDV during the whole long-term maintenance as shown by detection of the virus in the last host plant used in the corresponding procedures. The presence of WDV had no effect on the number of larvae produced ($P = 0.106$) and on the length of the long-term maintenance ($P = 0.587$).

Vibrational signals produced by Psammotettix *leafhoppers*

All registered vibrational signals were variations of the same basic pattern of regularly repeated pulse trains (figs 3 and 4). Vibrational songs had species- and sex-specific characteristics and differed substantially in pulse train duration and pulse train repetition time (figs 3 and 4, table 2).

According to existing information (Derlink *et al.*, 2016), the great majority of leafhoppers collected from the cereal fields in France emitted calling songs with the *P. alienus* temporal pattern (figs 3a and 4a). However, some males emitted calling songs that can be, according to Tishechkin (1999), clearly assigned to *P. confinis* (fig. 3b). In comparison with *P. alienus*, male calling song in *P. confinis* is characterized by shorter pulse train duration and pulse train repetition time (table 2). Female calling song of *P. alienus* is formed by longer pulse trains repeated with longer repetition time (Derlink *et al.*, 2016) (fig. 4a, table 2). Due to the low number of collected *P. confinis*, we did not obtain a recording that could be clearly assigned as *P. confinis* female calling song. However, one female collected in France (individual #51B) emitted calling song that did not correspond to any previously characterized *Psammotettix* species-specific vibrational song (fig. 4c). The emission of this song could not be reliably associated with any male calling song included in the stimulation sequence. In comparison with *P. alienus* female calling song, this song is characterized by longer, frequency-modulated pulse trains and longer pulse repetition time (table 2).

The analysis of vibrational signals registered from the leafhoppers collected in Slovenia revealed the presence of a leafhopper community composed by three main species including *P. alienus* and *P. confinis*. The third main species collected in Slovenian fields was *P. helvolus*. Vibrational signals of *P. helvolus* have not been, to our knowledge, described previously. In comparison with *P. alienus*, males of *P. helvolus* produced calling songs composed of longer pulse trains repeated with shorter repetition time (fig. 3c, table 2), while females emitted long pulse trains (fig. 4b, table 2). While female replies can be reliably associated with *P. helvolus* male calling song

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Table 4. Measurement of different parts of the male and female leafhopper body.

	Morphometric measurements[1]							
Male	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
34	3.98	0.48	0.22	0.56	0.56	0.98	0.7	3.28
117	4.14	0.51	0.25	0.56	0.5	0.95	0.72	3.38
169Aa	3.44	0.36	0.32	0.54	0.5	0.87	0.72	2.76
108	4.27	0.38	0.35	0.55	0.5	0.94	0.8	3.54
134	3.89	0.36	0.32	0.54	0.4	0.98	0.73	3.21
189	3.71	0.33	0.36	0.55	0.47	0.88	0.71	3.02
207	3.94	0.37	0.27	0.55	0.52	0.93	0.74	3.3
209	3.94	0.37	0.34	0.5	0.48	0.9	0.73	3.23
212	3.8	0.5	0.25	0.5	0.44	0.83	0.73	3.05
261	4.08	0.32	0.33	0.48	0.48	0.9	0.73	3.43
267	3.94	0.5	0.32	0.54	0.45	0.87	0.75	3.12
268	3.88	0.38	0.29	0.54	0.48	0.92	0.76	3.21
271	4.1	0.31	0.35	0.59	0.5	0.91	0.76	3.44
284	3.97	0.39	0.36	0.5	0.49	0.88	0.74	3.22
286	4.03	0.35	0.31	0.6	0.52	0.99	0.8	3.37
299	3.94	0.32	0.33	0.54	0.45	0.89	0.72	3.29
308	3.57	0.34	0.33	0.48	0.39	0.81	0.71	2.9
319	3.95	0.32	0.35	0.48	0.45	0.9	0.75	3.28
326	3.9	0.36	0.31	0.55	0.47	0.94	0.77	3.23
360	4.05	0.4	0.28	0.5	0.43	0.93	0.75	3.37
370	4.04	0.4	0.35	0.51	0.49	0.95	0.8	3.29
389	3.95	0.38	0.3	0.56	0.53	0.94	0.75	3.27
400	3.98	0.42	0.33	0.47	0.48	0.97	0.75	3.23
S-01	3.11	0.26	0.40	0.58	0.51	0.83	0.76	2.45
S-02	3.34	0.33	0.33	0.55	0.46	0.81	0.71	2.68
S-03	3.41	0.35	0.34	0.59	0.50	0.87	0.70	2.72
S-07	2.97	0.34	0.24	0.53	0.44	0.75	0.63	2.39
S-15	3.45	0.34	0.36	0.47	0.43	0.74	0.64	2.75
S-20	3.38	0.30	0.34	0.54	0.53	0.82	0.71	2.74
S-22	4.09	0.34	0.38	0.49	0.48	0.85	0.73	3.37
S-24	3.87	0.38	0.48	0.56	0.51	0.93	0.78	3.01
S-32	3.41	0.32	0.32	0.61	0.53	0.93	0.74	2.77
S-34	3.55	0.45	0.30	0.57	0.49	0.83	0.72	2.80
S-46	3.15	0.33	0.38	0.55	0.49	0.83	0.74	2.44
S-48	3.43	0.33	0.36	0.55	0.50	0.85	0.77	2.74
S-50	3.34	0.31	0.35	0.56	0.44	0.82	0.75	2.68
S-56	3.62	0.43	0.32	0.56	0.51	0.92	0.71	2.87
S-57	3.73	0.42	0.34	0.53	0.42	0.79	0.68	2.97
S-58	3.93	0.38	0.34	0.61	0.52	0.92	0.78	3.21
S-59	4.17	0.40	0.34	0.52	0.51	0.91	0.78	3.43
S-61	3.78	0.24	0.40	0.61	0.49	0.99	0.79	3.14
S-62	3.90	0.36	0.36	0.60	0.53	0.96	0.75	3.18
S-63	3.91	0.37	0.36	0.59	0.47	0.83	0.68	3.18
S-65	3.79	0.37	0.33	0.49	0.40	0.89	0.72	3.09
S-66	4.02	0.30	0.33	0.56	0.51	0.94	0.72	3.39

	Morphometric measurements[1]							
Female	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
20E	3.95	0.33	0.38	0.57	0.45	0.91	0.79	3.24
30A	4.01	0.41	0.33	0.62	0.55	1.02	0.84	3.27
30B	4.12	0.36	0.37	0.64	0.54	1.01	0.83	3.39
30G	4.19	0.39	0.3	0.62	0.56	1.06	0.79	3.5
34Gc	3.48	0.26	0.37	0.59	0.53	0.91	0.77	2.85
49	3.85	0.49	0.31	0.56	0.46	0.92	0.8	3.05
51B	3.57	0.36	0.35	0.57	0.49	0.85	0.77	2.86
57	4.03	0.42	0.34	0.68	0.42	1.03	0.84	3.27
104G	3.67	0.32	0.37	0.6	0.53	0.95	0.79	2.98
104H	3.96	0.28	0.39	0.64	0.52	1	0.8	3.29
169A	4.04	0.37	0.34	0.59	0.53	0.97	0.8	3.33
170B	4.16	0.46	0.3	0.54	0.5	1	0.81	3.4
170C	3.79	0.41	0.25	0.55	0.57	0.89	0.75	3.13
347	4.11	0.38	0.34	0.58	0.51	1.12	0.81	3.39
15	3.82	0.34	0.38	0.59	0.48	0.92	0.77	3.1
104	4.25	0.39	0.36	0.64	0.56	1.02	0.82	3.5
394	4.22	0.42	0.33	0.52	0.5	0.96	0.81	3.47
34G	3.55	0.43	0.25	0.59	0.55	0.96	0.76	2.87
S-09	3.45	0.33	0.42	0.62	0.50	0.82	0.78	2.70
S-11	3.11	0.26	0.40	0.65	0.52	0.84	0.76	2.45
S-12	3.31	0.38	0.41	0.61	0.55	0.87	0.69	2.52
S-13	3.51	0.30	0.35	0.56	0.48	0.88	0.79	2.86
S-16	3.45	0.38	0.30	0.48	0.40	0.68	0.54	2.77
S-17	3.32	0.37	0.32	0.57	0.53	0.82	0.75	2.63
S-41	3.86	0.44	0.29	0.61	0.56	0.97	0.80	3.13
S-45	3.21	0.32	0.38	0.62	0.55	0.87	0.71	2.51
S-67	3.21	0.28	0.42	0.65	0.51	0.93	0.77	2.51
S-68	3.68	0.33	0.41	0.66	0.60	0.99	0.82	2.94
S-69	3.54	0.44	0.34	0.51	0.42	0.74	0.76	2.76
S-70	3.74	0.30	0.54	0.54	0.43	0.89	0.82	2.90

Continued

Table 4. (*Cont.*)

Male	Morphometric measurements[1]								Female	Morphometric measurements[1]							
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*		*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
S-71	3.40	0.32	0.36	0.53	0.45	0.76	0.68	2.72	Mean ± SD for males	3.71 ± 0.33	0.36 ± 0.06	0.33 ± 0.04	0.54 ± 0.04	0.48 ± 0.04	0.88 ± 0.07	0.73 ± 0.04	3.02 ± 0.32
S-73	3.32	0.29	0.35	0.63	0.47	0.84	0.71	2.68									
S-74	3.43	0.34	0.35	0.56	0.43	0.77	0.72	2.74	Mean ± SD for females	3.74 ± 0.33	0.37 ± 0.06	0.35 ± 0.06	0.59 ± 0.05	0.51 ± 0.05	0.93 ± 0.09	0.78 ± 0.06	3.02 ± 0.32
S-75	3.18	0.30	0.36	0.51	0.42	0.75	0.73	2.52									
S-76	3.20	0.28	0.37	0.60	0.44	0.80	0.71	2.55									
S-78	3.31	0.26	0.35	0.57	0.48	0.84	0.73	2.70	*P* value	0.65	0.46	0.09	<0.0001	<0.001	<0.01	<0.001	0.87
S-79	3.25	0.33	0.34	0.52	0.40	0.71	0.70	2.58	(males vs. females)[2]								

[1]The measures associated to the different parts of the leafhopper's body (*a–h*, see fig. 2a) are listed in mm.
[2]Wilcoxon rank-sum test implemented in R version 3.2.1.

included in the stimulation sequence (fig. 4b), the resulting structure of a duet in which female reply overlapped with male song may be an artefact, since live male may stop emitting pulse trains, when he perceives female reply. One female collected in Slovenia (Ravnica area, individual #S-70) emitted vibrational song characterized by a unique song pattern not described previously (fig. 4d). The emission of this song could not be reliably associated with any male calling song included in the stimulation sequence. In comparison with *P. alienus*, in this song, pulse trains were shorter and repeated with longer repetition time (table 2).

Morphological characteristics of adult leafhoppers

Different morphological parameters, from description of the body to specific characteristics of the habitus (e.g. antennae, pronotum, apical cells of the wings and tergits…), the head (e.g. thyridia spots) and the apical part of the abdomen (e.g. ovipositor and pygofer), can be used to accurately describe members of the cicadomorpha families (Biedermann & Niedringhaus, 2009). The F_0 leafhoppers were all assigned to *Psammotettix* genus after observation of each individual in the laboratory. To better describe the morphological characteristics of these insects, eight morphometric characters associated with the length of the body and the wings and the size of specific parts of the head/pronotom were determined for 82 leafhoppers (i.e. 52 males and 30 females, tables 3 and 4, fig. 2a). While males and females did not differ in their body length (fig. 2a and table 4, *a*, *b*, *c* and *h*), the comparison of other characters showed statistical difference in parameters '*d*', '*e*', '*f*' and '*g*' (fig. 2a), which showed males were in general narrower than females (non-parametric Wilcoxon rank-sum test, $P < 0.01$).

Aedeagus general morphology enabled the assignment of seven males (#65A, #524, #S-32, #S-34, #S-56, #S-60 and #S-64) to the *P. confinis* species. However, male genital morphology of some species (e.g. *P. alienus* (Dahlbom, 1850), *P. helvolus* (Kirschbaum, 1868), *P. notatus* (Melichar, 1896) and *P. striatus* (Linnaeus, 1758)) is very similar and other 61 males collected in the present study were, after preliminary inspection of aedeagus under the microscope, in the first step assigned to the 'similar to *P. alienus*' group. To accurately evaluate the variations in aedeagus morphology between *P. alienus*-like leafhoppers, several aedeagus characters (length and width of the shaft, and morphological characteristics of the spoon) were measured (fig. 2b, table 6). However, raw data showed very small variations of the determined values. The length and the width of the shaft (fig. 2b, parameters *i* and *n*, respectively), and the size of the spoon (fig. 2b, parameter *m*) were 122.00 ± 21.78, 35.63 ± 3.70 and 111.67 ± 25.97 µm, respectively.

LDA based on 14 body and aedeagus characters grouped males of *P. alienus* and *P. helvolus* into two distinct, well-defined groups that corresponded with species assignment based on vibrational signals (fig. 5a). Males of *P. helvolus* had smaller body and wing length, as well as aedeagus shaft length (Fig. S1). However, LDA also split *P. alienus* males according to the geographical origin, the main difference between French and Slovenian males being the size of the head (Fig. S1). LDA based on eight body characters clearly divided females of *P. alienus* and *P. helvolus*, as well as separated female #S-70 (fig. 5b and fig. S2). However, it did place female #51B within *P. alienus* group and did not separate *P. alienus* females according to the geographical origin.

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Table 5. Data associated with the lineages produced using 35 gravid leafhopper females collected in 2013.

Sanitary status of leafhopper	Nb of lineages	Size of the F_0 progeny[1]	Nb of larvae produced	Maintenance duration[2]
Viruliferous	7	5.0 ± 1.83 (2–8)	38.9 ± 51.4 (1–143)	143.0 ± 70.8 (46–241)
Virus-free	28	14.1 ± 7.9 (2–33)	66.1 ± 54.2 (3–217)	187.7 ± 122.5 (72–470)
P value (viruliferous vs. virus-free)[3]		<0.001	0.106	0.587
All leafhoppers	35	12.3 ± 8.0	60.7 ± 54.0	170.7 ± 114.6

Mean and standard deviation are shown.
[1]Number of F_1 individuals in the progeny.
[2]Duration in days.
[3]Kruskal–Wallis rank test implemented in one of the packages of the XLSTAT software.

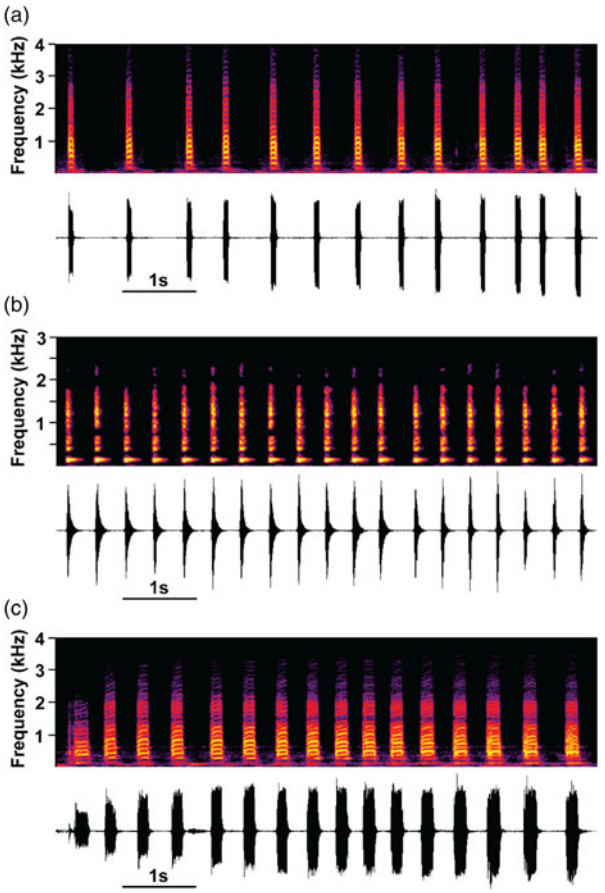

Fig. 3. Male calling songs of *Psammotettix* leafhoppers. (a) *Psammotettix alienus*, (b) *Psammotettix confinis*, (c) *Psammotettix helvolus*. For each signal, the spectrogram is shown above the corresponding waveform.

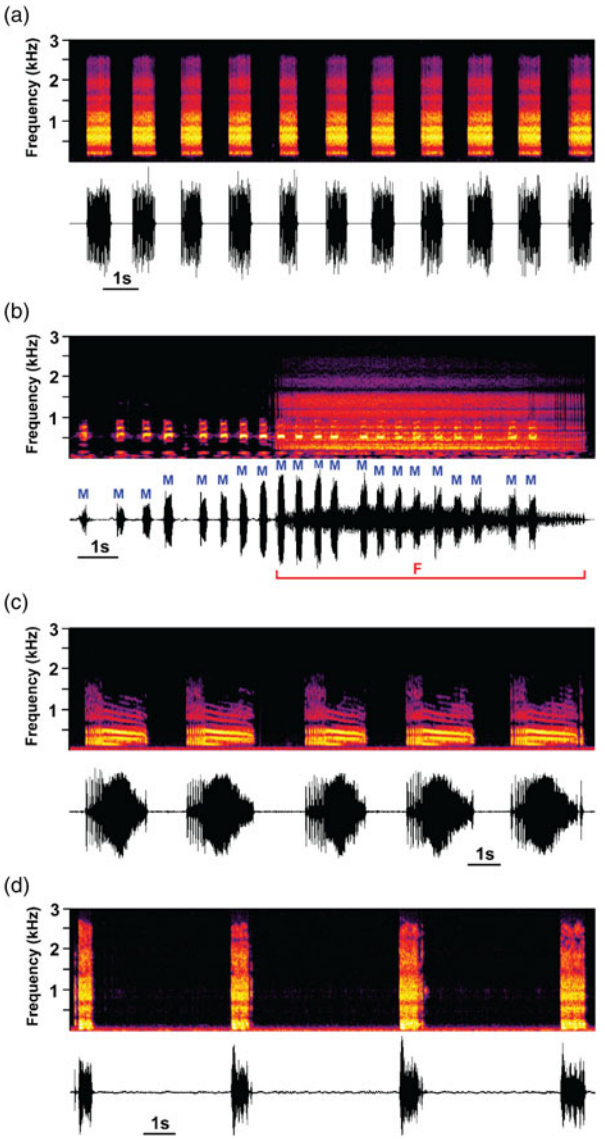

Fig. 4. Female calling songs of *Psammotettix* leafhoppers. (a) *Psammotettix alienus*, (b) *Psammotettix helvolus*, (c) female 51B, (d) female S70. In (b) female (F) signal overlaps male (M) pulse trains in male song used in playback stimulation. For each signal, the spectrogram is shown above the corresponding waveform.

Molecular characterization of individuals using COI data

Description of individuals using vibrational signals and morphometric data suggested the presence of *P. confinis* (sampled in both France and Slovenia), *P. helvolus* (not described among the *Psammotettix* individuals sampled in French cereal fields) and *P. alienus* (main species collected in French cereal fields) among leafhoppers included in this work. To complete the description of these insects, sequence

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Table 6. Measurement of aedeagus.

		Morphometric measurements[1]					
		i	*j*	*k*	*l*	*m*	*n*
Leafhopper[2]	34	142.5	230.63	168.13	161.25	107.50	38.13
	117	129.45	234.38	156.22	175.00	99.38	34.38
	108	107.5	261.25	161.25	201.88	126.25	32.50
	134	117.65	251.15	154.84	172.57	150.39	37.21
	189	137.00	221.15	166.78	189.37	82.50	37.83
	207	120.17	245.91	161.38	164.98	116.16	38.98
	209	147.90	228.64	167.99	174.97	82.37	38.13
	212	152.60	233.13	180.55	187.50	86.88	38.76
	261	139.38	239.49	171.63	194.52	96.38	38.13
	267	148.19	243.71	168.56	181.06	95.66	36.88
	268	154.64	233.19	182.13	177.75	83.76	37.01
	271	147.70	249.37	169.95	185.18	112.65	34.38
	284	130.63	256.25	167.11	196.97	120.79	38.13
	299	95.63	251.25	128.13	171.25	156.88	35.63
	308	149.38	208.91	165.83	160.63	64.38	35.00
	319	142.93	217.99	142.79	144.36	101.12	35.27
	326	149.90	239.70	177.36	192.91	84.65	40.01
	370	123.09	242.29	155.12	179.00	108.97	37.63
	389	149.38	225.63	169.11	165.27	77.37	35.01
	400	135.01	242.75	156.38	181.41	110.58	36.25
	A	102.57	187.52	113.73	108.13	83.94	23.75
	S-02	123.14	204.30	145.79	156.91	73.77	28.76
	S-03	122.73	219.48	148.86	163.92	94.00	31.41
	S-20	117.50	218.28	135.38	144.46	107.02	28.75
	S-58	155	235.08	171.36	157.51	93.75	39.45
	S-61	159.39	194.08	171.56	162.11	52.56	33.13
	S-62	140	235.21	164.43	183.80	105	38.13
	S-63	110.00	253.84	142.44	182.50	134.41	42.50
	S-75	96.25	221.05	109.57	123.75	108.75	28.13
	33A	110.52	239.35	130.85	165.59	123.52	34.49
	15B	92.80	234.40	113.93	160.01	138.13	29.38
	190A	109.56	258.09	140.46	181.62	139.80	35.29
	217	141.88	236.88	164.97	171.88	97.50	35.00
	11A	144.33	267.20	172.30	182.99	115.98	38.67
	12K	102.24	219.15	120.35	153.79	125.43	30.94
	15C	143.33	225.56	162.47	175.13	82.16	37.72
	15H	106.62	237.19	131.45	170.14	125.43	40.39
	188G	80.79	209.62	116.50	173.10	126.05	36.55
	20A	120.62	245.81	160.06	185.51	127.52	37.44
	27A	142.75	217.61	160.39	167.94	72.54	34.36
	28A	128.68	219.98	155.42	188.38	97.13	34.57
	30E	106.25	245.01	135.68	180.05	130.70	33.13
	30I	116.55	249.38	147.86	191.07	136.55	33.17
	31B	98.54	228.41	147.15	188.52	113.47	31.65
	31C	129.05	224.57	155.28	184.35	97.22	37.72
	31E	88.24	234.56	130.55	183.16	136.86	33.10
	34C	77.67	242.64	133.62	204.53	158.69	32.98
	35A	116.00	226.05	149.20	187.34	106.67	36.01
	93A	101.39	263.34	129.18	163.71	173.50	37.72
	97D	109.62	254.03	141.22	195.23	151.21	37.50
	97H	81.62	229.40	119.22	179.59	150.37	32.66
	190C	101.54	236.69	127.94	160.31	130.53	34.38
	190E	98.48	229.01	126.10	167.94	127.48	35.11
	190F	90.21	229.61	115.52	166.89	138.32	31.80
	S-15	114.38	197.50	133.56	131.25	86.26	33.75
	S-22	123.75	251.88	158.04	182.50	108.13	41.88
	S-24	98.13	253.13	135.94	189.38	146.25	45.00
	S-57	145.63	240.63	169.70	169.38	93.75	37.50
	S-59	144.38	238.13	175.27	179.38	85.63	36.88
	S-65	125.63	223.75	148.56	164.38	105.00	39.38
	S-66	103.75	246.89	144.52	190.00	146.28	38.13
	Mean	122.00 ± 21.78	234.12 ± 16.76	149.63 ± 19.34	173.38 ± 17.93	111.67 ± 25.97	35.63 ± 3.70

[1]The measures associated to the different parts of the leafhopper's *aedeagus* (*i*–*n*, see fig. 2b) are listed in μm.
[2]Leafhoppers with *aedeagus* corresponding to *Psammotettix confinis* (i.e. #524, #65A, #S-32, #S-34, #S-56, #S-60 and #S-64) have been excluded from the table.

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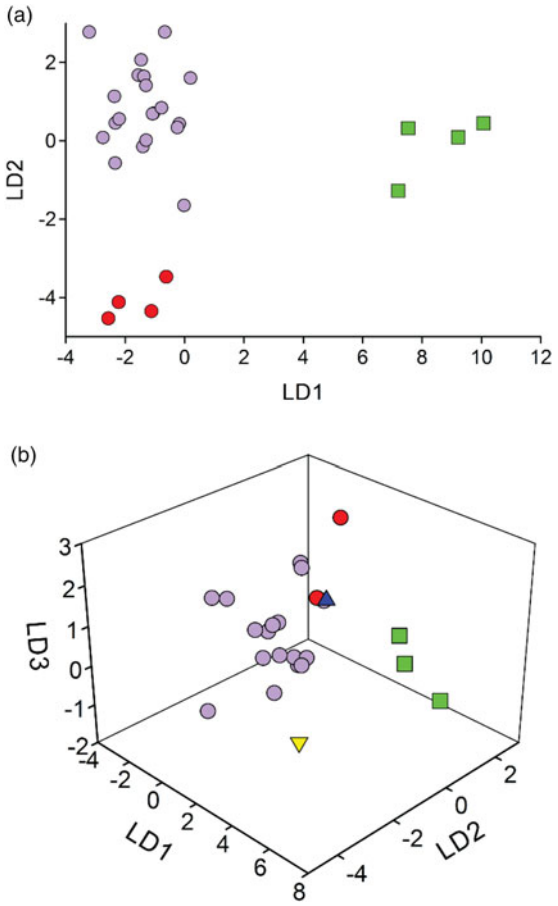

Fig. 5. Linear discriminant analysis (LDA) of (a) 14 morphometric characters for males and (b) eight morphometric characters for females of *Psammotettix* leafhoppers. (a, b) *Psammotettix alienus* from France, black circles; *P. alienus* from Slovenia, grey circles; *Psammotettix helvolus* from Slovenia, black squares. (b) female 51B; black triangle; female S70, white triangle.

analyses were carried out. An amplified DNA fragment of ±700 nt within the barcoding region was obtained for each of the tested leafhoppers. At the end of the cleaning procedure applied to raw sequence data, a sequence of up to 614 nt long was available for 70 individuals (table 3) including leafhoppers from South ($N = 5$) and North ($N = 26$) of France and from West Slovenia ($N = 39$). Alignment of these sequences was performed using the algorithm implemented into the Geneious software with standard parameters. The COI sequence from *Psammotettix* sp. (Gwiazdowski *et al.*, 2015) and from leafhopper *Macrosteles quadrilineatus* isolate J124LCO (Le Roux & Rubinoff, 2009) were retrieved from Genbank and included in the sample list. A 442-nt long sequence of COI, available for each leafhopper and corresponding to nucleotides 117–558 of the *Macrosteles* COI sequence, was used for phylogenetic analysis. Individuals with identical sequences were removed from the data and phylogenetic trees (Bayesian inference (fig. 6) and Neighbour-Joining (fig. S3)) were constructed using the 442 nt-long COI sequence from *Macrosteles* to root the tree. As expected from aedeagus morphology and vibrational signals, males #65A, #524, #S-32, #S-34, #S-56, #S-60 and #S-64 together with two individuals sampled in Slovenia (S-48 and S-67) and with Bioug05847-g02 (reported to be a member of the *P. confinis* species by Gwiazdowski *et al.*, 2015) formed a group that should be considered the *P. confinis* species (fig. 6). The 61 other *Psammotettix* leafhoppers included in this study have been assigned in three groups (fig. 6, groups A, B and C), which do not correspond to the *P. dentatus*, *P. attenuens*, *P. lividellus*, *P. beirnei* and *P. lapponicus* studied by Gwiazdowski *et al.* (2015). The group B included most of the individuals collected during our surveys ($N = 41$) and contains sequences from leafhoppers collected in France and in Slovenia. Surprisingly, this group, which contains males with *P. alienus*-like aedeagus includes the leafhopper CNC#HEM403450, initially described by Gwiazdowski *et al.* (2015) as a member of the *P. confinis* species. Group C ($N = 19$) included individuals belonging to *P. helvolus* collected in Slovenia. The female #S70 was placed on its own branch (group A). We were not able to obtain the sequence for the female #51B. Our results also revealed that the *P. attenuens* clade, which is well separated from the *P. lividellus* clade, contains an individual (CNC#HEM403476) described, according to Gwiazdowski *et al.* (2015), to be a member of the *P. lividellus* species.

Discussion

Results of the present study show that although *P. alienus* was the dominant species collected in the cereal field, it was found syntopically with other congeners. Such information is crucial for providing an insight into the epidemiology of WDD and, if needed, to adapt current pest management strategies.

During field surveys carried out at different periods of the cereal growing season, it was easy to catch male and female adult leafhoppers of the genus *Psammotettix*. The embryonic development of overwintering eggs occurs when the temperature and the duration of the day increase. The first eggs hatch in early spring and the duration of each generation is, from egg to adult, of about 50 days at an average temperature of 20°C (Manurung *et al.*, 2005). As the fecundation and first egg laying has been reported to occur after the seventh day of the adult stage, the duration of a complete *Psammotettix* life cycle from egg to egg is about 2 months. Taking into account the environmental conditions of French cereal growing regions, *Psammotettix* leafhoppers can complete up to four life cycles during the spring-to-autumn period. The presence of gravid females in fields surveyed at the end of spring and the beginning of autumn (rate of gravid females = 48%) indicated that these insects are, at these times of the year, actively involved in the production of their progenies. However, no obvious correlation was identified between field locations, sex ratio and proportion of gravid females suggesting that local biotic and/or abiotic parameters strongly impacted biological characteristics, including the efficiency of the mating process, of studied leafhoppers. It was surprising to collect only few gravid females in the surveys performed during late August in Slovenia. It has been reported that biological activity of adults declines with decreasing temperatures (Lindblad & Areno, 2002) and that all individuals die during the cold winter season. However, adults can be caught in newly sown crops until the third week of December (the beginning of winter period) in Saxony-Anhalt, Germany (Manurung *et al.*, 2005) suggesting, according to the lifetime of adult leafhoppers, their involvement in reproduction at least until the mid-autumn in a

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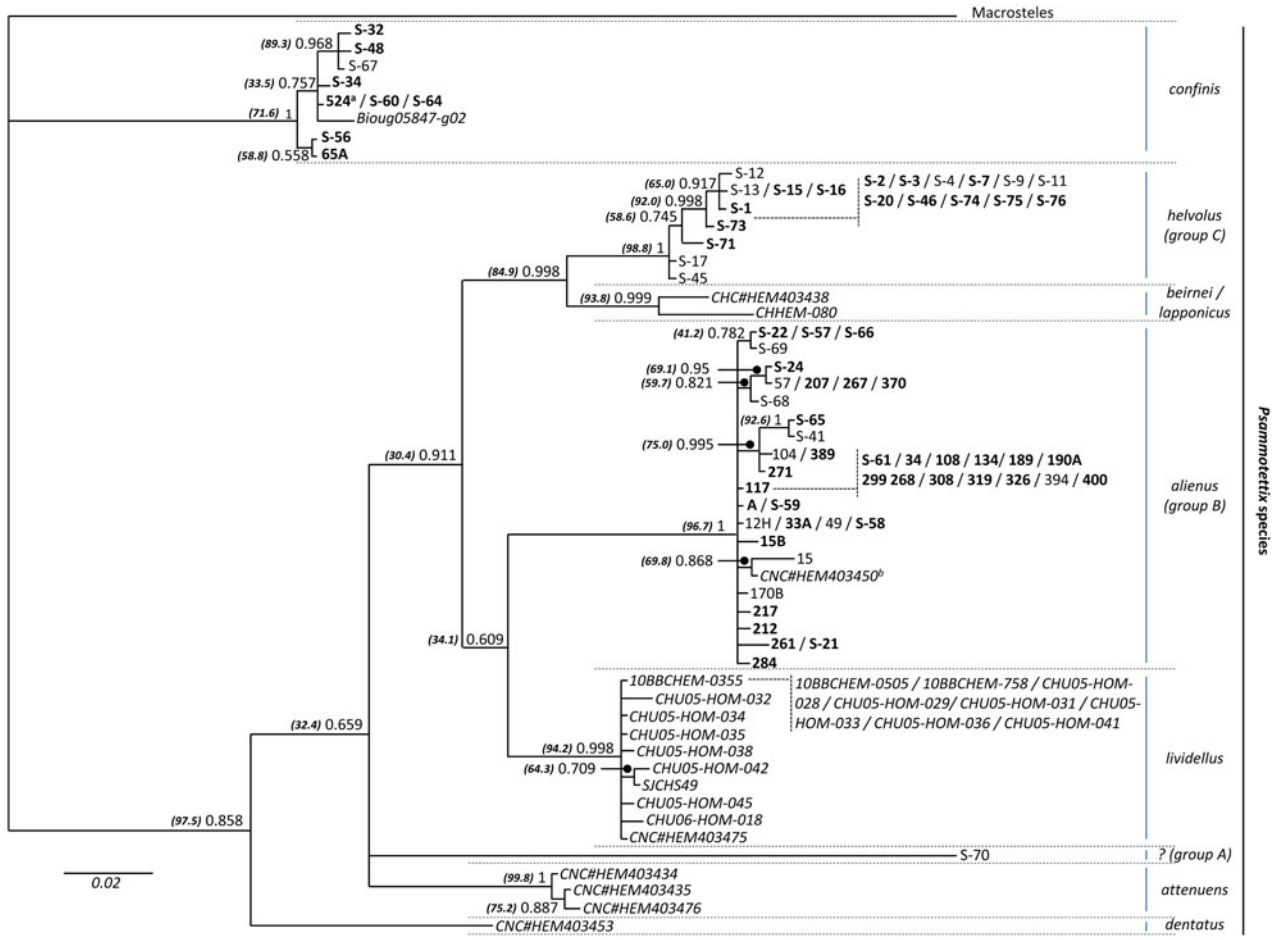

Fig. 6. Phylogenetic tree obtained from alignment of 442 nucleotides of Cytochrome Oxydase I (COI) sequences. Macrosteles COI sequence (GenBank accession number: EU981892.1) was used as outgroup to root the tree. Phylogenetic tree was constructed using MrBayes method (Huelsenbeck & Ronquist, 2001) implemented into Geneious software (Biomatters) with the HKY85 nucleotide substitution model. Posterior probability obtained for the 73 nodes are reported on the branches. The scale bar represents the relative genetic distance (number of substitutions per nucleotide). Males from this study are presented in bold. COI sequences from *Psammotettix* retrieved from Genbank are listed in italic. Accession numbers are listed in table S1. The PHYML (Guindon *et al.*, 2010) methods with the TN93 (Tamura & Nei, 1993) substitution model implemented into Geneious software was applied to the alignment to construct a phylogenetic tree based on maximum likelihood. As the ML tree has a topology similar to the MrBayes tree, bootstrap values from ML tree are presented between brackets on the illustrated MrBayes tree. (A) The aedeagus of theses leafhoppers are illustrated in fig. 3. (B) The insect CNC#HEM403476 and CNC#HEM403450 have been described to be members of the *Psammotettix confinis* and to the *Psammotettix livedillus* species, respectively.

Northern-European environment like Germany. As the biology of leafhoppers is temperature-dependent, in locations with a cold winter period, a delayed beginning of spring season and a rapid transition from warm to cold temperatures in early autumn, only two-to-three leafhopper generations are produced per year (Schiemenz, 1969). Slovenia has continental weather conditions and the biological activity of leafhoppers in late summer can be already reduced, resulting in a lower-than-expected rate of gravid females observed in this study. It should also be mentioned that in Slovenia, *Psammotettix* individuals were collected in grassland and not in the wheat fields.

The WDV is exclusively transmitted from plant to plant by *Psammotettix* leafhopper vectors in a persistent non-replicative manner. This indicates that: (i) particles do not replicate in their vectors and (ii) the viruliferous status of vectors, once acquired, lasts for several days/weeks (Brault *et al.*, 2010; Kvarnheden *et al.*, 2016). Thus, the detection of WDV in a leafhopper reflects the sanitary status of host(s) visited by the insect prior being caught. *Psammotettix* adults visit numerous hosts (healthy and infected) during their life. Consequently, the proportion of viruliferous insects sampled in a field is an overestimation of the proportion of infected plants in the surveyed area. Wheat dwarf epidemiological data showed that prevalence of WDV varies from field to field, region to region and year to year (Manurung *et al.*, 2004). In France in 2008, 2009 and 2010, mean prevalence of WDV in cereals was reported to be in 13.05, 3.94 and 6.14%, respectively (Abt & Jacquot, 2015), which is in broad agreement with the 14.9% of viruliferous leafhoppers described in this study for French samples. WDV has been reported in numerous European countries including Slovenian neighbouring countries Italy (Conti, 1994), Hungary (Bisztray *et al.*, 1989) and Austria (Schubert *et al.*, 2014). Although all *Psammotettix* leafhoppers

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randomly collected for our study in 2014 in the surveyed grassland in West Slovenia were virus-free, in 2016 WDV has been detected in few plants exhibiting disease symptoms in a wheat field in the North-East of the country (Viršček Marn & Mavrič Pleško, 2017).

The number of eggs produced by a gravid leafhopper females has been described to be determined by both abiotic (e.g. temperature) and biotic (plant host species) parameters (Van Rensburg, 1982; Okoth *et al*., 1987). The rearing conditions used in this work to maintain leafhoppers in the laboratory facilities allow a mean production of few tens of larvae per gravid female (mean size of F_1 populations = 7.29; minimum = 2, maximum = 50). The fecundity of *Psammotettix* females observed under our experimental conditions is in agreement with previous reports (size of progenies in the range 1–100) and with low peak of population density (maximum = 43 adults m^{-2}) monitored from self-sown winter barley areas (Manurung *et al*., 2004, 2005). In addition to the successful long-term maintenance of several populations of *Psammotettix*, some of the field-collected gravid females produced progenies that died few hours after hatching. It has been reported that inter-specific mating can alter qualitatively (bias of the sex ratio) and/or quantitatively (number of offsprings) the characteristics of the produced progeny (Delpuech *et al*., 2010). Thus, it may suggest that the sudden death few days after hatching of F_1 progenies produced by some of the F_0 field-collected gravid females may be a consequence of inter-specific mating of *Psammotettix* occurring in fields. Since our rearing procedure was standardized, at present, we do not have other explanation for the early extinction of some leafhopper progenies.

For many insect species, the identification of males and females can be achieved by several parameters including sex-dependent size or morphological characteristics of the insect body. For some species of the *Psammotettix* genus, females are bigger than males (e.g. *P. maritimus* (Perris, 1857), *P. sabulicola* (Curtis, 1837), *P. pallidinervis* (Dalhbom, 1850), *P. albomarginatus* (Wagner, 1941), *P. putoni* (Then, 1898) and *P. dubius* (Ossiannilsson, 1974)). However, for other species belonging to the this genus, including *P. nardeti* (Relane, 1965), *P. kolosvarensis* (Matsumura, 1908), *P. unciger* (Ribaut, 1938), *P. inexpectatus* (Remane, 1965), *P. angulatus* (Then, 1899), *P. poecilus* (Flor, 1861), *P. alienus* (Dahlbom, 1850), *P. cephalotes* (Herrich-Schäffer, 1835), *P. helvolus* (Kirschbaum, 1868), *P. excises* (Matsumura, 1906), *P. nodosus* (Ribaut, 1925), *P. notatus* (Melichar, 1896) and *P. confinis* (Dalhbom, 1850), males and females are of similar size (Biedermann & Niedringhaus, 2009). The characterization of the sex of collected *Psammotettix* leafhoppers was determined by observations of the apical part of the abdomen. This procedure was performed after the transfer of individuals, randomly sampled in fields, in the laboratory facilities. Consequently, the sex ratios calculated from our data can be considered as estimates of the male/female proportions in the sampled locations at the collection times (i.e. June and September for France and August for Slovenia), which correspond to periods with numerous overlapping leafhopper generations. Data indicate a light preponderance of females (mean sex ratio = 0.7 (minimum = 0.2; maximum = 3), i.e. 1.42 females per male) in *Psammotettix* populations with some (four out of the 39 surveyed fields) local sex ratio above 1, i.e. prevalence of males. These results are in broad agreement with the sex ratios reported by Guglielmino & Virla (1997) for two *P. alienus* generations (47/60 and 59/49 males/females for generations 1 and 2, respectively) produced under controlled conditions.

While sex determination of *Psammotettix* adults is reliable, species identification within this genus has been so far carried out exclusively through the observation of the male's aedeagus, (Tishechkin, 1999; Biedermann & Niedringhaus, 2009; Borchard & Fartmann, 2014). However, all individuals (males, females and nymphs) are able to transmit the virus and, in particular, nymphs are likely to play an important role in the WDV transfer in the spring (Abt & Jacquot, 2015). Moreover, aedeagus form appears to be highly variable (Tishechkin, 1999) and, therefore, based exclusively on aedeagus morphological characters, *Psammotettix* males can also be assigned to the wrong species. The characterization of *Psammotettix* individuals using, as shown in this work, recordings of vibrational signals, sequence of the COI gene and morphometric data made it possible to confirm the presence of *P. alienus* in both French wheat fields and Slovenian agroecological grasslands. Moreover, this comprehensive analysis of *Psammotettix* species present in the surveyed areas revealed that *P. confinis* and *P. helvolus* individuals were found syntopically with *P. alienus*. Furthermore, combining body and aedeagus characters of individuals initially identified by vibrational signals in LDA analysis also revealed geographic differences between species. Moreover, although LDA analysis based on body characters placed the female #51B, which emitted distinct vibrational song, within *P. alienus* group, it clearly separated females of the other three species. Taken together, these results indicate that future studies should include individuals from different countries in order to provide the necessary robustness to morphometric data.

Our results on diversity of vibrational calling songs in the genus *Psammotettix* confirm previous observations that, in general, behavioural characters are the most accurate ones to delimit species (Schlick-Steiner *et al*., 2010; Henry *et al*., 2013). Mating behaviour in leafhoppers is associated with the emission of species- and sex-specific vibrational signals (Heady *et al*., 1986; Gillham, 1992; Tishechkin, 2000; Percy *et al*., 2008; Bluemel *et al*., 2014). Comparison of temporal parameters of male calling song of *P. alienus* obtained in the present and previous (Derlink *et al*., 2016) studies shows a complete overlap with parameters previously attributed to males of *P. striatus* (Linnaeus, 1758) (Tishechkin, 1999, 2000). Recently, it has been suggested that the concept of *P. striatus* (L.) sensu Ribaut (1925, 1952) is erroneous and therefore should not be used (Wilson *et al*., 2015) and our data support the view of these authors that records of *P. striatus* refer to *P. alienus*. *Psammotettix alienus* is considered a serious pest of wheat crops through transmission of wheat blue dwarf phytoplasma (Zhang *et al*., 2012; Li *et al*., 2014). Although included in one barcoding study (Kamitani, 2011), sequences from specimens referred to as *P. striatus* are not available in public databases, and therefore, the conclusive resolution whether individuals attributed to *P. striatus* correspond to *P. alienus* within the present study was not possible. However, this result highlights the need for reliable species identification in this genus, in particular, since taxonomic errors in identifying vectors can have direct economic consequences.

Molecular approach used in the present study clearly confirmed the presence of four *Psammotettix* species at our colleting sites. However, it also showed that some individuals included in the previous study by Gwiazdowski *et al*. (2015) were not correctly identified. It has been highlighted previously in the leafhoppers from the genus *Aphrodes* that misidentifications are common in museum collections (Bluemel *et al*.,

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2011) and that species identification in molecular studies may also be questionable (Bluemel *et al.*, 2014). Since biodiversity data and DNA barcodes are now commonly shared via Internet databases (Patterson *et al.*, 2010; Gibson *et al.*, 2012; Foottit *et al.*, 2014; Gwiazdowski *et al.*, 2015), mistakes may be rapidly disseminated and individuals should be validated based on reliable standards.

In summary, our study shows that, although *P. alienus* was the most common *Psammotettix* species collected in the wheat fields, it can occur syntopically with *P. confinis and P. helvolus*, as well as another unidentified species from this genus. Since epidemiology of WDD is still poorly understood (Abt & Jacquot, 2015), future studies should take into account whole *Psammotettix* wheat field communities. While molecular approach enables fast and reliable determination of all *Psammotettix* individuals, identification by vibrational signals appears to be at present the only available approach in pathogen transmission studies, when live individuals are needed.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485317000669.

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