



<http://dx.doi.org/10.11646/zootaxa.3741.2.3>

<http://zoobank.org/urn:lsid:zoobank.org:pub:3BEA5254-C4C0-47D9-9C5E-DA0448C3259E>

## ***Pachybrachis holerorum* (Coleoptera: Chrysomelidae: Cryptocephalinae), a new species from the Apennines, Italy, identified by integration of morphological and molecular data**

MATTEO MONTAGNA<sup>1,2,3</sup>, DAVIDE SASSI<sup>1</sup> & ANNAMARIA GIORGI<sup>1,2</sup>

<sup>1</sup>Centro Interdipartimentale di Studi Applicati per la Gestione Sostenibile e la Difesa della Montagna - Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

<sup>2</sup>Dipartimento di Scienze Agrarie e Ambientali - Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

<sup>3</sup>Corresponding author. E-mail: [matteo.montagna@unimi.it](mailto:matteo.montagna@unimi.it)

### **Abstract**

*Pachybrachis holerorum* n. sp. is described from the northern Apennines, Italy. The new species is related to *P. karamani* (Weise, 1893), from which it differs in the shape of the median lobe of the aedeagus and in small differences in chromatic pattern. The close relationship with *P. karamani* is confirmed by molecular analyses performed on a fragment of 829 nucleotides of the mitochondrial gene Cytochrome *c* oxidase subunit 1 (*cox1*). The general mixed Yule-coalescent model, developed for species delimitation using single-locus molecular data, was applied to a *cox1* phylogeny in order to test the hypothesis of *P. holerorum* as a separate species. Information on the host plants, acquired during specimen collection, was confirmed from gut content, targeting a fragment of the plastid large subunit of the ribulose-bisphosphate carboxylase gene and the *trnL*(UAA) intron. Besides, the lectotype of *P. karamani* is designated.

**Key words:** Leaf beetle, internal sac, rectal apparatus, species delimitation, molecular ecology

### **Introduction**

*Pachybrachis* Chevrolat, 1836, is a widely distributed Holarctic and Neotropical genus of phytophagous insects that reaches maximum species diversity in the Neotropical region. The genus comprises more than 350 described species, with 156 species and subspecies in the Palearctic (Schöller *et al.* 2010; Sassi 2012). Remarkably, 26 species of *Pachybrachis* have been reported from Italy, 19 in peninsular territories, four in Sardinia and seven (three endemic) in Sicily (Sassi 2012). The Mediterranean area and especially the island territories seem to be particularly rich for *Pachybrachis* (Burlini 1957; Burlini 1959; Daccordi and Ruffo 1971; Daccordi and Ruffo 1975; Sassi 2006), with high endemism in this region (about 20%; Montagna 2011). There are still surprising discoveries to be made, such as the recent discovery of a new taxon in areas thoroughly investigated by entomologists (Montagna 2011). Molecular characters, as well as being useful in the resolution of relationships between taxa, also shed light on their ecology, for example revealing associations between phytophagous insects and their host plants, as well as the role played by symbionts in the host metabolism (e.g. Jurado-Rivera *et al.* 2009; Sabree *et al.* 2012; García-Robledo *et al.* 2013). At present, the knowledge of the host plants of *Pachybrachis* is limited to few species but the genus is regarded as being polyphagous (Jolivet and Hawkeswood 1995; Bienkowski 1999).

Careful study of specimens of *Pachybrachis* from various localities in the North Apennine (Italy), has revealed what appears to be a new species. Subsequent collecting trips were carried out to determine the range of this putative new taxon and obtain fresh samples of several *Pachybrachis* species for molecular analyses. The new species is compared with *P. karamani* Weise, 1893, which is the most similar from morphology, and with the less similar *P. salfilii* Burlini, 1957, and other species. Molecular comparison of the species is based on the general mixed Yule-coalescent model (Pons *et al.* 2006; Fontaneto *et al.* 2007), developed for species delimitation using single-locus

molecular data, applied to a *cox1* phylogeny. The shape of genitalia and the molecular comparisons show that *P. karamani* and the new species, are closely related, but different. The description of the new species is set out below.

## Material and methods

47 specimens belonging to the new taxon were collected directly on the host plants by net sweeping in six localities (see type series). Examination, dissection, measurement and drawings were completed with the use of a stereo microscope with an ocular micrometer. Photographs were developed by photo-montage with Zerene Stacker (Richland, WA, USA). SEM micrographs of the aedeagus were acquired using a Jeol JSM-5610LV scanning electron microscope.

19 individuals belonging to further six species of *Pachybrachis* were collected in different localities of the Mediterranean basin and stored in absolute ethanol since 2009 (Table 1). These samples were used for molecular comparison. About 30 specimens of the morphologically most similar *P. karamani* were examined and compared with the new species.

**TABLE 1.** Species of *Pachybrachis* analyzed in the present study.

<i>Pachybrachis</i> species	collecting site	latitude N longitude E	collecting date	altitude <sup>a</sup>	accession numbers <sup>b</sup>
<i>holerorum</i>	IT-Emilia Romagna-PC, P.sso del Pellizzone	44°40'48.84" 9°44'42.66"	8-jun-2011	1000	HF947529- HF947535
<i>karamani</i>	IT-Emilia Romagna-BO, Castel d'Aiano	N.A.	3-jul-2012	950	HF947526, HF947528
<i>burlinii</i>	IT-Lazio-LT Isola di Ponza	40°54'5.4036" 12°57'27.3594"	6-apr-2010	35	HF947547, HF947548
<i>hippophaes</i>	FR-Champoléon, Pont des Eyrauds - Drac Blanc	44°43'11" 6°15'27"	7-jun-2011	1200	HF947538
	IT-Lombardia, CO, Merone, Laghi Baggero	45°46'11.41" 9°14'7.97"	24-may-2012	280	HF947539, HF947540
	IT-Piemonte-AL, Cabelle Ligure	44°39'31.95" 09°06'48.35"	14-jun-2011	520	HF947536, HF947537
<i>hieroglyphicus</i>	IT-Lombardia-LC, Costamasnaga	45°45'49.8306" 9°15'59.8212"	25-may-2011	300	HF947542, HF947543
	IT-Lombardia-LC, Primaluna	45°59'26.10" 9°24'3.57"	jun-2012	484	HF947541
<i>exclusus</i>	IT-Liguria- SP, Monterosso al Mare	44°9'42.03" 9°39'34.78"	21-jun-2011	453	HF947544
	IT-Toscana-MS Passo del Brattello	44°27'23.39" 9°49'22.38"	21-jun-2011	950	HF947545
	IT-Liguria-GE, Piani di Creto	44°27'53.77" 9° 0'3.57"	18-may-2011	535	HF947546
<i>salfii</i>	IT-Abruzzo-Majella	42°12'0.27" 13°47'6.74"	N.A.	757	HF947523- HF947525

<sup>a</sup>altitude is expressed in meters above sea level; <sup>b</sup>*cox1* sequence accession number.

**DNA extraction, PCR amplifications and sequencing.** 19 individuals belonging to six species of *Pachybrachis* and seven individuals of the new species, all from Passo del Pellizzone, were processed for molecular analyses (see Table 1). Total genomic DNA was extracted through non-destructive procedure from each individual and purified using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). All the specimens preserved in ethanol were dried before DNA extraction, the abdomen removed and then left for 12 h at 56°C in 180 µL of ATL lysis buffer (Qiagen) with 200 ng/mL proteinase K (Sigma Aldrich, St. Louis, MO, USA). Extractions were performed according to the manufacturer's instructions. After DNA extraction the specimens

were dry mounted on pins together with genitalia and kept for future reference in the authors' collections. A fragment of 829 nucleotides at the 3'-end of the insect mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was selected as marker for comparative genetic analyses and then amplified with primer C1-J-2183 / TL2-N-3014 (Simon *et al.* 1994).

To confirm the host plant of the new species, molecular characterization of the gut contents was performed. Two plant loci were amplified and sequenced from total DNA extracted from three specimens of the new species. The two amplified loci are used in plant DNA barcoding studies (Kress and Erickson 2007; Jurado-Rivera *et al.* 2009; García-Robledo *et al.* 2013): the plastid large subunit of the *ribulose-bisphosphate carboxylase rbcL* gene and the plastid *trnL* (UAA) intron. The gene regions were amplified using primers *rbcL* 1F/*rbcL* 724R and c A49325/d B49863 respectively for *rbcL* and *trnL* (Taberlet *et al.* 1991).

PCR amplifications were performed in 25  $\mu$ L reaction mix containing: 1X GoTaq reaction Buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>), 0.2 mM of each deoxynucleoside triphosphate, 0.5 pmol of each primer, 0.6 U of GoTaq DNA Polymerase and 10 ng of template DNA. PCR conditions used 3 min at 95°C followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 50°C/57°C (respectively for *cox1* and *trnL* genes), 57°C (for *rbcL* gene) and 1 min 20 s extension at 72°C, with a final single extra extension step of 10 min at 72°C. Successful amplification was determined by gel electrophoresis. PCR products were directly sequenced for both strands using the marker-specific primers from ABI technology (Applied Biosystems, Foster City, CA, USA). The obtained sequences were edited and primers removed using Geneious Pro 5.3 (Biomatters Ltd., Auckland, New Zealand) and deposited in the European Nucleotide Archive (ENA). The obtained *Pachybrachis* species *cox1* gene sequences were deposited under accession numbers HF947523-HF947548, while the plant sequences were under HF947549 (*rbcL*) and HF947550 (*trnL*).

**Bioinformatic and genetic analyses.** The obtained 26 *cox1* sequences plus one of *Cryptocephalus zambanellus* MARSEUL, considered as outgroup (accession number HE600388; Gómez-Zurita *et al.* 2012), were assembled in a dataset of 27 taxa and 829 nucleotides. The sequences were aligned at codon level using MUSCLE (Edgar 2004) with default parameters. The obtained dataset was used as input for the following analyses: (i) estimation of within and between species nucleotide mean distances; (ii) phylogenetic inference based on maximum likelihood; and (iii) delimiting the species from single-locus data (Generalized Mixed Yule Coalescent Approach; Pons *et al.* 2006; Fontaneto *et al.* 2007).

The evolutionary nucleotide model best-fitting to the analyzed datasets was selected using jModelTest 2 (Darriba *et al.* 2012), according to the Bayesian Information Criterion (BIC). The Hasegawa-Kishino-Yano model (Hasegawa *et al.* 1985), considering a proportion of invariable sites (I) and site-heterogeneity in evolutionary rates ( $\Gamma$ -distribution parameter), was selected as most appropriate. Within and between species nucleotide mean distances were calculated on the aligned sequence dataset with MEGA 5 (Tamura *et al.* 2011) using *p*-distances, gaps treated with partial deletion and standard deviation estimated by 500 bootstrap pseudoreplicates. Maximum likelihood analysis was performed on the *cox1* dataset using PhyML version 3.0 (Guindon *et al.* 2010) with the following options: evolutionary nucleotide model as obtained by model selection procedure; the best of NNI and SPR tree searching operation. The maximum likelihood tree, after removal of branches with length equal to zero, was converted to ultrametric using r8s 1.7 (Sanderson 2003) and the penalized likelihood strategy with a smoothing parameter of 1, selected after cross-validation of values between 0.1 to 100. The generalized mixed Yule coalescent (GMYC) (Pons *et al.* 2006; Fontaneto *et al.* 2007), developed to delimit species using single-locus molecular markers, was applied to the maximum likelihood ultrametric tree, after the removal of the outgroup, obtained using the mitochondrial *cox1* gene. The hypothesis tested assumed that the samples were divided into *n* independently evolving units. Comparison between the GMYC model to the null hypothesis (all samples belong to a single entity) was performed using log-likelihood ratio test. The GMYC method was implemented in the R package "splits" (SPeCies LImits by Threshold Statistics, available at <http://r-forge.r-project.org/projects/splits/>).

The obtained plant gene sequences (i.e. *rbcL* and *trnL*) were subjected to BLAST search (<http://www.ncbi.nlm.nih.gov/blast>) and compared with homologous sequences available in GeneBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The phylogenetic approach proposed by Jurado-Rivera *et al.* (2009) was adopted to perform taxonomic identification.

**Abbreviations.** MSNM: Museo civico di Storia naturale, Milano, Italy; DSPC: Davide Sassi personal collection, Castelmarte, Italy; MDPC: Mauro Daccordi personal collection, Verona, Italy; MMPC: Matteo Montagna personal collection, Inverigo, Italy; MSPC: Matthias Schöller personal collection, Berlin, Germany.

## Results and discussion

**Molecular analysis. species delimitation.** The *cox1* dataset obtained in this work encompasses 26 *Pachybrachis* gene sequences (see table 1) plus one of *Cryptocephalus zambanellus* for a total of eight species (seven *Pachybrachis*). No insertions/deletions are present within the dataset. Values of nucleotide mean distances between and within species based on *cox1* sequence dataset, after the removal of *C. zambanellus*, are presented in Table 2. Nucleotide mean distance between species was 13.8% (sd: 3.2%) with minimum and maximum values of 2% to 16.7%. The lowest value of between species nucleotide mean distance is recorded for specimens belonging to *P. karamani* and *P. holerorum* (2%, sd: 0.5%). This indicates that the two species are genetically closely related as hypothesized by morphological evidence. The value of the nucleotide mean distance within species is 0.5% (sd: 0.41%; min-max range 0% to 1.2%) with the specimens of *P. holerorum* from Passo del Pellizzone (Italy) showing 0.4% (sd: 0.13%; min-max range 0% to 0.8%). The close relationship of *P. holerorum* and *P. karamani*, is also supported by the maximum likelihood phylogeny based on *cox1* gene (figure 1). This result confirms the monophyly of the new species and of *P. karamani*, with incomplete lineage sorting absent.

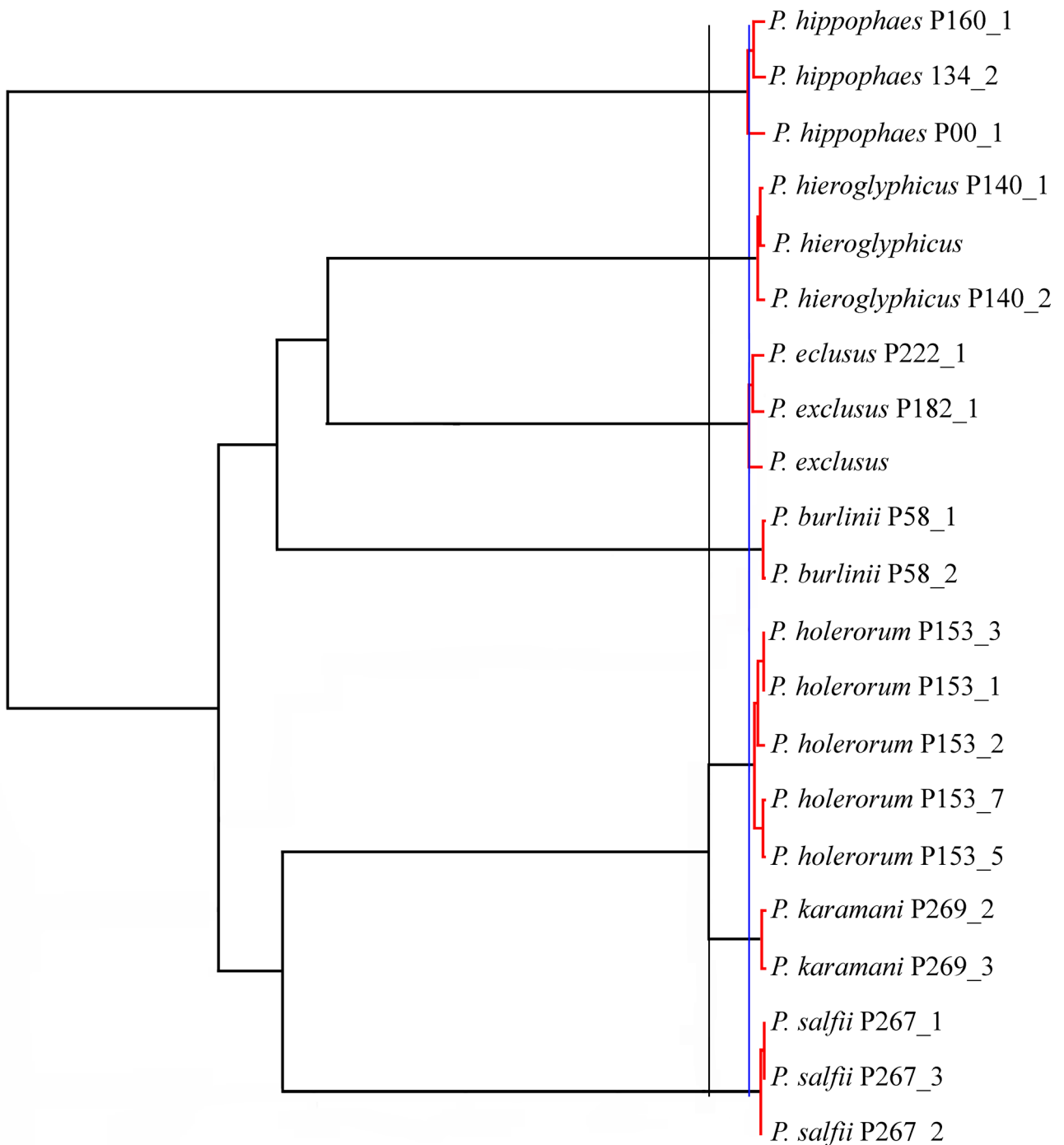
**TABLE 2.** Within and between *Pachybrachis* species nucleotide mean distances<sup>a</sup>.

Species <sup>b</sup>	<i>holerorum</i>	<i>karamani</i>	<i>salfii</i>	<i>burlinii</i>	<i>exclusus</i>	<i>hieroglyphicus</i>	<i>hippophaes</i>
<i>holerorum</i> (7)	<b>0.4 (0.13)</b>						
<i>karamani</i> (3)	2 (0.5)	<b>0 (0)</b>					
<i>salfii</i> (3)	11 (1.1)	10.5 (1)	<b>0.12 (0.09)</b>				
<i>burlinii</i> (2)	13.3 (1.1)	12.7 (1.1)	13.3 (1.1)	<b>0.13(0.12)</b>			
<i>exclusus</i> (3)	14.6 (1.1)	13.5 (1.1)	15.1 (1.2)	13.5 (1.1)	<b>1.2 (0.29)</b>		
<i>hieroglyphicus</i> (3)	14.1 (1.1)	14.2 (1.2)	15.1 (1.2)	14.8 (1.2)	15.9 (1.2)	<b>0.41 (0.18)</b>	
<i>hippophaes</i> (5)	16.3 (1.2)	15.1 (1.2)	14.8 (1.2)	16.3 (1.2)	16.4 (1.2)	16.7 (1.3)	<b>0.78(0.22)</b>

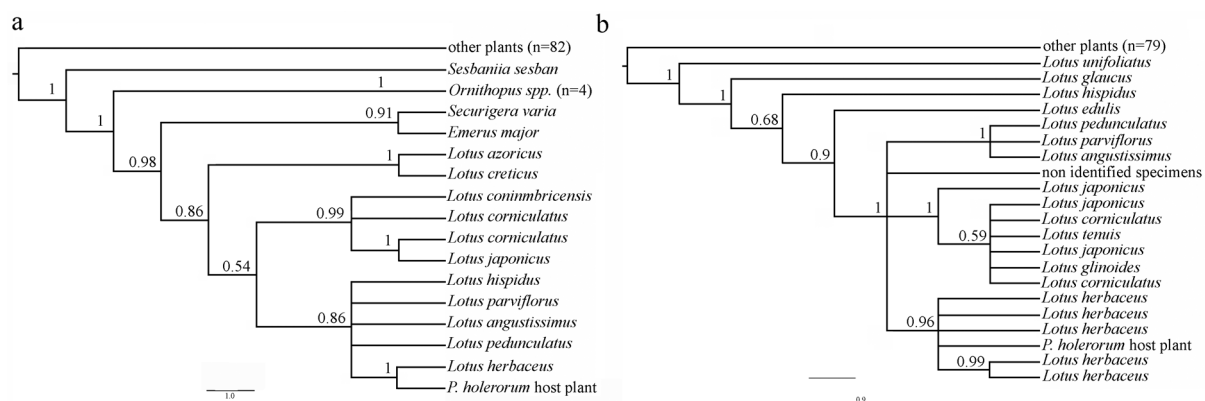
<sup>a</sup>Nucleotide mean distances are expressed as percentage; below the diagonal: values of *p* distances between the seven species of *Pachybrachis*; on the diagonal, in bold: values of *p* distances within the seven species of *Pachybrachis*; within brackets: standard deviation.

The results of the GMYC analysis performed on the *cox1* maximum likelihood tree are reported in figure 1. The GMYC model exhibited a significantly better likelihood than the null model ( $\log L_{\text{GMYC}} = 79.3$ ,  $\log L_{\text{NULL}} = 69$ ;  $2\Delta L = 20.7$ ,  $\chi^2$  test,  $P = 0.00012$ ). The model identified a transition in the tree branching rate from Yule to Coalescent model, indicating the boundary between and within species, at threshold time of -0.027. This threshold identifies seven maximum likelihood clusters (confidence intervals 6-7) that fit the six known species of *Pachybrachis* plus the new species. Nevertheless, the minimum value of the confidence interval identifies six clusters ( $\log L_{\text{GMYC}} = 78.8$ ), suggesting the specimens of *P. holerorum* and *P. karamani* belong to the same entity. The small nucleotide mean distance between the two morphological species (2%, sd: 0.5%), indicates that morphology evolves more rapidly than neutral genetic markers (Paterson 1993; Arnqvist 1998; McPeck *et al.* 2008).

**Molecular analysis. characterization of the host plants.** The amplification of chloroplast genes (i.e. *rbcL* and *trnL*), from total host DNA resulted in a single gel electrophoresis band pattern for the specimens tested. The *rbcL* sequences obtained from three different specimens of *P. holerorum* were identical at nucleotide level; the same results have also been obtained for the *trnL* sequences. BLAST analyses performed on the *rbcL* and *trnL* gene sequences provided identity with 100% (E-value = 0) and 99% (E-value = 0) probability respectively, with *Dorycnium pentaphyllum* (now *Lotus herbaceus* (Vill.) Peruzzi (Peruzzi 2010)). The two gene sequences clustered within the species belonging to the genus *Lotus* (with 0.86 BPP for *rbcL* gene and 1 BPP for *trnL*), as sister to *L. herbaceus* supported by 1.0 BPP (*rbcL* gene) and 0.96 BPP (*trnL* gene) (Fig. 2).



**FIGURE 1.** *Cox1* maximum likelihood ultrametric rooted tree obtained by GMYC analysis depicting the seven identified species of *Pachybrachis* (in red). The outgroup (*Cryptocephalus zambanellus*) and all branches with 0 length were removed in order to perform the analysis (see Materials and Methods). The vertical blue line depicts the estimated threshold between and within species, with the highest value of maximum likelihood; the vertical black line depicts the lowest value of the identified maximum likelihood confidence interval.



**FIGURE 2.** Synthetic cladogram of the *P. holerorum* Montagna & Sassi diet obtained with Bayesian Inference on *rbcL* and the *trnL*(UAA) gene alignments (respectively **a** and **b**). Values of Bayesian Posterior Probabilities > 0.5 are reported above the branches.

### *Pachybrachis holerorum* Montagna & Sassi, new species (figure 3)

**Material examined.** *Holotype*. Male, deposited in MSNM. Original label: Emilia-Romagna, Parma, Passo del Pellizzone, 1000 m, 8.VI.2011, Montagna & Sassi leg., 44°40'48.84"N 9°44'42.66" E [white label, printed] / *Pachybrachis holerorum* n. sp. holotypus Montagna & Sassi des. [red, printed]. DNA extracted to perform the amplification through PCRs of the genes: *cox1*, *rbcL* and *trnL*.

*Paratypes*. 46 specimens: Emilia-Romagna, Parma, Passo del Pellizzone, 1000 m, Montagna & Sassi leg., 44°40'48.84"N 9°44'42.66" E [white labels, printed], 8♂ and 19♀, DNA extracted from 4♂ (gene *cox1*) and 2♀ (genes: *cox1*, *rbcL* and *trnL*); Lombardia, PV, dint. Brallo di Pregola, Cima Colletta, 1366 m, 16. VI. 2011, Sassi leg., 44°42'37.59"N 9°15'36.55"E [white labels, printed], 3♂ and 1♀; Em. Romagna, MO, Nirano, Salse di Nirano, 30.V.2007, D. Sassi leg. [white label, handwritten], 1♂ and 2♀; LOMB. Emilia, Lago di Trebecco, 11.6.1997, leg. D. Sassi, [white label, printed], 2♂; Lombardia, PV, R, de' Giorgi, 16.6.1990, D. Sassi, 3♂ and 6♀ [white label, printed]; Em. Romagna, MO, Sassatella, 800 m, 30.V.2007, D. Sassi leg., [white label, printed], 1♂. All paratypes with our label: *Pachybrachis holerorum* n. sp. paratypus Montagna & Sassi des. [red, printed]. Paratypes in MSNM, DSPC, MMPC, MSPC, MDPC.

**Etymology.** The name is genitive plural of *holus*, used by the Roman poet Lucilius to indicate leguminous forbs.

**Description of male.** Total length: male = 2.9±0.1 mm. Head yellow except vertex, a median longitudinal stripe with bifurcated apex along frons, antennae sockets, anterior margin of clypeus black. Frons shining, covered with fairly impressed punctation, denser on clypeus and above insertion of antennae, sparser on frons. Antennae filiform, brownish, segments 1–5 partly yellowish.

Pronotum black with yellow bands along anterior and lateral borders; anterior band lightly thickened at anterior angles and with short median posteriorly directed vitta; two anteriorly directed yellow lines from basal margin at sides of scutellum, 1.5 times wider than long, regularly curved at sides, with maximum width at about middle; punctation deep, denser at sides, slightly sparser on disc. Scutellum elevated, black, minutely punctate, apically truncate. Elytra coarsely punctured, partially arranged in striae; interstices raised, black with yellow pattern slightly raised from black surface, arranged as follows (spots and vittae may be interrupted or absent): narrow bands and vittae along anterior, lateral and posterior margins; narrow vitta along posterior half of sutural margin; two elongated spots near suture, plus one behind scutellum and one larger, in median position; longitudinal vitta on anterior margin lateral to humeral callus; post-median spot on disc; several smaller spots variously arranged on elytral surface. Epipleura black in posterior half, partly yellow anteriorly, with one or two series of irregularly aligned punctures on edges. Venter black, mesepimera with yellow spot, sometimes indistinct. Abdominal ventrites sparsely punctured and covered with rather sparse, long whitish hairs; ventrite 5 with shallow depression, glabrous.



FIGURE 3. *Pachybrachis holerorum* Montagna & Sassi, dorso-lateral view.

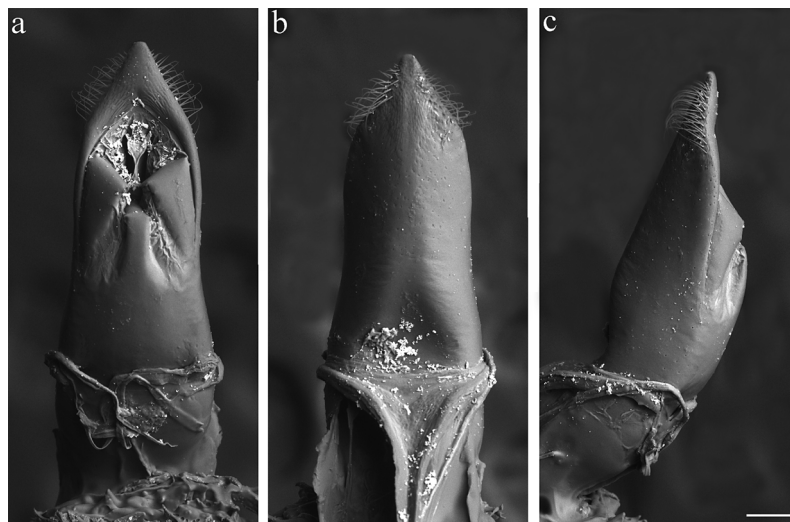


FIGURE 4. *Pachybrachis holerorum* Montagna & Sassi, holotype, median lobe of aedeagus, scale bar = 100  $\mu$ m, magnification 130x. a: dorsal, b: ventral, c: lateral view.

Legs yellow, fore femora blackish along posterior edge; median and hind femora largely darkened along basal half, fore tibiae yellow; meso and metatibiae darkened at apex; tarsi mostly yellow, more or less darkened towards apex. First protarsomere moderately broadened, as wide as apex of tibia.

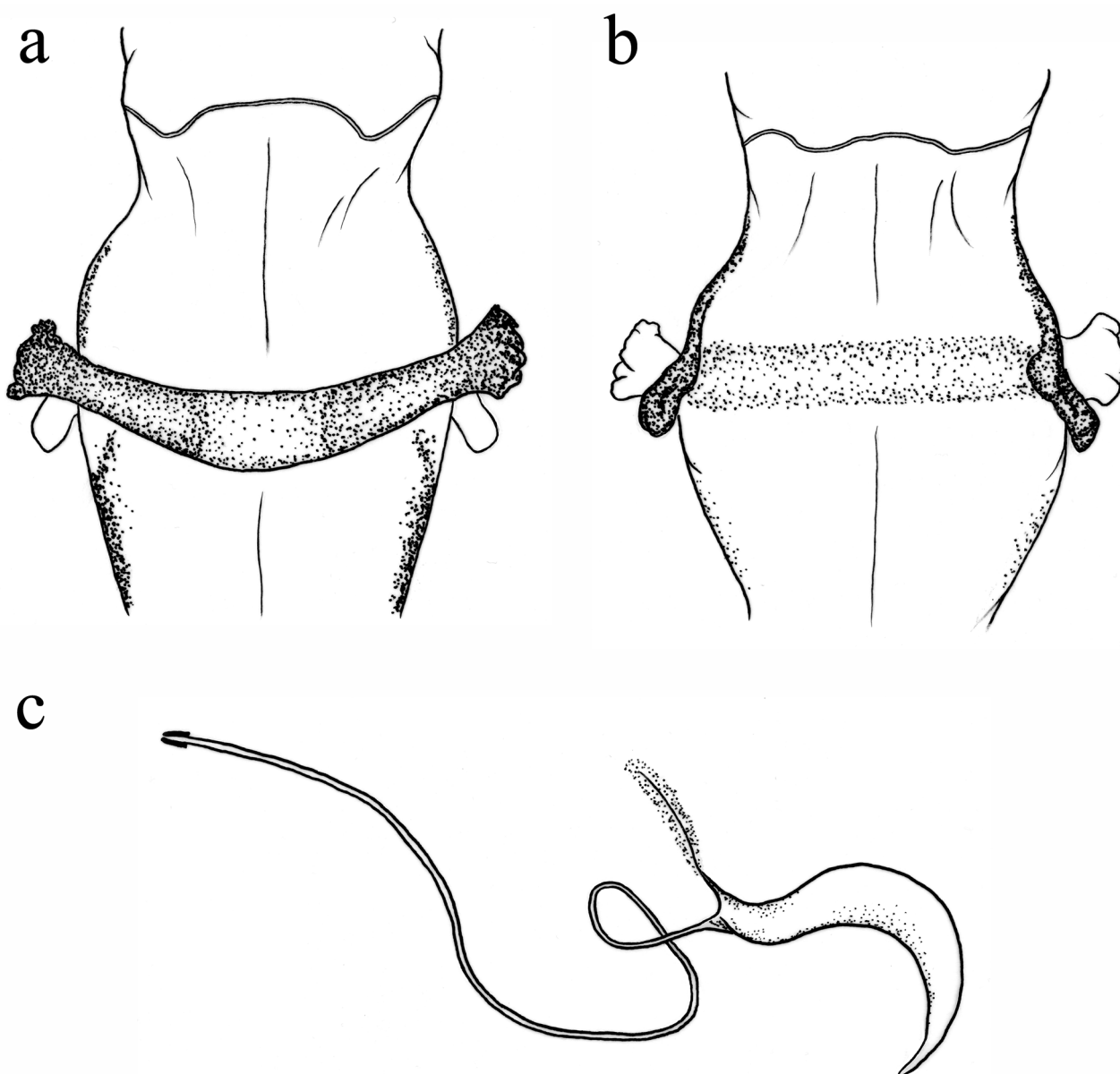
Apex of aedeagal median lobe (figure 4) acute, lateroventrally with row of white hairs, shaft thin and elongated, slightly careened along ventral surface, venter straight in lateral view.

Female differs from male in: larger and stouter body (length  $3.3 \pm 0.1$  mm); frons broader and, as result, eyes more separated; generally reduced yellow pattern; first protarsomeres significantly narrower than tibiae apically; rectal apparatus (figure 5a,b) with two dorsal and one ventral sclerites; dorsal sclerites short and narrow, slightly wider than rectum, transverse connection across dorsal fold not perceptible, so sclerites essentially reduced to the apodemes only; ventral sclerite ribbon-like, evenly pigmented in the middle, with large and rounded apodemes at both ends, wider than rectum; dorsal and ventral sclerotizations of lateral fold present; spermatheca (figure 5c) sickle-shaped, lightly pigmented, basal part not swollen; base reflexed, with gland and duct insertions well

sclerotized, so that it seems bifurcated; duct not coiled, quite short, its insertion on bursa copulatrix not enlarged and only feebly and briefly pigmented.

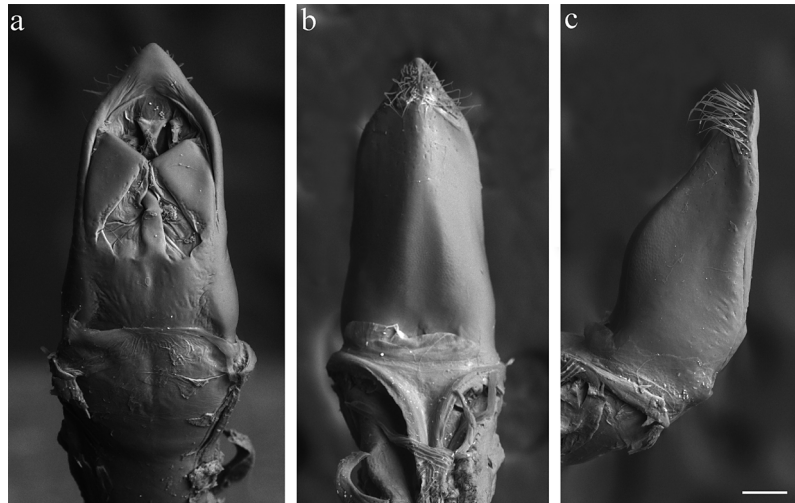
**Diagnosis.** *Pachybrachis* of medium-small size, characterized by elytral pattern, with marginal yellow stripe from anterior edge, to and around posterior margin, then along apical half of suture. This stripe interrupted only at humeral callus. Similar elytral marginal stripes are present in *P. karamani* Weise and *P. fimbriolatus* Suffrian, with which the new species forms a group, based on morphology. In *P. karamani*, the elytral yellow spots are generally smaller, and the irregularly distributed small spots that characterize *P. holerorum* are almost entirely missing. *Pachybrachis fimbriolatus* is distinguished from the new species by: less transverse and more minutely and densely punctured pronotum, reduced elytral yellow spotting, particularly, postmedian dot nearly always absent. The new species clearly differs from the all *Pachybrachis* species in the shape of the median lobe of aedeagus (figures 4 and 6).

**Distribution.** The new species is endemic to Northern Apennines, Italy. The type locality is Emilia Romagna, Piacenza Prov., Passo del Pellizzone (also written “Pelizzzone”) (44°40'48.84"N 9°44'42.66"E).



**FIGURE 5.** *Pachybrachis holerorum* Montagna & Sassi, female rectal apparatus. **a:** ventral, **b:** dorsal, **c:** spermatheca.





**FIGURE 6.** *Pachybrachis karamani* Weise, lectotype, median lobe of aedeagus, scale bar = 100  $\mu$ m, magnification 140x. **a:** dorsal, **b:** ventral, **c:** lateral view.

**Remarks.** The biology of *P. holerorum* is poorly known. At Passo del Pellizzone it was collected in early June on *Lotus herbaceus*. This possible host was confirmed by the gut analysis of plant DNA from specimens at this locality. The preference of many species of the genus *Pachybrachis* for Fabaceae is well known (Jolivet and Hawkeswood 1995).

*Pachybrachis holerorum* is restricted to the north and west Apennines and *P. karamani* is on the Adriatic slopes of North and Central Apennines (Sassi 2006). Molecular and morphological evidence show that *Pachybrachis holerorum* and *P. karamani* are recently diverged sister species in adjacent allopatric ranges, suggesting vicariant origin for the two species.

We take the opportunity here to designate a lectotype for *P. karamani*, to fix the identity of this species which is similar to *P. holerorum*. The syntypic series of *P. karamani* consists of five specimens. We designate a male as lectotype, labelled as follows: Spalato (handwritten, white label) / Typus (printed, red label) / *karamani* Ws. (handwritten, white label) / Zool. Mus. Berlin (printed, yellow label) / Sintypus (printed, red label) / *Pachybrachis karamani* Weise, 1893 labelled by MNHUB 2012 (printed, red label). *Pachybrachis karamani* Weise, 1893 LECTOTYPUS Montagna & Sassi des. (Printed / red label). The remaining paralectotypes are labelled as follows: 2 ♂, 1 ♀ : Spalato (handwritten, white label) / Typus (printed, red label) / Zool. Mus. Berlin (printed, yellow label) / Sintypus (printed, red label) / *Pachybrachis karamani* Weise, 1893 labelled by MNHUB 2012 (printed, red label). *Pachybrachis karamani* Weise, 1893 PARALECTOTYPUS Montagna & Sassi des. (Printed / red label). Female: Spalato Karam (handwritten, white label) / Karamani det. Burlini 1968 (partly printed, white label) / Zool. Mus. Berlin (printed, yellow label) / Karamani (handwritten, white label) / Typus (printed, red label) / *Pachybrachis karamani* Weise, 1893 labelled by MNHUB 2012 (printed, red label). *Pachybrachis karamani* Weise, 1893 PARALECTOTYPUS Montagna & Sassi des. (Printed / red label).

### Concluding remarks

In our study of these *Pachybrachis* populations, the combination of three different approaches (morphological features of the aedeagus, nucleotide distance values and a method delimiting species based on single-locus molecular data), has provided evidence for a new species and its sister taxon.

The results contained in this work strongly confirm the urgent need to increase efforts to uncover the real biodiversity of the European fauna, in particular the Mediterranean region.

## Acknowledgements

We cordially thank Dr. Johannes Frisch and Joachim Willers of the Museum für Naturkunde (Berlin) which give us the opportunity to study the type series of *P. karamani* Weise, 1893. Moreover we thank Dr. Matthias Schöller (Berlin) for the helpful comments, Michele Zilioli of the Museo civico di Storia naturale di Milano for the SEM support, Loris Colacurcio (Bologna) for his precious help during the collecting campaigns, the Editor and two anonymous reviewers for their comments and suggestions to the preliminary version of the manuscript.

This work was partially supported by "Accordo di Programma, affermazione in Edolo del Centro di Eccellenza Università della Montagna" MIUR-Università degli Studi di Milano, prot. n. 1293-05/08/2011.

## References

- Arnqvist, G. (1998) Comparative evidence for the evolution of genitalia by sexual selection. *Nature*, 393, 784–786.  
<http://dx.doi.org/10.1038/31689>
- Bienkowski A.O. (1999) *Key to identification of leaf-beetles (Coleoptera Chrysomelidae) of the European part of Russia and neighboring European countries*. Mikron-print, Moscow, 204 pp. [in Russian, English abstract]
- Burlini, M. (1957) Un nuovo *Pachybrachis* dell'Isola di Cefalonia (Col. Chrysomelidae). *Bollettino della Società Entomologica Italiana*, 87, 25–26.
- Burlini, M. (1959) Note su alcuni *Pachybrachys* appenninici e descrizione di una nuova razza (Coleoptera Chrysomelidae). Ricerche sulla fauna appenninica XXXVI. *Memorie del Museo Civico di Storia Naturale di Verona*, 7, 199–201.
- Daccordi, M. & Ruffo, S. (1971) Coleotteri crisomelidi raccolti nelle isole Ponzie e descrizione di una nuova specie del genere *Pachybrachis* Redt. *Fragmenta Entomologica*, 8, 41–48.
- Daccordi, M., & Ruffo, S. (1975). Coleotteri crisomelidi delle Isole Egadi e descrizione di una nuova specie del genere *Pachybrachis* Chev. *Bollettino del Museo Civico di Storia Naturale di Verona*, 1, 427–437.
- Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772.  
<http://dx.doi.org/10.1038/nmeth.2109>
- Edgar, R.C. (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, 5, 113.
- Fontaneto, D., Herniou, E.A., Boschetti, C., Caprioli, M., Melone, G., Ricci, C. & Barraclough, T.G. (2007) Independently evolving species in asexual bdelloid rotifers. *PLoS Biology*, 5, e87.  
<http://dx.doi.org/10.1371/journal.pbio.0050087>
- García-Robledo, C., Erickson, D.L., Staines, C.L., Erwin, T.L. & Kress, W.J. (2013) Tropical plant-herbivore networks: reconstructing species interactions using DNA barcodes. *PLoS One*, 8, e52967.
- Gómez-Zurita, J., Sassi, D., Cardoso, A. & Balke, M. (2012) Evolution of *Cryptocephalus* leaf beetles related to *C. sericeus* (Coleoptera: Chrysomelidae) and the role of hybridization in generating species mtDNA paraphyly. *Zoologica Scripta*, 41, 47–67.  
<http://dx.doi.org/10.1111/j.1463-6409.2011.00500.x>
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, 59, 307–321.
- Hasegawa, M., Kishino, H. & Yano, T. (1985) Dating of human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, 22, 160–174.  
<http://dx.doi.org/10.1007/bf02101694>
- Jolivet, P. & Hawkeswood, T.J. (1995) *Host-plants of Chrysomelidae of the World. An Essay about the Relationships between the Leaf Beetles and their Food-plants*. Backhuys Publishers, Leiden, 281 pp.
- Jurado-Rivera, J.A., Vogler, A.P., Reid, C.A.M., Petitpierre, E. & Gomez-Zurita, J. (2009) DNA barcoding insect-host plant associations. *Proceedings of the Royal Society B*, 276, 639–648.  
<http://dx.doi.org/10.1098/rspb.2008.1264>
- Kress, W.J. & Erickson, D.L. (2007) A Two-locus global DNA barcode for land plants: The coding rbcL gene complements the non-coding trnH-psbA spacer region. *PLoS One*, 2, e508.  
<http://dx.doi.org/10.1371/journal.pone.0000508>
- McPeck, M.A., Shen, L., Torrey, J.Z. & Farid, H. (2008) The tempo and mode of three-dimensional morphological evolution in male reproductive structures. *American Naturalist*, 171, 158–178.  
<http://dx.doi.org/10.1086/587076>
- Montagna, M. (2011) *Pachybrachis sassii*, a new species from the Mediterranean Giglio Island (Italy) (Coleoptera, Chrysomelidae, Cryptocephalinae). *Zookeys*, 155, 51–60.  
<http://dx.doi.org/10.3897/zookeys.155.1951>

- Paterson, H.E.H. (1993) *Evolution and the Recognition Concept of Species*. Johns Hopkins University Press, Baltimore, 234 pp.
- Peruzzi, L. (2010) Notulæ: 1734-1735. *Lotus germanicus* (Fabaceae), *L. herbaceus* (Fabaceae). *Informatore Botanico Italiano: Bollettino della Società Botanica Italiana*, 42, 528–529.
- Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun, S., Sumlin, W.D. & Vogler, A.P. (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, 55, 595–609.
- Sabree, Z.L., Huang, C.Y., Okusu, A., Moran, N.A. & Normark, B.B. (2012) The nutrient supplying capabilities of *Uzinura*, an endosymbiont of armoured scale insects. *Environmental Microbiology*, 15 (7), 1988–1999.  
<http://dx.doi.org/10.1111/1462-2920.12058>
- Sanderson, M.J. (2003) r8 s: inferring absolute rates of molecular evolution, divergence times in the absence of a molecular clock. *Bioinformatics*, 19, 301–302.  
<http://dx.doi.org/10.1093/bioinformatics/19.2.301>
- Sassi, D. (2006) Insecta Coleoptera Chrysomelidae Cryptocephalinae. In: Ruffo, S. & Stoch F. (Eds.), *Checklist and distribution of the Italian fauna*. Memorie del Museo Civico di Storia Naturale di Verona, pp. 221–223.
- Sassi, D. (2012) The Cryptocephalini of the Maltese Islands (Coleoptera, Chrysomelidae). *Bulletin of Entomological Society of Malta*, 5, 87–92.
- Schöller, M., Löbl, I. & Lopatin, I. (2010) Cryptocephalini. In: Löbl, I. & Smetana, A. (Eds.), *Catalogue of Palaearctic Coleoptera, Vol. 6. Chrysomeloidea*. Apollo Books, Stenstrup, pp. 606–617.
- Simon, C., Frati, F., Beckenbach A., Crespi B, Liu, H. & Flook, P. (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87, 651–701.
- Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. *Plant Molecular Biology*, 17, 1105–1109.  
<http://dx.doi.org/10.1007/bf00037152>
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.  
<http://dx.doi.org/10.1093/molbev/msr121>
- Weise, J. (1893) *Pachybrachys karamani*. *Deutsche Entomologische Zeitschrift*, II, 348.