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Description and DNA barcoding of a new Iberian species of *Pijnackeria* (Scali, 2009) from Sierra Nevada, Spain (Phasmida: Diapheromeridae)

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Abstract

Male, female and egg of *Pijnackeria recondita* **sp. n.** are described from specimens collected at about 2,000 m in Sierra Nevada (Spain) feeding on *Cytisus scoparius*. The number of antennae segments in males, the smooth thorax in females and the different sculpturing of the egg capsule are the main differences from the other species of the genus. In addition, DNA barcode sequences (COI and COII) clearly differ from the other Iberian species of the genus. For COI, K2P minimum distance between the new species and the most morphological related species, *Pijnackeria hispanica* (Bolívar, 1878), showed a mean of 8%. In the case of COII, comparison with the other species of *Pijnackeria*, showed a K2P minimum distance range from 8 to 10.5% (mean 9.2%); and comparison with the species of the related genus *Leptynia,* showed a K2P minimum distance range from 7.1 to 10.5%.

Key words: Phasmida, Diapheromeridae, New species, Insect morphology, Insect barcoding, Sierra Nevada Spain

Abbreviations.

MNCN	National Museum of Natural Sciences, Madrid, Spain.
RCBA-UMU	Research Collection of Biología Animal, University of Murcia, Spain.

Introduction

Diapheromeridae Kirby, 1904 (Phasmida, Verophasmatodea) are small- to medium-sized and of a mixture of winged and wingless stick insects species with their legs usually without spines. Their antennae are often longer than their forelegs, with indistinct segments, or very short ones. Globally, 180 genera and approximately 1,600 species of diapheromids are known although numerous species still await formal description.

In the Iberian Peninsula, four stick insect genera are present: *Bacillus* and *Clonopsis* (Bacillidae) and *Leptynia* and *Pijnackeria* (Diapheromeridae). While the former two genera are distributed in the Mediterranean region, *Leptynia* and *Pijnackeria* are only distributed in the Iberian Peninsula up to Southern France. The genus *Pijnackeria* Scali, 2009 was disaggregated from *Leptynia* Pantel, 1890, on the basis of several distinguishing features (Scali 2009). Later on, this complex was also split into 6 species based mainly on karyological and mtDNA (COII) differences (Scali *et al.* 2013). Broad distributional range was pointed out for the parthenogenetics *Pijnackeria hispanica* (Bolívar, 1878) and *P. masettii* Scali, Milani & Passamonti, 2013 while the other four species (*P. lucianae* Scali *et al.,* 2013*, P. barbarae* Scali *et al.,* 2013*, P. lelongi* Scali *et al.,* 2013 and *P. originis* Scali *et al.,* 2013) were located in restricted mountainous areas in the Eastern Iberian Peninsula.

The mitochondrial gene COI has been widely used by taxonomists as a standard DNA barcode sequence for the identification of many animal species and usually has considerable congruence with morphology-based identifications (e.g., Hebert *et al*. 2003; Hausmann *et al.* 2011; Huemer & Hebert 2011; Park *et al.* 2011). DNA barcoding has its disadvantages (Taylor & Harris 2012), as it is of limited use to identify certain species and is not efficiently amplified by PCR in all animal taxa, but it may be used as an additional, and apparently very powerful, method in taxonomy (Schlick-Steiner *et al.* 2010). Despite the wide use of DNA barcodes in the current taxonomy

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and biodiversity studies, the method has only scarcely been used in taxonomic studies except for the Australian and New Zealand stick insect (e.g., Trewick *et al.* 2005, 2008; Buckley *et al.* 2009, 2010; Morgan-Richards *et al.* 2010; Schwander *et al.* 2011; Nosil *et al.* 2012). The COII mitochondrial gene has been sequenced over a wide variety of taxa and has proven to be useful for phylogenetic research, especially in Phasmida (Mantovani *et al*. 2001; Passamonti *et al.* 2004; Ghiselli *et al.* 2007; Scali 2009; Scali *et al.* 2012, 2013). Species identification using COII sequences had a higher frequency of success and yielded lower intra- and higher interspecific genetic divergence values than the other markers such as in Hemiptera Aphidae (Chen *et al*. 2012) and Siphonaptera Pulicidae (Lawrence *et al.* 2014). Roe & Sperling (2007) recommend that researchers should maximise sequence COI-COII length to increase the probability of sampling regions of high phylogenetic information, and to minimise stochastic variation in estimating total divergence.

In this article, we provide a description of *Pijnackeria recondita* **sp. n.**, collected from Sierra Nevada (Spain). Both sexes and egg of the new species are richly illustrated. All collected samples have some consistent morphological features which differ from the other *Pijnackeria* species, especially the smooth meso- and metanotum in females (granulated in the other species except for *P. lelongi*) and a constant number of 11 antennae segments in males (usually 16 in males from other species). In addition, mtDNA sequences (COI-COII) were used to assess genetic divergence between the new species and species of related genera *Pijnackeria* and *Leptynia*.

Material and methods

Collecting and maintenance in laboratory. Eight females and nine males of *P. recondita* **sp. n.** were picked by hand in Sierra Nevada (Spain); two females and one male were collected alive in late July 2013 and the rest of them in late July 2014. The females collected in 2013 survived for few days, and laid some eggs before they died, which have been incubated on silica sand at room temperature (20–25ºC) and 70–80% relative humidity (RH). The specimens collected in 2014 survived for 3 months in captivity, feeding on *Dorycnium pentaphyllum,* as seen with other species of *Pijnackeria* in their natural habitat (e.g. *P.hispanica*, *P. masettii,* etc). Matings were frequently observed (Fig. 6A) and 40 eggs were dropped during July–September. These eggs were incubated on silica sand at room temperature and brought to the refrigerator (4ºC and 70–80% RH), from November to March, to simulate a winter period.

Morphological procedures. Specimens were preserved, dried and pinned in RCBA-UMU. One of the females captured in 2013 has been designated as holotype and the rest of the females, males and ten eggs were designated as paratypes.

The right middle-leg of three males and three females, including the holotype were removed and fixed in 100% ethanol at -20ºC to provide molecular samples. The same procedure was done with five *P. hispanica* females from the RCBA-UMU.

Macro-photographs of the insects and eggs were taken with a Canon EOS 550D camera equipped with a Tamron 90 mm f2.8 DI USD VC Macro lens and Yongnuo Speedlite YN 568EX-II external flash. Measurements were taken using a digital calipers. The terminology used to describe egg structures generally follows that of Clark-Sellick (1997).

The collection of Iberian phasmids from the MNCN has been revised. A total of 149 *Pijnackeria* females from 39 different locations and 20 *Pijnackeria* males from 6 different locations were checked. For *P. lelongi*, Philippe Lelong kindly shared information and pictures of the featured species types.

Molecular procedures. *Pijnackeria* specimens used for mitochondrial gene cytochrome oxidase subunit 1 (COI) and cytochrome oxidase subunit 2 (COII) sequencing are reported in Table 1.

Total DNA was extracted from the right middle-leg by mechanical mincing of the tissue in buffer solution using Invisorb Spin Tissue Mini Extraction Kit (Invitek, Germany). DNA was stored in bidistilled water at -20°C. Amplification of DNA was done using a direct method with primers that amplified a sequence between 650 and 750 bp in length. COI and COII were sequenced using the primers LCO1490 and HCO2198 for COI (Folmer *et al.* 1994) and TL2-J-3034 and TK-N-3785 for COII (Simon *et al.* 1994) (Table 2).

COI and COII PCR's were carried out in a total volume of 136.5 µl and 73.5 µl respectively, with Kapa Taq PCR kit (Kapa Biosystems, Wilmington, USA). The reaction mixture contained 10X Kapa Taq buffer, 5 U/µl Kapa TAQ DNA Polymerase, 10 mM dNTP, 10 µl of each primer (10 µM) and 2 µl of extracted DNA (concentrations varied from 2.67 nµ/µl to 16.55 nµ/µl).

TABLE 1. *Pijnackeria* specimens used in DNA barcoding (COI and COII). Coordinates are given in geographic coordinate system format.

Species	Storage code in RCBA-UMU	Gender	Capture location	Gene target	GenBank
P. recondita	PVRFAS0001	Male	Sierra Nevada (Granada). 37º 6.652'N, 3º 25.983W	COI	KT799537
				COII	KT799543
P. recondita	PVRFAS0002	Female	Sierra Nevada (Granada). 37º 6.652'N, 3º 25.983W	COI	KT799538
				COII	KT799544
P. recondita	PVRFAS0024	Female	Sierra Nevada (Granada). 37º 6.367'N, 3º 25.241W	COI	KT799539
				COII	KT799545
P. recondita	PVRFAS0025	Female	Sierra Nevada (Granada). 37º 6.367'N, 3º 25.241W	COI	KT799540
				COII	KT799546
P. recondita	PVRFAS0027	Male	Sierra Nevada (Granada). 37º 6.367'N, 3º 25.241W	COI	KT799541
				COII	KT799547
P. recondita	PVRFAS0028	Male	Sierra Nevada (Granada). 37º 6.367'N, 3º 25.241W	COI	KT799542
				COII	KT799548
P. hispanica	PVRFAS0005	Female	Sierra de Huetor (Granada). 37º 17.845'N, 3º 27.000'W	COI	KT799532
P. hispanica	PVRFAS0007	Female	Sierra Nevada (Granada). 37º 6.644'N, 3º 26.157'W	COI	KT799533
P. hispanica	PVRFAS0008	Female	Sierra Nevada (Granada). 37º 6.644'N, 3º 26.157'W	COI	KT799534
P. hispanica	PVRFAS0021	Female	Serrania de Cuenca (Cuenca). 39º 59.527'N, 1º 40.326'W	COI	KT799535
P. hispanica	PVRFAS0022	Female	Serrania de Cuenca (Cuenca). 39º 59.527'N, 1º 40.326'W	COI	KT799536

TABLE 2. Primers used in the amplification of COI and COII.

Gene	Name of primer	Sequence of primer	Max/Min length (bp) of sequenced product
COI	LCO1490	5'-ggtcaacaaatcataaagatattgg-3'	664/716
	HC02198	5'-taaacttcagggtgaccaaaaaatca-3'	
COII	TL2-J-3034	5'-aatatggcagattagtgca-3'	730/740
	TK-N-3785	5'-gtttaagagaccagtacttg-3'	

PCR was carried out on a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA), with the following cycling parameters for COI: a 3 min denaturing step at 94°C, followed by 37 cycles of 30 s at 94°C, 30 s at 48°C, and 60 s at 72°C, and a subsequent 10-min final extension at 72°C. The PCR conditions for the amplification of COII was 5 min denaturing step at 95°C, followed by 38 cycles of 60 s at 94°C, 60 s at 54°C, and 90 s at 72°C, and a subsequent 10-min final extension at 72°C.

The presence of PCR products were checked in agarose gel with 3% RedSafe™ Nucleic Acid Staining Solution (Intron Biotechnology, Seongnam, South Korea) and then 2 µl of PCR product were diluted in 3 µl bidistilled water and 0.5 µl of 0.5 µm primers were added. Sequencing was carried out by SECUGEN (Madrid, Spain).

The rest of the COII sequences of Iberian *Pijnackeria* and *Leptynia* species were obtained from Genbank. *Clonopsis gallica* Charpentier, 1825, *Bacillus rossius* Rossi, 1787, *B. atticus* Brunner von Wattenwyl, 1882, *B. grandii* Nascetti & Bullini, 1981 and *Medaura scabriuscula* (Wood-Mason, 1873) were utilised for comparison as outgroups. Representatives from each of these genera were solely used to provide a root for the topology of the

putative ingroup and were included in the analysis without an *a priori* assumption regarding their outgroup status. In total, 16 species including three subspecies and 119 specimens were used for the taxonomic analysis.

COI and COII sequences were aligned with the CLUSTAL algorithm of MEGA6 program (Tamura *et al.* 2013). COII diagnostic sites from species alignments are presented as Supplementary material (S1). In order to assess COI divergence between the new species and *P. hispanica* and COII divergence between the new species with the other 12 Iberian stick insect species and subspecies, we used the Kimura 2-Parameter (K2P) algorithm with MEGA6 software, including all sites with the pairwise deletion option (Table 3).

TABLE 3. Interspecific mean K2P (Kimura 2-Parameter) divergences (mean pairwise distances) based on the analysis of COII fragments (>500 bp) between *Pijnackeria recondita* n. sp. and 12 Iberian *Leptynia* and *Pijnackeria* species or subspecies and *Bacillus rossius*, *Clonopsis gallica* and *Medaura scabriuscula* as outgroup taxa.

	Pijnackeria recondita
Medaura scabriuscula	16,00%
Clonopsis gallica	14,60%
Bacillus rossius	11,40%
Leptynia attenuata attenuata	11,40%
Leptynia montana	10,80%
Leptynia attenuata iberica	10,70%
Leptynia caprai	10,40%
Leptynia attenuata algarvica	10,20%
Leptynia annaepaulae	7,90%
Pijnackeria originis	10,20%
Pijnackeria lucianae	10,20%
Pijnackeria barbarae	10,10%
Pijnackeria hispanica	10,00%
Pijnackeria lelongi	9,90%
Pijnackeria masettii	9,90%

For tree construction based on COII K2P distances, a random specimen sequence of each of the species of *Leptynia* and *Pijnackeria* genera and the six outgroup species sequences in Passamonti *et al.* (2004) and Ghiselli *et al.* (2007) were used. Neighbor-Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) trees were calculated to visualise similarity among Iberian stick insect species. All trees presented the same topology and were practically identical; therefore, only the ML tree is presented (Fig. 1). Because one gene is far too little for reasonable phylogenetic analysis (Gatesy *et al.* 2007), the trees presented here do not reliably illustrate evolutionary relationships among the sequenced taxa.

Results

Diagnosis of the new species. Typical morphology for *Pijnackeria* genus, but differing from the other species of the genus because of the smooth meso- and metathorax for females (Fig. 2), and the constant number of 11 antennal segments for males and females (Figs. 3D, 4D). Females of *P. lelongi* also have a smooth meso- and metathorax but this species presents 16 antennae segments. Moreover, the egg of *P. recondita* n. sp. does not present the typical chorionic netting pattern as the other taxa of the genus (Fig. 5).

Holotype: ♀, Sierra Nevada, Granada, Spain, 37º 6.652' N, 3º 25.983' W, 1903 m, on *C. scoparius*, 31.VII.2013, P. Valero & J.J. Guerrero leg. (RCBA-UMU). For body part measurements see table 4.

Paratypes (9 ♂♂, 7 ♀♀ and 10 eggs): 1♂, 1♀, Sierra Nevada, Granada, Spain, 37º 6.652' N, 3º 25.983' W, 1903 m, on *C. scoparius*, 31.VII.2013, P. Valero & J.J. Guerrero leg. (RCBA-UMU); 8 ♂♂, 6 ♀♀, Sierra Nevada, Granada, Spain, 37º 6.367' N, 3º 25.241' W, 2026 m, on *C. scoparius*, 31.VII.2014, P. Valero & J.J. Guerrero leg. (RCBA-UMU). For body part measurements see table 4.

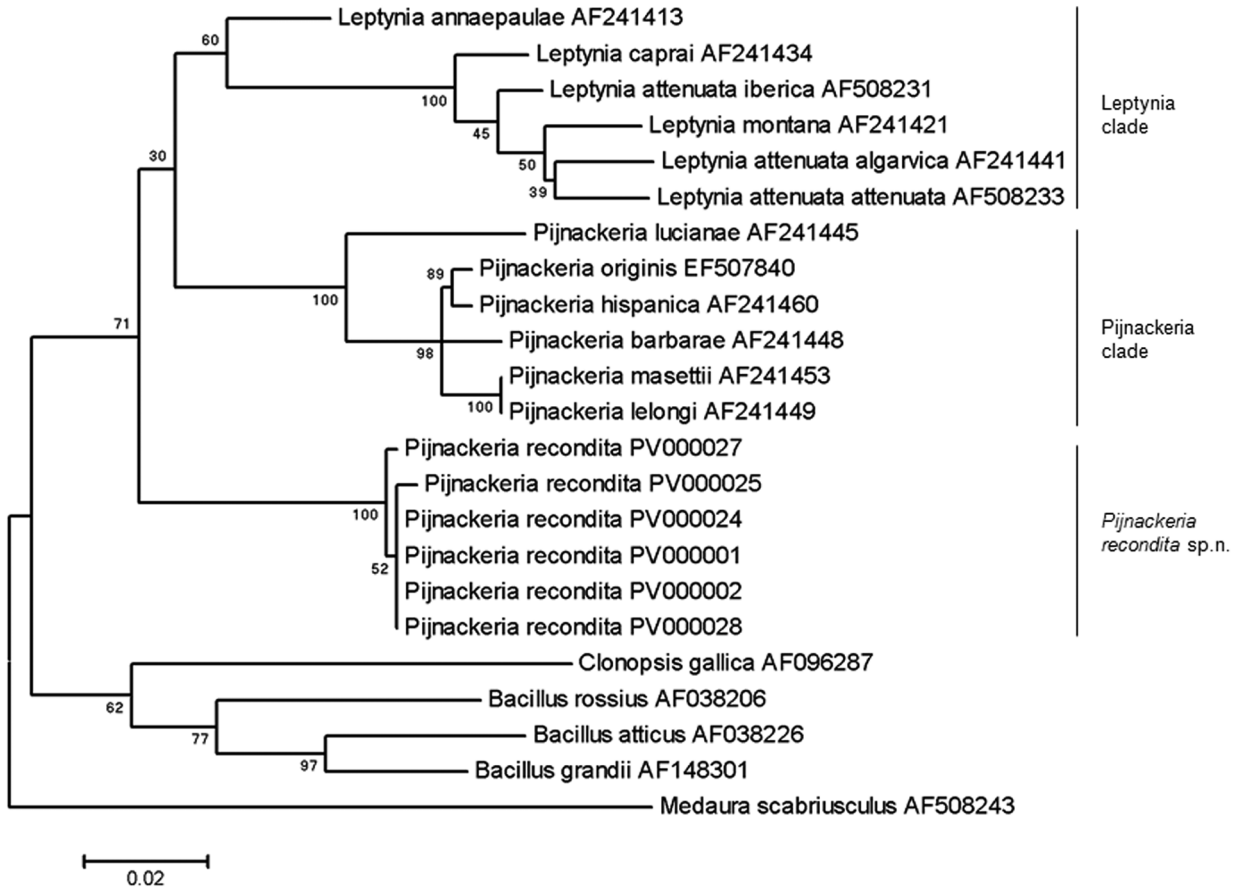

FIGURE 1. Maximum Likelihood tree based on COII sequences (mtDNA) of 6 *Leptynia* species and subspecies and 6 *Pijnackeria* species using *Clonopsis gallica, Medaura scabriuscula* and three *Bacillus* species, as outgroups. Numerical values denote Bootstrap values after 1,000 replications. Scale bar: nucleotide substitution per site.

TABLE 4. Measurements of body parts and their comparison between female holotype and male or female paratypes (values in mm).

	Holotype ♀	Paratype ♂♂	Paratype ♀♀
Body	58.6	41.5–47.4	52.1–59
Antenna	3.6	5.1–7	2.7–3.7
Head	3.4	2.1–2.9	3–3.4
Pronotum	2.3	1.4–1.8	1.7–2.2
Mesonotum	9.9	7.1–8.6	8.7–10
Metanotum	9.6	7.3–8.4	8.1–9.5
Median segment	1.3	0.7–1	0.9–1.3
Abdomen (excluding median segment and cerci)	32.3	21.3–24	29.3–32.3
Profemora	15.1	15.5–19	13.8–16.5
Mesofemora	9.8	10.3–12.1	9–10.3
Metafemora	12.3	13.6–16	11.2–13
Protibiae	16.2	15.9–20.5	13.6–16.6
Mesotibiae	9.9	11–13.2	9.1–10.6
Metatibiae	12.5	14.5–17.6	11.3–13.6
Protarsus	5.0	4.6–7.3	4.6–5.6
Mesotarsus	3.1	3.4–4.6	2.7–3.6
Metatarsus	4.3	4–5.4	3.1–4.3

FIGURE. 2. Thorax surface comparison; **A**: *Pijnackeria recondita* **sp. n.** with smooth surface; **B**: *Pijnackeria hispanica* as example of the typical granulated surface shown by the additional described taxa of the genus (excepting *Pijnackeria lelongi,* which differs from *P. recondita* **sp. n.** by having 16 antennal segments).

FIGURE. 3. *Pijnackeria recondita* **sp. n.** female, holotype. **A**: Body, dorsal view; **B**: Body, lateral view; **C**: Body, ventral view; **D**: Anterior part of body, dorsal view (not to scale); **E**: Apex of the abdomen, ventral view; **F**: Apex of the abdomen, lateral view; **G**: Apex of the abdomen, dorsal view; **H**: Egg, dorsal view; **I**: Egg, lateral view; **J**: Operculum; **mp** = micropilar plate, **sp** = subgenital plate; **ms** = mesothorax; **a**: antennae.

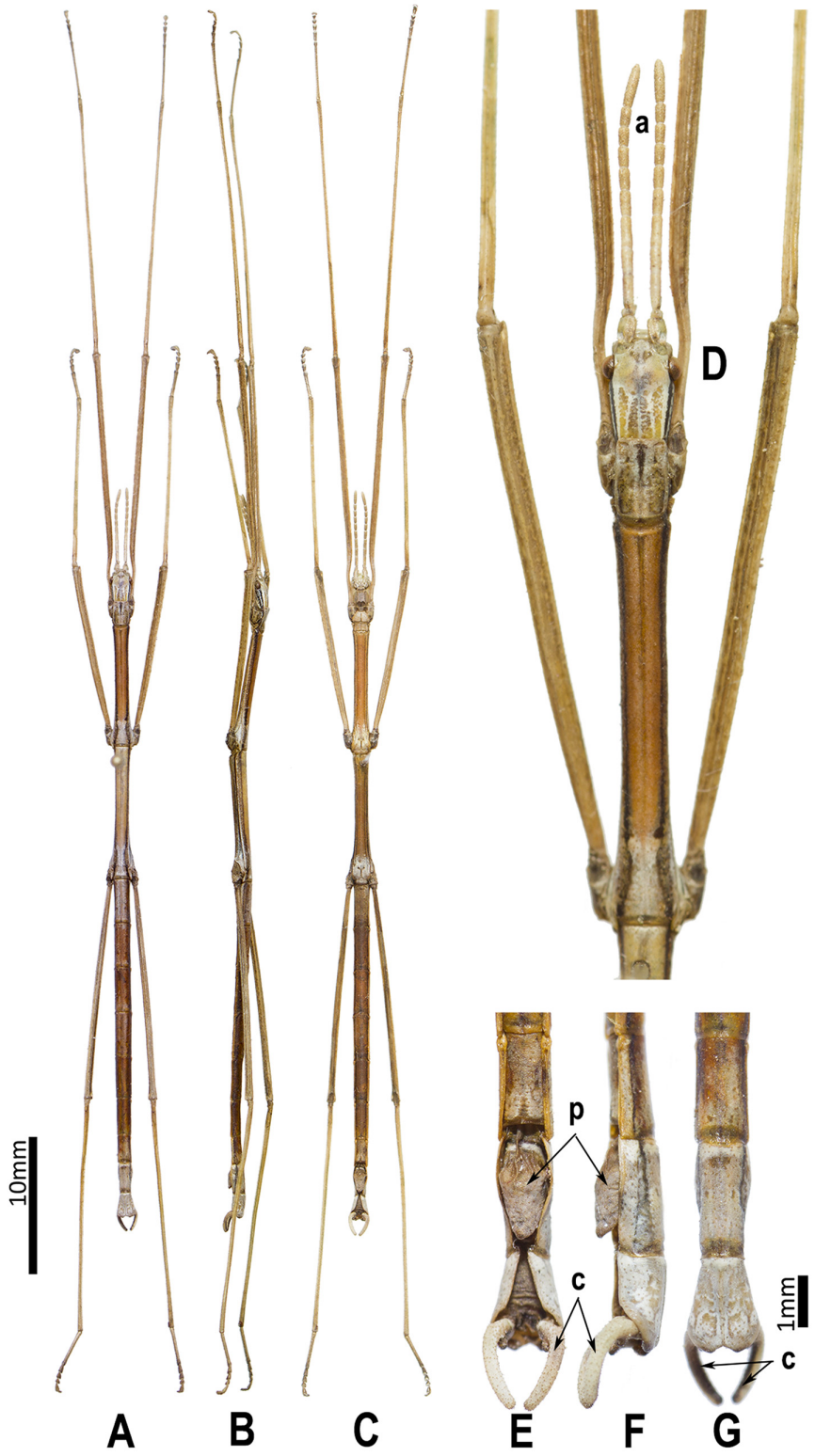

FIGURE. 4. *Pijnackeria recondita* **sp. n.** male, paratype. **A**: Body, dorsal view; **B**: Body, lateral view; **C**: Body, ventral view; **D**: Anterior part of body, dorsal view (not to scale); **E**: Apex of the abdomen, ventral view; **F**: Apex of the abdomen, lateral view; **G**: Apex of the abdomen, dorsal view; **a** = antennae; **p** = poculum; **c** = cerci.

FIGURE. 5. Eggs comparison. **A**: Egg of *Pijnackeria recondita* **sp. n.**, with a dense pitting pattern covering all the surface. **B**: Egg of *Pijnackeria hispanica*, with a netting pattern covering all the surface.

Etymology. The species epithet is from *reconditus* (Latin, adjective) meaning hidden. These words refer to the apparent low detectability of the new species in Sierra Nevada in the Iberian Peninsula.

Distribution and biology. So far only known from the type locality in a small area covered by *C. scoparius* near the Botanic Garden "Hoya de la Pedraza", a shrubbery area at approximately 2,000 m above sea level. *P. recondita* **sp. n.** was not found in any other surrounding plants nor at lower altitudes, although three *P. hispanica* females were found on *Medicago sativa* near the type locality. *P. hispanica* were also found in other places of Sierra Nevada, at lower altitudes and feeding on *Dorycnium pentaphyllum* (Scali *et al.* 2013).

Female morphology. Medium to high size for the genus, smooth, slender and unarmed. Body colour usually green, in some cases light brown. With a white latero-longitudinal line which begins at the pedicellus and reaches the end of the abdomen (Figs. 3, 6A, B).

Head: Flattened dorso-ventrally. Eyes spherical, brown, strongly projecting from capsule. Antennae pink and setose, composed by 11 segments: Scapus rectangular, approximately 2 times longer than wide. Pedicellus 0.3 times the length of the scapus, spherical in cross section; Antennomere IX longer than scapus; the remainders of smaller length. Antennomere I and III shorter than IX but longer than the remainder.

Thorax: Surface smooth. Median carina present. Pronotum narrower and shorter than head, 1.3 times longer than wide. Mesothorax and metathorax slightly widening towards the posterior, with mesonotum 4.3 times longer than pronotum and metanotum 4.1 times longer than pronotum.

Abdomen: Surface smooth. Longer than the head and complete thorax combined (including median segment). Abdominal segments progressively narrowing to apex but III–IV wider if the female is ripe. Median segment 0.1

times the length of the metanotum. Segments II–VII almost equal in length and 3 times longer than median segment. Tergite VIII shorter than II–VII but longer than IX and anal segment. Tergite IX shorter than anal segment. Anal segment with pointed apex. Subgenital plate shovel-shaped, goes beyond the articulation between sternite IX and X, and has a rounded distal border, truncated at apex. Straight cerci, setose and cylindrical, that not exceed the apex of the anal segment.

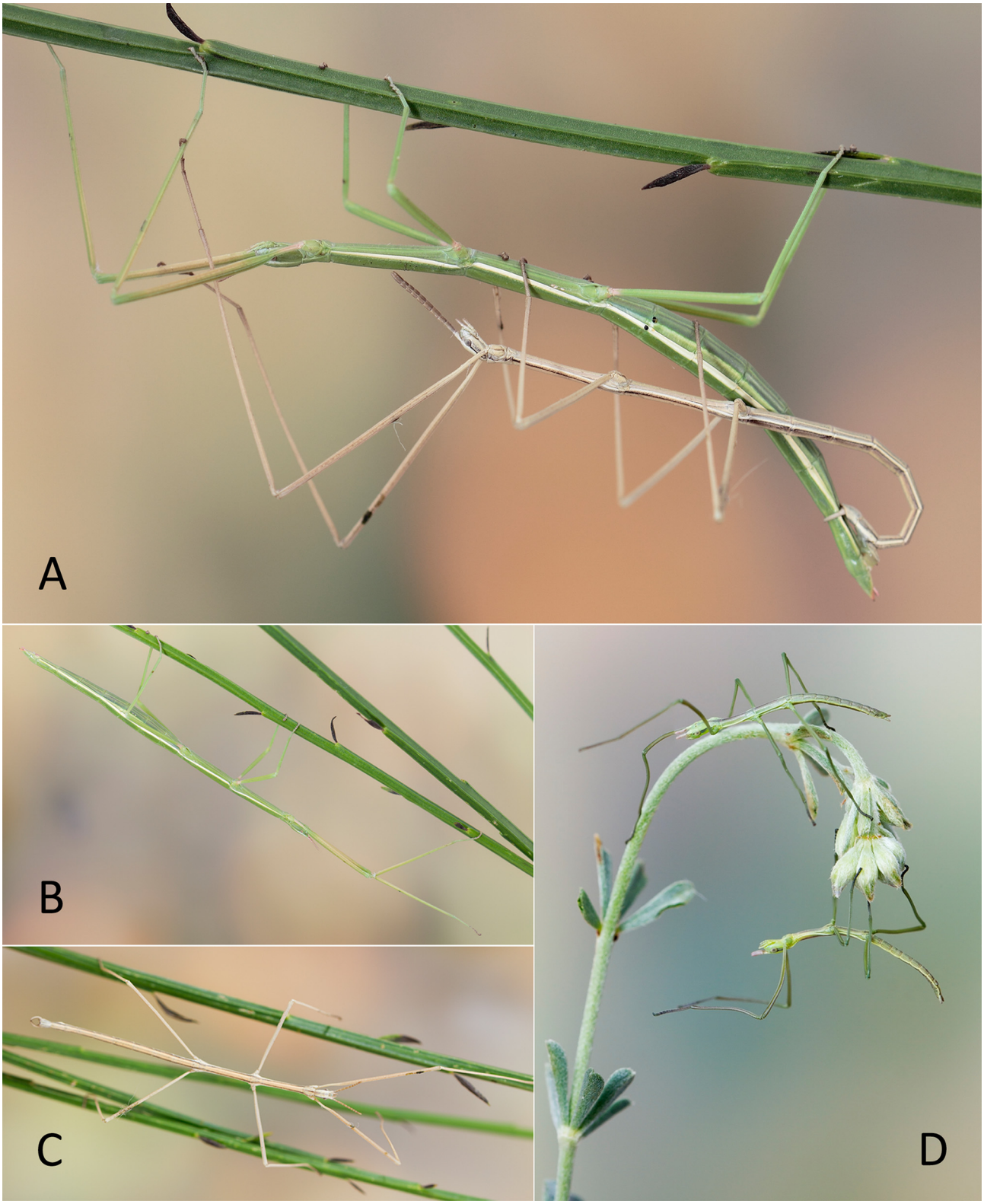

FIGURE. 6. Alive *Pijnackeria recondita* **sp. n.** specimens. **A**: Mating; **B**: Adult female on *Cytisus scoparius*; **C**: Adult male on *Cytisus scoparius*; **D**: Nymphs born in captivity on *Dorycnium pentaphyllum*.

Legs: Same color as the body. Slender and unarmed except from a variable number (2–4) of small black teeth at posteroventral meso- and metafemora; with small black hairs along the carinae. Pro-, meso- and metafemora pentagonal in cross-section. Hind legs usually do not reach beyond the end of abdomen. Profemora compressed medially and curved basally. Mesofemora shorter than pronotum and metanotum combined. The hind femur/tibiae articulation reaches half of the abdominal tergite V. Basitarsus shorter than tarsomeres combined.

Male morphology. Medium sized for the genus, smooth, very slender and unarmed. General body colour is usually light brown, although we found one greenish adult male. With a thin dark brown and a thin white latero-longitudinal parallel lines. Both begin behind the eye and reach the end of the abdomen (Figs. 4, 6A, C).

Head: Flattened dorso-ventrally. Eyes spherical, brown, strongly projecting from capsule. Antennae brown and setose, composed by 11 segments: scapus rectangular, approximately 2 times longer than wide, almost of uniform height from base to apex. Pedicellus 0.3 times the length of the scapus, spherical in cross section; first, third, and last antennomere slightly longer than scapus; the remainders shorter.

Thorax: Surface smooth. Pronotum narrower and shorter than head, 1.7 times longer than wide; usually with a dark brown median-longitudinal line. Mesothorax and metathorax slightly widening towards the posterior, with mesonotum 5.3 times longer than pronotum, and metanotum 5.1 times longer than pronotum.

Abdomen: Surface smooth. Longer than the head and complete thorax (including median segment) combined. Almost parallel-sided from segments II to VIII. IX tergite and anal segment slightly wider. Median segment 0.1 times longer than metanotum. Segments II–VI equal in length and each one 3.08 times longer than median segment, VII slightly shorter than previous but longer than segments VIII–X; tergites VIII and IX equal in length and 0.8 times the length of the terguites II–VI. Anal segment (tergite X) 0.83 times the length of the tergite IX, truncated and convex, pointing downwards, with a median carina on its distal part. Vomer absent. Poculum cup-like, with triangular tip. Cerci well developed, clearly projecting beyond posterior margin of anal segment, setose, with a small tooth at base and bent to act as claspers.

Legs: Same colour as the body. Slender and unarmed; with small black hairs along the carinae. Pro-, meso- and metafemora pentagonal in cross-section. Hind legs reach beyond the end of abdomen. Fore femora compressed and curved basally. Mesofemora approximately equal in length to head, pronotum, and metanotum combined. The hind femur/tibia articulation reaches abdominal tergites VI–VII. Basitarsus longer than tarsomeres combined.

Nymph and Egg morphology. Nymph: (Fig. 6D). Newly hatched nymph are green and very slender, with pink antennae and about 12 mm in length.

Egg: (Figs. 3H–J, 5A). Capsule almost cylindrical, rounded basally, usually about 3 times longer than wide. Surface, included operculum and micropylar plate, covered by a dense pitting pattern but not prominent; colour dark brown. Micropylar plate elongate and lanceolate, beige to cream, outlined, placed in the centre of capsule but slightly displaced to polar area. Without capitulum. Median line distinct and outlined, dark brown. Operculum convex, circular, tilted towards the dorsal. Measurements (in mm) of the eggs designated as paratypes are: length, 3.43–4.06: width, 1.22–1.33 and height, 1.3–1.56.

No nymphs from 2013 were born after more than 15 months but, by breaking some of the eggs, it was observed that they were fully developed, although they were already dead. In contrast, from eggs laid in 2014, the first nymph was born 30 days after removing the eggs from the refrigerator and being incubated at 20ºC and 70–80% RH.

Discussion

Based on COI divergence, the new species is apparently rather isolated from *P. hispanica* with interspecific distances varying from 7.8 % to 8.2 %. Between these *Pijnackeria* species vs. *B. rossius*, interspecific distances varied from 13 % to 14.7 %.

In relation to COII divergence, *P. recondita* **sp. n.** is apparently rather isolated from members of both genera *Leptynia* and *Pijnackeria* (Fig. 1). Interspecific distances of the new species vs. the other *Pijnackeria* species spanned from 9.9 % (*Pijnackeria masettii-lelongi* complex) to 10.2 % (*P. lucianae-P. originis*) (Table 3). Mean of the minimum interspecific distances was 10.0 %.

In addition, between the new species distance vs. *Leptynia* species varied from 7.9 % (*Leptynia annaepaulae* Scali, Milani & Passamonti, 2012) to 11.4 % (*Leptynia attenuata attenuata* Scali *et al.,* 2012) (Table 3). Mean of

the minimum interspecific distances was 10.2 %. COII divergence between the new species vs. *B. rossius*, *C. gallica* and *M. scabriuscula* were 11.4 %, 14.6 % and 16.0 % respectively (Table 3).

Pair-wise comparisons of COII sequences do not indicate a close relationship betwen the different *Leptynia* and *Pijnackeria* species. Much lower interspecific K2P distances were found between *Pijnackeria* species varying from 0.8 % to 5.8 % whereas *Leptynia* species vary from 2.7 % to 10.1 %. At any rate owing to the morphological similarity of the new species (including their eggs) to *Pijnackeria* species, we include the new species into genus *Pijnackeria*.

Roe & Sperling (2007) considered that it is more important to maximise fragment length than to target specific regions within COI–COII, to ensure that a fragment length will produce consistent divergence estimates across a range of taxa, while minimising variance within a fragment is important. Small fragments are more likely to be skewed by localised regions of unusual nucleotide divergence, whereas increased fragment length would reduce this risk.

The new species is probably a very rare stick insect. After checking more than 500 specimens of *Pijnackeria* and *Leptynia*, this species has hitherto remained unnoticed. Thus, despite this relatively large search no data was found and, although we had found more than twice the number of specimens in the type locality, only 17 specimens have been caught to try to minimise the population damage. This new species seems to be an endemism from Sierra Nevada and sympatric to *P. hispanica*, at least in the type locality.

Distinguishing the other *Pijnackeria* species by their morphology is at first glance very difficult, because, in most cases, the range of intraspecific variability overlaps between the different species. This new species differs from the other ones by the smooth meso- and metathorax for females, the constant number of 11 antennae segments for males and females, and the different pattern of the eggshell. After inspecting the MNCN and RCBA-UMU *Pijnackeria* collections we observed that, excepting *P. recondita* **sp. n.** specimens, all of the females had a granulated meso- and metathorax, at least partially, and all of the males had 13–16 antennal segments. This is consistent with the genus description, which describes the meso- and metathorax of the females as constantly covered by a conspicuous granulation, but now we can conclude that the variability of this feature is higher for the genus, as *P. recondita* **sp. n.** and *P. lelongi* show.

Imagoes were found in late July and as other *Pijnackeria* species it only lives during warm months of spring and summer. The type area is usually covered by snow in winter and *C. scoparius* is a deciduous plant. Because of this, we think that *P. recondita* **sp. n.** is probably closely associated and dependent on *C. scoparius,* which does not seem to be a plentiful plant in Sierra Nevada, so it could be a relict species.

In relation to the laid eggs, the results from incubations in captivity suggest that a hibernation period is needed to get a successful development and to complete their biological cycle.

Disclosure of interest. The authors declare that they have no conflicts of interest concerning this article.

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SUPPLEMENTARY MATERIAL S1. Diagnostic sites of *cox2* sequence alignments from Iberian *Picnakeria* species.

	42	45	72	75	78	108	110	114	129	136	141	146	147	163	168	192	198	201	210	211
P. recondita PV000025	T	A	T	T	C	T	A	A	C	G	T	A	C	C	T	T	A	T	A	A
P. recondita PV000024	T	A	T	T	C	T	A	A	C	G	T	A	T	C	T	T	A	T	A	A
P. recondita PV000001	T	A	T	T	C	T	A	A	C	G	T	A	T	C	T	T	A	T	A	A
P. recondita PV000002	T	A	T	T	C	T	A	A	C	G	T	A	T	C	T	T	A	T	A	A
P. recondita PV000028	T	A	T	T	C	T	A	A	C	G	T	A	T	C	T	T	A	T	A	A
P. recondita PV000027	T	A	T	T	C	T	A	A	C	G	T	A	T	C	T	T	A	T	A	A
P. lucianae AF241445	A	C	T	C	T	C	A	A	T	A	T	A	T	C	A	C	T	A	T	T
P. lelongi AF241449	A	T	C	T	T	C	C	T	T	A	C	T	T	T	A	A	T	A	A	A
P. masettii AF241453	A	T	C	T	T	C	C	T	T	A	C	T	T	T	A	A	T	A	A	A
P. barbarae AF241448	A	T	T	T	T	C	C	A	T	A	A	T	T	T	A	C	T	A	A	A
P. hispánica AF241460	A	T	T	T	T	C	C	A	T	A	C	T	T	T	A	C	T	A	A	A
P. originis EF507840	A	T	T	T	T	C	C	A	T	A	C	T	T	T	A	C	T	A	A	A

	216	222	231	240	241	247	249	250	255	256	261	279	288	291	297	300	303	339	343	363	369
P. recondita PV000025	A	C	T	A	T	T	G	T	T	T	A	C	A	T	A	T	A	T	A	T	T
P. recondita PV000024	A	C	T	A	T	T	G	T	T	T	A	C	A	T	A	T	A	T	A	T	T
P. recondita PV000001	A	C	T	A	T	T	G	T	T	T	A	C	A	T	A	T	A	T	A	T	T
P. recondita PV000002	A	C	T	A	T	T	G	T	T	T	A	C	A	T	A	T	A	T	A	T	T
P. recondita PV000028	A	C	T	A	T	T	G	T	T	T	A	C	A	T	A	T	A	T	A	T	T
P. recondita PV000027	A	C	T	A	T	T	A	T	T	T	A	C	A	T	A	T	A	T	A	T	T
P. lucianae AF241445	T	T	A	T	C	C	A	C	C	T	A	T	C	A	T	A	T	C	A	C	C
P. lelongi AF241449	T	T	A	C	C	T	A	T	T	C	A	T	C	A	A	A	C	T	G	T	T
P. masettii AF241453	T	T	A	C	C	T	A	T	T	C	A	T	C	A	A	A	C	T	G	T	T
P. barbarae AF241448	T	T	A	C	C	C	A	T	C	C	A	T	T	A	A	A	T	T	G	T	T
P. hispánica AF241460	T	T	A	C	A	T	A	T	C	C	G	T	C	A	A	A	T	T	G	T	T
P. originis EF507840	T	T	A	C	C	T	A	T	C	C	A	T	C	A	A	A	T	T	G	T	T

	379	380	381	393	400	408	417	421	423	424	426	429	459	462	468	471	474	487	489
P. recondita PV000025	-	-	-	T	T	A	A	G	T	A	A	T	A	T	C	A	T	T	A
P. recondita PV000024	A	A	T	T	T	A	A	G	T	A	A	T	A	T	C	A	T	T	A
P. recondita PV000001	A	A	T	T	T	A	A	G	T	A	A	T	A	T	C	A	T	T	A
P. recondita PV000002	A	A	T	T	T	A	A	G	T	A	A	T	A	T	C	A	T	T	A
P. recondita PV000028	A	A	T	T	T	A	A	G	T	A	A	T	A	T	C	A	T	T	A
P. recondita PV000027	A	A	T	T	T	A	A	A	T	A	A	T	A	T	C	A	T	T	A
P. lucianae AF241445	A	A	T	T	T	T	A	G	A	T	A	T	A	A	T	C	C	C	T
P. lelongi AF241449	G	A	G	C	C	T	A	G	A	T	G	T	G	A	T	A	T	C	T
P. masettii AF241453	G	A	G	C	C	T	A	G	A	T	G	T	G	A	T	A	T	C	T
P. barbarae AF241448	G	A	G	C	A	T	T	G	A	T	A	T	G	A	T	A	T	C	T
P. hispánica AF241460	G	A	G	C	C	T	A	G	A	T	A	C	G	A	T	A	T	C	T
P. originis EF507840	G	A	G	C	C	T	A	G	A	T	A	C	G	A	T	A	T	C	T

	492	495	516	525	534	543	550	558	565	570	582	594	597	606	612	625	627	632	633
P. recondita PV000025	A	A	A	A	C	T	T	C	T	T	T	T	T	T	T	A	T	G	A
P. recondita PV000024	A	A	A	A	C	T	T	C	T	T	T	C	T	T	T	A	T	G	A
P. recondita PV000001	A	A	A	A	C	T	T	C	T	T	T	C	T	T	T	A	T	G	A
P. recondita PV000002	A	A	A	A	C	T	T	C	T	T	T	C	T	T	T	A	T	G	A
P. recondita PV000028	A	A	A	A	C	T	T	C	T	T	T	C	T	T	T	A	T	G	A
P. recondita PV000027	A	A	A	A	C	T	T	C	T	T	T	C	T	T	T	A	T	G	A
P. lucianae AF241445	T	A	C	T	T	A	A	A	C	T	T	T	A	C	T	G	A	G	T
P. lelongi AF241449	C	T	A	T	T	A	A	T	C	T	T	T	A	T	T	G	A	G	T
P. masettii AF241453	C	T	A	T	T	A	A	T	C	T	T	T	A	T	T	G	A	G	T
P. barbarae AF241448	C	T	A	T	T	A	A	T	C	T	T	T	A	T	C	G	A	G	T
P. hispánica AF241460	C	T	A	T	T	A	A	T	C	T	T	T	A	T	T	G	A	A	T
P. originis EF507840	C	T	A	T	T	A	A	T	C	C	G	T	A	T	T	G	A	G	T