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DNA Barcoding reveals sexual dimorphism in *Isotrias penedana* Trematerra, 2013 (Lepidoptera: Tortricidae, Chlidanotinae)

MARTIN FRANCIS VANNER CORLEY[1, 2, 3] & SÓNIA FERREIRA[2]

[1]*Pucketty Farm Cottage, Faringdon, GB-Oxfordshire SN7 8JP Great Britain. E-mail: martin.corley@btinternet.com*
[2]*CIBIO/InBio - Centro de Investigação em Biodiversidade e Recursos Genéticos da Universidade do Porto, Vairão, 4485–661 Vairão, Portugal. E-mail: hiporame@gmail.com*
[3]*Corresponding author*

Abstract

Isotrias penedana Trematerra, 2013 was described from north Portugal based on males alone. Unidentified females were associated with the males using DNA barcoding, revealing sexual dimorphism in the species. Males and females differ in forewing shape, markings, and size, with females significantly smaller than males. The female is described and illustrated for the first time. We also document the species' occurrence in northern Spain.

Key words: biodiversity, Insect, cytochrome c oxidase I (COI), Portugal, Spain, systematics

Introduction

In June 2013, the first author collected four males of an unidentified tortricid in the Castro Laboreiro area of north-western Portugal. One of the males was taken in a steeply sloping meadow at Podre, a few kilometres south of Castro Laboreiro along with a single female that was recognised as belonging to the genus *Isotrias* Meyrick, 1895.

Following dissection of the genitalia, it became clear that the males belonged to the genus *Isotrias*, although they differed from any known species in having broader forewings with a reticulate pattern and no trace of the fasciae characteristic of other *Isotrias* species. Specimens were sent to Professor Pasquale Trematerra (Italy), who recognized that the species was new; he used the four males as the type series of *Isotrias penedana* Trematerra, 2013.

Dissection of the genitalia of the female *Isotrias* specimen was inconclusive. The habitus, with yellow fasciae, suggested *I. stramentana* (Guenée, 1845), which is known from Spain, but the genitalia did not appear to match this species. The possibility that this might be the female of *I. penedana* was considered, but the difference in habitus and size suggested that this was unlikely, despite the fact that both were collected in the same locality on the same date.

The locality at Podre was visited again two years later on 30 June 2014, when two more females were collected, although no males were observed. Subsequent DNA barcoding of the specimens revealed that indeed the two forms are sexually dimorphic members of the same species.

The genus *Isotrias* currently includes nine described species, eight in Europe, two of which extend east to Asia Minor, and one species in Morocco. Sexual dimorphism in some members of the genus is mentioned by Razowski (1984, 1987, 2002), but these differences are small. Males and females of all species show the characteristic external appearance of the genus, apart from the male of *I. penedana*, in which the forewing markings are completely atrophied or indistinct (Trematerra, 2013).

In *I. penedana*, males and females differ in forewing shape, markings, and size, with females significantly smaller than males. The female and its genitalia are described and figured herein for the first time. We also report the species from Spain for the first time and discuss its distribution.

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Material and methods

DNA extraction and sequencing. Genomic DNA was extracted from leg tissue (Table 1) using EasySpin Genomic DNA Tissue Kit (Citomed, Lisboa, Portugal) following manufacturer's protocol, except for the lysis period which was extended to enhance extraction success.

TABLE 1. Specimens of *Isotrias penendana* sequenced. [Code = sample code; S = sex; Date = date of collection; Locality = collecting locality; Lat = latitude; Long = longitude; Genbank = GenBank code for cytochrome c oxidase I (COI); and Gen. Prep. Code = slide number. All specimens in M. Corley personal collection.

Taxa	Code	S	Date	Locality	Lat	Long	GenBank	Gen. Prep. Code
Isotrias penedana	INV00190	♀	16-06-2012	Podre	42.002	-8.169	KY053460	Corley Gen. Prep. 3750
Isotrias penedana	INV00196	♂	14-06-2012	Podre	42.002	-8.169	KY053461	
Isotrias penedana	INV00197	♀	16-06-2012	Podre	42.002	-8.169	KY053463	B. Goodey Gen. Prep
Isotrias penedana	INV00198	♀	30-06-2014	Podre	42.002	-8.169	KY053462	Corley Gen. Prep. 4237
Isotrias hybridana	INV03034	♀	31-05-2016	Sambade	41.422	-7.004	KY053464	

The cytochrome c oxidase I (COI) barcoding fragment was amplified as two overlapping fragments using two sets of primers. For the first fragment, primers LepF (Hebert *et al.* 2004) and MlepR (Hajibabaei *et al.*, 2006) were used, while primers LepR (Hebert *et al.*, 2004) and MlepF (Hajibabaei *et al*. 2006) were used to amplify the second fragment.

Both PCR reactions had 10 μL of final volume, containing 5 μL of Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.4μM of each primer, and 1-2μL of DNA. PCR amplification was carried out on a T100 Thermal Cycler (BioRad, Hercules, CA, USA) using the following conditions: initial denaturation at 95ºC for 15 min; 5 cycles at 95ºC for 30 s, 47ºC for 45 s, 72ºC for 45 s; then 40 cycles at 95ºC for 30 s, 51ºC for 45 s, 72ºC for 45 s; and a final elongation step at 60ºC for 10 min. The amplified product was cleaned with ExoSap (ExoSAP-IT® PCR Product Cleanup and FastAP Thermosensitive Alkaline Phosphatase, ThermoFisher Scientific, Waltham, MA, USA), and sequenced for both directions. Sequencing reaction was performed using BigDye® Terminator v3.1 Cycle Sequencing Kits (AB Applied Biosystems, Carlsbad, CA, USA) following manufacture's protocol on a T100 Thermal Cycler (BioRad) and sequenced on an ABI 3130xl Genetic Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequences were assembled and edited in Geneious Pro v8.1.7 (http://www.geneious.com/).

Phylogenetic analyses. Sequences available in BOLD for three species of *Isotrias* (i.e. *I. rectifasciana* (Haworth, 1811), *I. cuencana* (Kennel, 1899) and Spanish specimens of *I. penedana*) were included, and *Lobesia physophora* (Lower, 1901), *Ancylis sciodelta* (Meyrick, 1921) and *Sparganothis distincta* (Walsingham, 1884) were used as outgroups. The best-fitting model of sequence evolution was determined using jModeltest v.2.1.3 (Darriba *et al.* 2012) under the Akaike Information Criterion (AIC) (Akaike, H. 1973). Haplotype alignments were analysed using Maximum Likelihood (ML) method. ML trees were built in PhyML (Guindon *et al.*, 2010) with 1,000 bootstrap replicates and searching for the best-scoring ML tree. The average divergence (uncorrected p-distance) between species was calculated in MEGA v.5.2.1 (Tamura *et al.* 2011) for the COI sequence data.

Results

Molecular results. All samples amplified the partial COI gene sequence (658 bp). The final COI dataset consisted of 19 sequences from four *Isotrias* species and five sequences from three outgroup Tortricidae species (Fig. 1). The COI alignment for phylogeny reconstruction yielded two distinct haplotypes for *I. penedana* (Fig. 1). Only one haplotype was found in the Portuguese specimens (n = 4). *Isotrias* species pairs exhibited moderate levels of genetic divergence in the COI dataset (2% < uncorrected p-distance ≥ 4%) (Table 2), with Spanish specimens of *I. penedana* exhibiting a second haplotype with less than 0.4% divergence from specimens from the type locality. The most appropriate model for the COI dataset was GTR. *Isotrias penedana* is recovered as more closely related with the widespread *I. rectifasciana* than with the widespread *I. hybridana* and the Iberian endemic *I. cuencana*.

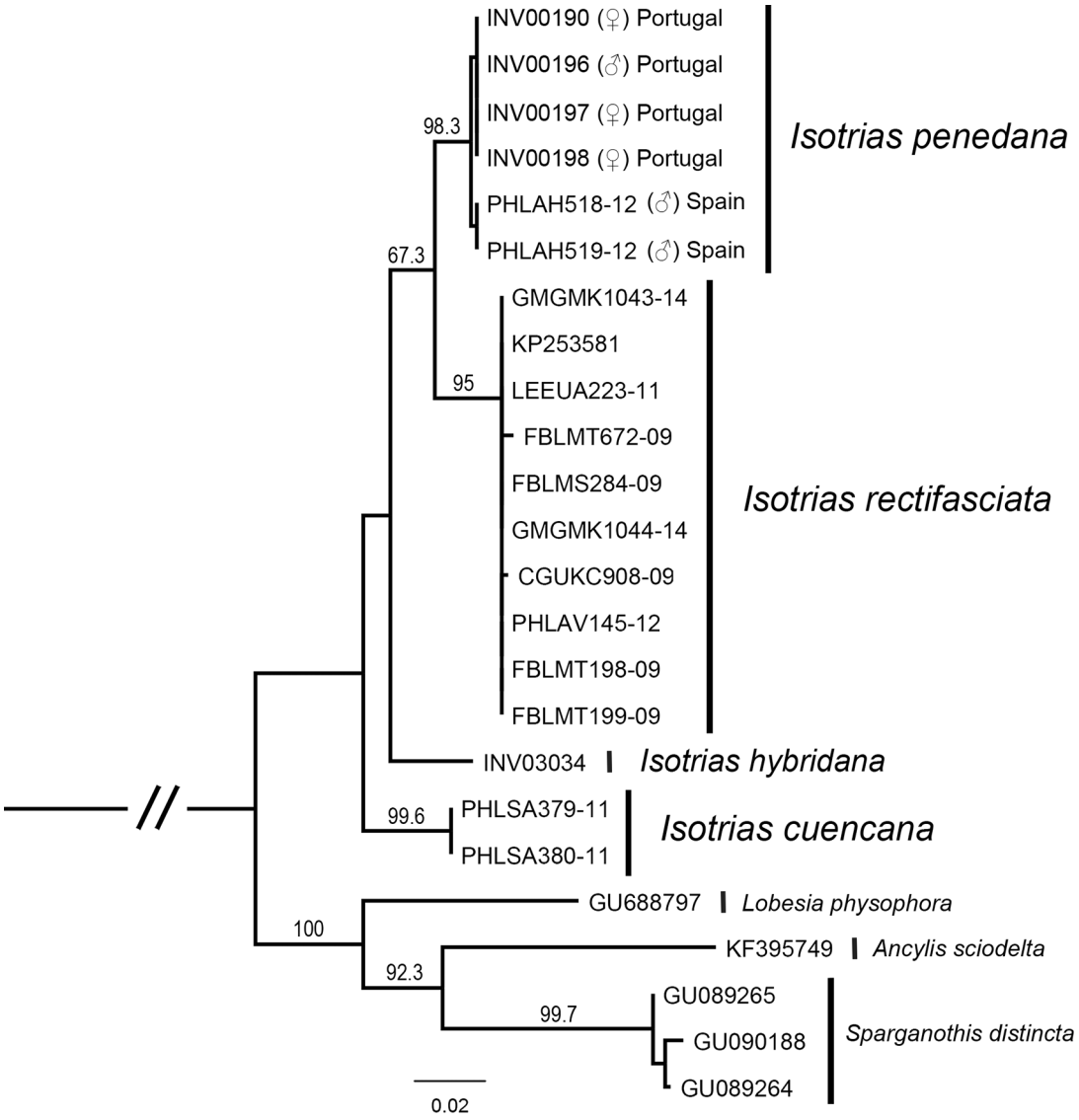

FIGURE 1. Maximum Likelihood (ML) tree of species of *Isotrias* based on sequences of cytochrome c oxidase I gene (COI) (n = 23; 658 bp); bootstrap values (>80%) indicated at nodes.

TABLE 2. Mean (below diagonal) and standard deviation (above diagonal) sequence divergence (uncorrected p-distances) of 658 bp fragment of cytochrome c oxidase I (COI) among pairs of species of *Isotrias*, and representative outgroup species of other Tortricidae.

	I. penedana	*I. rectifasciata*	*I. hybridana*	*I. cuencana*	*Lobesia physophora*	*Ancylis sciodelta*	*Sparganothis distincta*
I. penedana		**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**
I. rectifasciata	0.02		**0.01**	**0.01**	**0.01**	**0.01**	**0.01**
I. hybridana	0.04	0.04		**0.01**	**0.01**	**0.01**	**0.01**
I. cuencana	0.04	0.03	0.04		**0.01**	**0.01**	**0.01**
Lobesia physophora	0.09	0.09	0.09	0.07		**0.01**	**0.01**
Ancylis sciodelta	0.09	0.09	0.10	0.10	0.09		**0.01**
Sparganothis distincta	0.09	0.09	0.10	0.09	0.08	0.07	

Description of the female of *Isotrias penedana* (Fig. 2A). Wingspan 13.0–13.5 mm. Head and thorax pale buff. Tegulae buff with some ochreous scales. Forewing creamy white with yellow-ochreous fasciae at one-quarter and one-half, both with irregularly waved or angled inner and outer margins; additional yellow-ochreous patches, one on dorsum at two-fifths, triangular, sometimes extended as a fine line to costa, one near tornus and one before apex, which has a tail extending towards termen above tornus; fasciae with some brown scales near costa and forming brown spots on costal margin; cilia creamy white. Hindwing grey with irregular paler patches; cilia light grey with a distinct line. Abdomen buff.

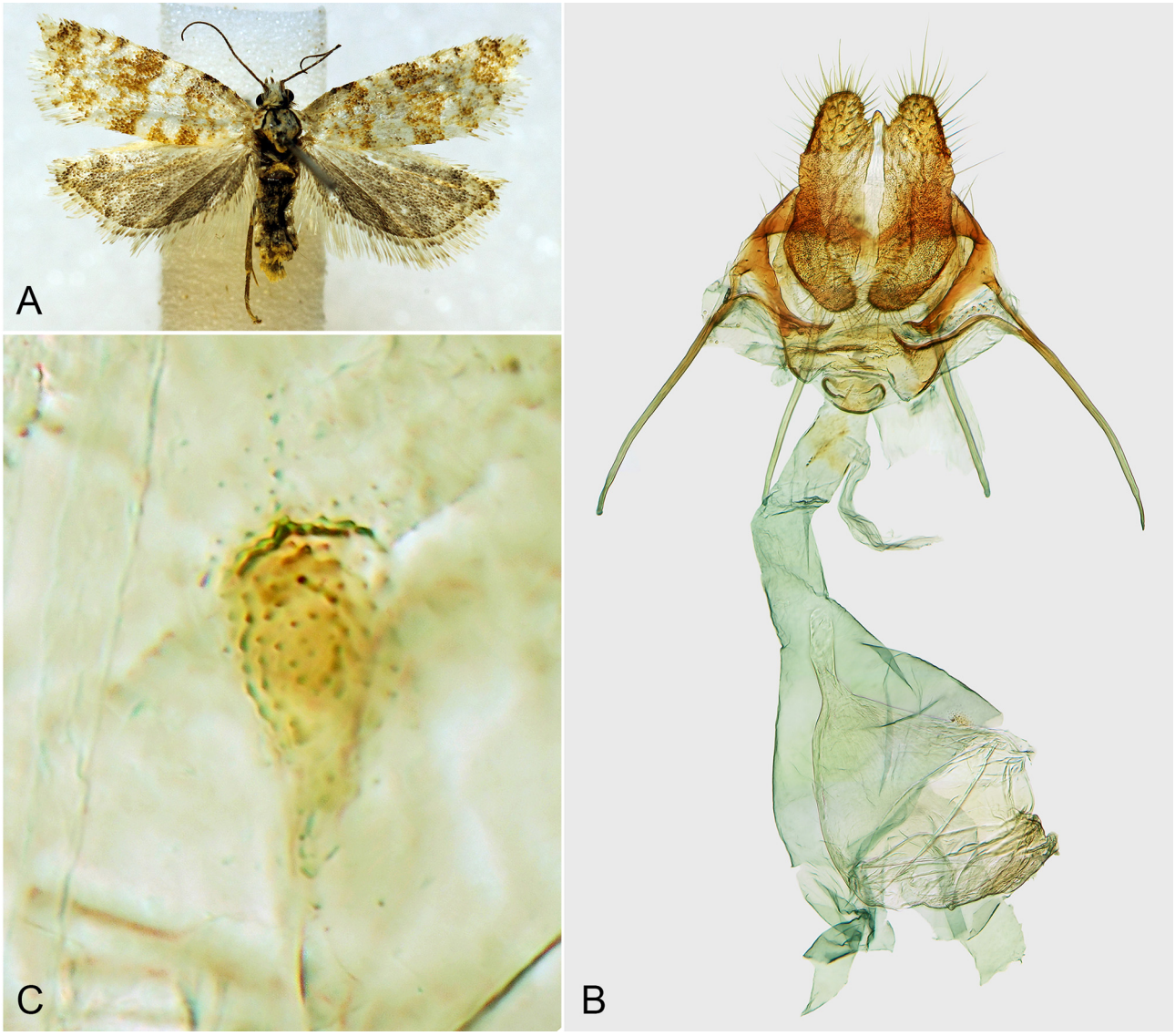

FIGURE 2. *Isotrias penedana*, Portugal, Podre, 42.002º N, 8.169º W, 770 m. A. Female, 30.vi.2014, coll. British Museum (Natural History); B. Female genitalia, 16.vi.2012; C. Signum 30.vi.2014, M. Corley genitalia preparation 4237. Photos by Pedro Pires (A) and.Brian Goodey (B and C).

Comparison of the female of *I. penedana* with other species of *Isotrias*, as illustrated in Razowski (2002), shows it to be externally very similar to *I. stramentana* and *I. joannisana* (Turati, 1921), but the forewing fasciae of these species are distinctly narrower than those of *I. penedana*. Furthermore, in *I. stramentana* the fasciae lack brown scales on the costa.

The adult male was described and illustrated by Trematerra (2013), so only the contrast with the female is mentioned here. Wingspan much greater, 16–20 mm, forewing broader, and forewing markings consisting only of a fine reticulation with no hint of fasciae.

Female genitalia (Figs. 2B–C). Similar to those of other species in the genus, but sterigma with distinct antero-lateral bulges, and signum consisting of a weakly sclerotised papillose plate, unlike the belt-shaped signum of the other species.

Habitat (Fig. 4A). In the Castro Laboreiro area, *I. penedana* flies diurnally in small, sloping, slightly acid meadows with a mosaic of marshy and drier ground and a rich flora of Poaceae, *Carex* spp., *Juncus* spp., *Centaurea nigra*, *Cirsium filipendulum*, *Achillea millefolium*, *Conopodium majus*, *Lotus uliginosus* and others. In all the sites, shrubs of *Genista florida* grow around the drier edges of the meadows. At Podre (770 m) and Portos (1170 m; 42.0293º N, 8.1198º W) the ground slopes towards the east, whereas at Rodeiro (1060 m; 42.0544º N, 8.1384º W) it slopes to the west.

Material examined. One male, Cantabria, Picos de Europa, Fuente Dé, 43.145º N, 4.812º W by day on 9.vii.1999, leg. R.J. Heckford in coll. Heckford. Three males, Cantabria, Picos de Europa, Portillas de Poqueion, 43.149° N, 4.776° W, 1340 m, 11.vii.2012, leg. P. Huemer and T. Mayr. Two specimens in coll. TLMF, one in coll. Mayr (Fig. 3).

FIGURE 3. *Isotrias penedana*, male, Spain, Portillas de Poqueion, 43.149° N, 4.776° W, 1340 m, 11.vii.2012, T. Mayr coll. Photo by Peter Huemer.

***Isotrias penedana* in Spain.** After the description of *I. penedana*, Robert Heckford (UK) and Peter Huemer (Austria) both reported that they had seen the species in northern Spain. Heckford had collected a single male in July 1999 which he determined as *Isotrias*, but he did not pursue its identity further. It has now been confirmed as *I. penedana*. Huemer sent two specimens to Guelph, Canada, for barcoding, which after dissection were found to be an unknown *Isotrias*. The sequences are in the BOLD database and have been included in our analysis (Fig. 1). TLMF Lep 08337 (PHLAH518-12) and TLMF Lep 08338 (PHLAH519-12) have 99.68% correspondence with the Portuguese specimens.

The male from Spain in Figure 3 is darker than other Spanish males and all Portuguese males. Not only is the scaling darker, the individual reticulations are slightly broader, as well.

Discussion

Females of *I. penedana* are remarkably distinct from the males, both in size and in forewing markings. According to Razowski (2002), *I. rectifasciana* (Haworth, 1811) and *I. stramentana* show some sexual dimorphism. In *I. rectifasciana* the male forewing is broader, darker, and less distinctly marked than in the female. In *I. stramentana*

the females are smaller, darker, and the markings more contrasting than in the male. Nevertheless, these differences are small compared with the differences between male and female in *I. penedana*. Whereas females of *I. penedana* resemble females of other *Isotrias* species, the signum of *I. penedana* is unique within the genus, consisting of a weakly sclerotised papillose plate. All other species figured by Trematerra (1991) and Razowski (2002) have a signum in the form of a belt with a few transverse folds.

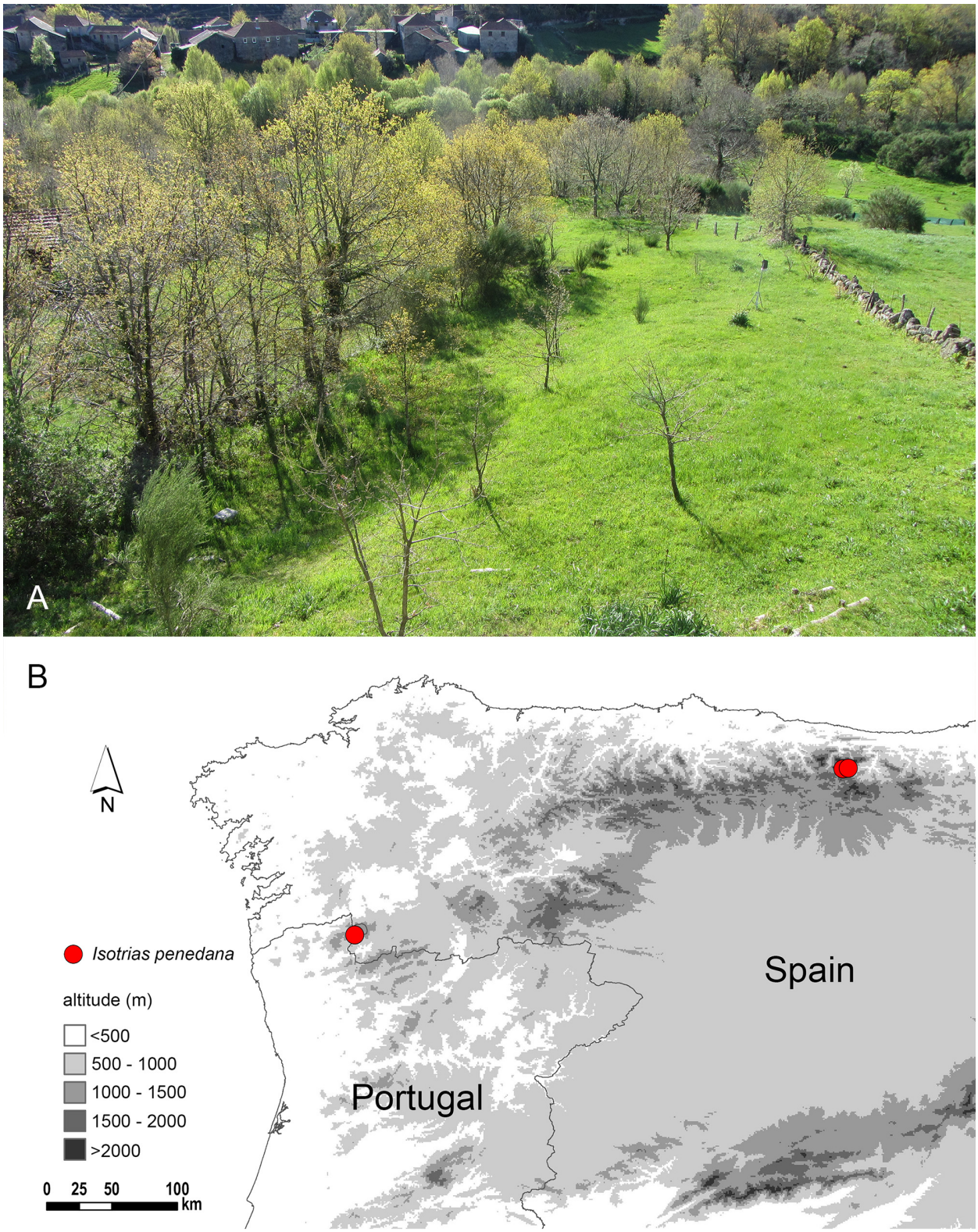

FIGURE 4. A. Habitat of *Isotrias penedana*, Podre, 42.002º N, 8.169º W, 770 m, 27.vii.2013; B. Distribution map of *Isotrias penedana.* Photo by Henrique Pereira.

The two known areas of distribution of *I. penedana* are separated by about 300 km (Fig. 4B), ranging from 770 m elevation in Portugal to 1340 m in Spain. The species distribution is likely to be much wider than currently known as many suitable sites for the species are sure to be present in the Cordillera Cantabrica and in Galicia.

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