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***Ochthebius* (*Enicocerus*) *aguilerai* sp.n. from central Spain, with a molecular phylogeny of the Western Palaearctic species of *Enicocerus* (Coleoptera, Hydraenidae)**

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Abstract

We describe *Ochthebius* (*Enicocerus*) *aguilerai* **sp.n.** from central Iberia. The species belongs to the *O. exsculptus* group, including *O. legionensis* Hebauer & Valladares, *O. halbherri* Reitter, *O. colveranus* Ferro and *O. exsculptus* Germar. There are few consistent differences in external morphology among species of the group, but characters of the aedeagus allow the unequivocal recognition of the new species. A molecular phylogeny of the European species of subgenus *Enicocerus* based on fragments of four mitochondrial and two nuclear genes recognised *O. aguilerai* **sp.n.** as sister to the remaining species of the *O. exsculptus* group, which according to molecular clock estimations seems to have an Iberian origin in the Late Miocene (Tortonian). There are two genetically distinct lineages within *O. exsculptus*, one in South Spain and a second from the Pyrenees to Ireland, but the lack of consistent morphological differences between them, and the incomplete sampling of other Iberian populations of the species, prevents the recognition of distinct taxa.

Key words: Hydraenidae, *Ochthebius*, *Enicocerus*, *Ochthebius aguilerai* **sp.n.**, Iberian peninsula, molecular phylogeny

Introduction

Enicocerus Stephens is currently considered a subgenus of *Ochthebius* Leach (Coleoptera, Hydraenidae) (e.g. Jäch 1992, 2004), although it has been treated as genus by different authors (e.g. Perkins 1997; Hansen 1998), including Stephens (1829) in his original decription. *Enicocerus* has been traditionally divided in two groups of species, one distributed in the Westerm Palaearctic (Europe and Middle East), including the type species *Ochthebius exsculptus* Germar; and a second in the Himalayas and East Asia (Jäch 2004). The latter was excluded from *Enicocerus* by Skale & Jäch (2009) (see also Jäch 1992), as they do not share some of the putative synapomorphies of the Western Palaearctic clade: cup-shaped second antennal segment, short postocular brush-like setae and ocelli almost contiguous with the eyes (Skale & Jäch 2009). Molecular data agree with the inclusion of the Eastern clade within *Ochthebius* s.str., not closely related to *Enicocerus* (I. Ribera, unpublished data). The western clade was revised by Jäch (1992), and two new species from Iran and Greece have recently been added (Skale & Jäch 2009 and Ferro 2008 respectively). In total, it currently includes 14 species from West Iberia to Iran, being conspicuously absent from northern Africa (Jäch 2004; Ferro 2008; Skale & Jäch 2009).

In this work we describe a new species of *Enicocerus* in its restricted sense (i.e. including only the Western Palaearctic species) from the central mountains in the Iberian Peninsula. It belongs to what could be considered the *O.* (*Enicocerus*) *exsculptus* group, including *O. exsculptus* (Western Europe), *O. colveranus*

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Ferro (Central & Eastern Europe, Anatolia), *O. legionensis* Hebauer & Valladares (NW Iberia) and *O. halbherri* Reitter (Italy and Switzerland) (Jäch 1992). All species share a very similar external morphology, and the same general structure of the aedeagus (Jäch 1992). We also provide a phylogeny inferred from molecular data, including all species of the *O. exsculptus* group (as defined above) plus some other representatives of the genus as outgroups.

Molecular methods

Taxon sampling, DNA extraction and sequencing

Specimens were preserved in absolute ethanol in the field (Table 1). Extractions of single specimens were non-destructive, using a standard phenol-chloroform method or the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). Vouchers and DNA samples are kept in the collections of the Museo Nacional de Ciencias Naturales, Madrid (MNCN) and the Institut of Evolutionary Biology, Barcelona (IBE).

We included multiple examples of all species of the *O. exsculptus* group, plus two specimens each of *O. gibbosus* Germar, *O. melanescens* Dalla Torre and *O. granulatus* Mulsant. The subgenus *Enicocerus* has a rather isolated position within Ochthebiinae, without close relatives in the Holarctic region (I. Ribera, unpublished molecular data). Trees were thus rooted in *O. granulatus*, which is clearly outside the *O. exsculptus* group (Jäch 1992).

We amplified fragments of six genes, four mitochondrial and two nuclear: 3' end of *cox1*; 5' end of *rrnL*; full *tRNA-Leu*; 5' end of *nad1*; 5' end of *SSU*; and an internal fragment of *LSU* (see Table 2 for primers used, and Ribera *et al*. 2002 for general PCR conditions). Sequences were assembled and edited using Sequencher TM 4.1.4 (Gene Codes, Inc., Ann Arbor, MI). New sequences have been deposited in GenBank (NCBI) with Accession Numbers GU143725-GU143783 (Table 1).

Phylogenetic analyses

We aligned the sequences using the MAFFT online v.6 and the Q-INS-i algorithm (Katoh & Toh 2008), a progressive pair-wise method with secondary refinement. Bayesian analyses were conducted on a combined data matrix with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), which runs two independent, simultaneous analyses, using six partitions corresponding to the six genes and with a GTR+I+Γ evolutionary model. MrBayes ran for 6x10^6 generations using default values, saving trees at each 500th generation. "Burn-in" values were established after visual examination of a plot of the standard deviation of the split frequencies between two simultaneous runs.

We also conducted maximum likelihood searches in Garli v.0.951 (www.bio.utexas.edu/faculty/antisense/garli/Garli.html), which uses genetic algorithms (Zwickl 2006), with an estimated GTR+I+Γ model for the combined sequence and the default settings. Support was measured with 1,000 bootstrap replicates, reducing the number of generations without improving the topology necessary to complete each replicate to 5,000. We did an additional analyses with Garli using the nuclear sequence only (*SSU* and *LSU* fragments), to check for possible incongruence between the nuclear and mitochondrial datasets.

Estimation of the ages of diversification

To calibrate the tree we use molecular clock-methods, with a combined rate for the mitochondrial genes of 2% per MY, based on a calibration using the separation between the Sardinian and the continental lineages of a related group of Coleoptera (Leiodidae, Cholevidae, Leptodirini; Ribera *et al*. 2010). To obtain an ultrametric tree we used Bayesian estimations as implemented in Beast 1.4.7 (Drummond & Rambaut 2007) with a matrix including only specimens with the complete sequence for both mitochondrial genes (Table 1). Well supported nodes according to the results with the combined data were constrained to be monophyletic (see below), and a GTR+I+Γ model was enforced with an uncorrelated lognormal relaxed clock and a Yule process speciation model. Priors and other parameters were left with default values, with the exception of the prior of the evolutionary rate, which was set to a normal distribution with mean of 0.01 substitutions/site/MY and a standard deviation of 0.001 (i.e. a 95% confidence interval of 0.0084 and 0.0116). The results of two independent runs were merged with Tracer v1.4 and TreeAnnotator v1.4.7 (Drummond & Rambaut 2007).

TABLE 1. Material used in the molecular study, with locality data, collector, voucher and MNCN ref. numbers, and GenBank Accession Numbers (all numbers preceded by GU1437). See Text for detailed locality data of type material. “16S” includes the gen fragments of *rrnL+tRNA-Leu+nad1*.

species	DNA voucher	ref MNCN	Country	Locality	cox1	16S	SSU	LSU
aguilerai	MNCN-AI387	23733	Spain	Ávila, Arenas de San Pedro, 15.5.2005, IR & CH leg.	61	28	70	78
aguilerai	MNCN-AI648	23994	Spain	Cáceres, S. de Sta. Bárbara, 9.8.2002, AC leg.	45	29		
aguilerai	MNCN-AI957	24303	Spain	Madrid, Arroyo de la Laguna Grande de Peñalara, 1.7.2006, IR & A. Cieslak leg.	46	30		
colveranus	MNCN-AI791	24137	Turkey	Bartin, road to Topallar from Kakraz, 25m N41º45’50.5” E32º29’11”, 25.4.2006 IR, AC, CH & P. Aguilera leg.	44	33	68	76
colveranus	MNCN-AI800	24146	Turkey	Kastamonu, road 759 to Senpazar, Çocukören Çoyl, 591m N41º49’38” E33º25’03”, 26.4.2006, IR, AC, CH & P. Aguilera leg.	57			
colveranus	MNCN-AI818	24164	Turkey	Kastamonu, road Agli to Azdavay, E cross to Senpazar, 982m N41º42’41.5” E33º30’47”, 28.4.2006, IR, AC, CH & P. Aguilera leg.	56			
exsculptus	MNCN-AC28		Ireland	Galway, Lough Briskeen, 30.7.2007, D.T. Bilton leg.	54	42		
exsculptus	MNCN-AI374	23720	Spain	Barcelona, Guardiola del Berguedà, Te. Gavarrós, 850m N 42º15’05.9” E1º55”20.1”, 25.7.2005, IR leg	63	41	72	80
exsculptus	MNCN-AI565	23911	Spain	Guipuzkoa, Ereñozu, river Urmua, 37m 3º13’26”N 1º55’33”W, 12.3.2005, IR leg.	53	40		
exsculptus	MNCN-AI655	24001	Spain	Lleida, Llinars, St. Pere de Graudescales, riu Aiguadora, 31T0391665 46621838, 10.2005, J. Fresneda leg.	58			
exsculptus	MNCN-AI1069	24415	Spain	Huesca, Espés de Baix, Riu Blanc, 14.7.2006, J. Fresneda leg.	60			
exsculptus	MNCN-AI925	24271	Spain	Albacete, E-No. 10, N Sierra de Segura, 10 km N Yeste, Rio Tús, 38º25'N, 02º19W, 10.4.2003, V. Assing & P. Wunderle leg.	50	37		
exsculptus	MNCN-AI991	24337	Spain	Granada, Río Lanjarón, 214, 14.10.2005, F.M. Cabezas leg.	59			
exsculptus	MNCN-AI994	24340	Spain	Granada, Río Alhama, 118, 14.10.2005, F.M. Cabezas leg.	52	39		
exsculptus	MNCN-AI1087	24433	Spain	Granada, Sierra Nevada, Río Genil, 29.7.2006, AC leg.	51	38		
gibbosus	MNCN-AI365	23711	Italy	Piamonte, V. Móngia, trib. Te. Móngia before Castello, 724m N44º17’35.8” E7º58’34.5”, 31.7.2005, IR & A. Cieslak leg.	55	27	69	77
gibbosus	MNCN-AI794	24140	Turkey	Bartin, road to Yeniköy from Kapisuyu I, 161m N41º47’08” E32º43’07”, 25.4.2006, IR, AC, CH & P. Aguilera leg.	67	26		
granulatus	MNCN-AI291	23637	France	Tributary river Bevera, 5 km n Moulinet, 28.7.2000, IR & A. Cieslak leg.	47	31		

continued next page

TABLE 1. (continued)

species	DNA voucher	ref MNCN	Country	Locality	cox1	16S	SSU	LSU
granulatus	MNCN-AI427	23773	Austria	Niederösterreich, Kleiner Ötscherbach, ca. 700 m a.s.l., Langau, 3.5 km SSW Lackenhof, 15º08'04"E 47º50'27"N, 25.9.2005, M.A. Jäch leg.	65	25	74	82
halbherri	MNCN-AH190		Italy	Emilia Romagna, rd. Bosco-Mucino, Te. Parma, 818m N44º27'18.0" E10º02'25.8", 24.5.2008, IR leg.	64	36	73	81
legionensis	MNCN-AI507	23853	Spain	León, Puerto de Panderrueda, 12.07.2005, L.F. Valladares leg.	62	35	71	79
legionensis	MNCN-AI906	24252	Spain	Cantabria, Beges, 13.07.2005, L.F. Valladares leg.	49	34		
melanescens	MNCN-AI344	23690	Austria	Niederösterreich, Schwarzenbach, lat. rd. 18 W Sankt Veit an der Gölsen, 448m N48º04'42.4" E15º40'42.9", 6.8.2005, IR & A.Cieslak leg.	66	43	75	83
melanescens	MNCN-AI556	23902	Germany	Thuringia, Thuringian Forest, Floh, Ickersbach, July 2001, R.G. Beutel leg.	48	32		

TABLE 2. Primers used in the study. F, forward; R, reverse.

Gene	Name	Sense	Sequence	Reference
cox1	Jerry (M202)	F	CAACATTTATTTTGATTTTTTGG	Simon *et al.* (1994)
	Pat (M70)	R	TCCA(A)TGCACTAATCTGCCATATTA	Simon *et al.* (1994)
	Chy	F	T(A/T)GTAGCCCA(T/C)TTTCATTA(T/C)GT	Ribera *et al.* (2010)
	Tom	R	AC(A/G)TAATGAAA(A/G)TGGGCTAC(T/A)A	Ribera *et al.* (2010)
rrnL-nad1	16saR (M14)	F	CGCCTGTTTA(A/T)CAAAAACAT	Simon *et al.* (1994)
	16Sa	R	ATGTTTTTGTTAAACAGGCG	Simon *et al.* (1994)
	ND1A (M223)	R	GGTCCCTTACGAATTTGAATATATCCT	Simon *et al.* (1994)
SSU	5'	F	GACAACCTGGTTGATCCTGCCAGT	Shull *et al.* (2001)
	b5.0	R	TAACCGCAACAACTTTAAT	Shull *et al.* (2001)
LSU	Ka	F	ACACGGACCAAGGAGTCTAGCATG	Ribera *et al.* (2010)
	Kb	R	CGTCCTGCTGTCTTAAGTTAC	Ribera *et al.* (2010)

Abbreviations

CAC	Collection A. Castro, Córdoba, Spain
CDS	Collection J.A. Díaz, Universidade de Santiago, Spain
CDTB	Collection D.T. Bilton, Plymouth, UK
CGV	Collection J. Garrido, Universidad de Vigo, Spain
COV	Collection O. Vorst, Utrecht, The Netherlands
CVL	Collection L.F. Valladares, Universidad de León, Spain
IBE	Institut de Biología Evolutiva (UPF-CSIC), DNA&Tissue collection, Barcelona, Spain
MNCN	Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain
NMW	Naturhistorisches Museum Wien, Austria
RMNH	National Museum of Natural History Naturalis, Leiden, The Netherlands

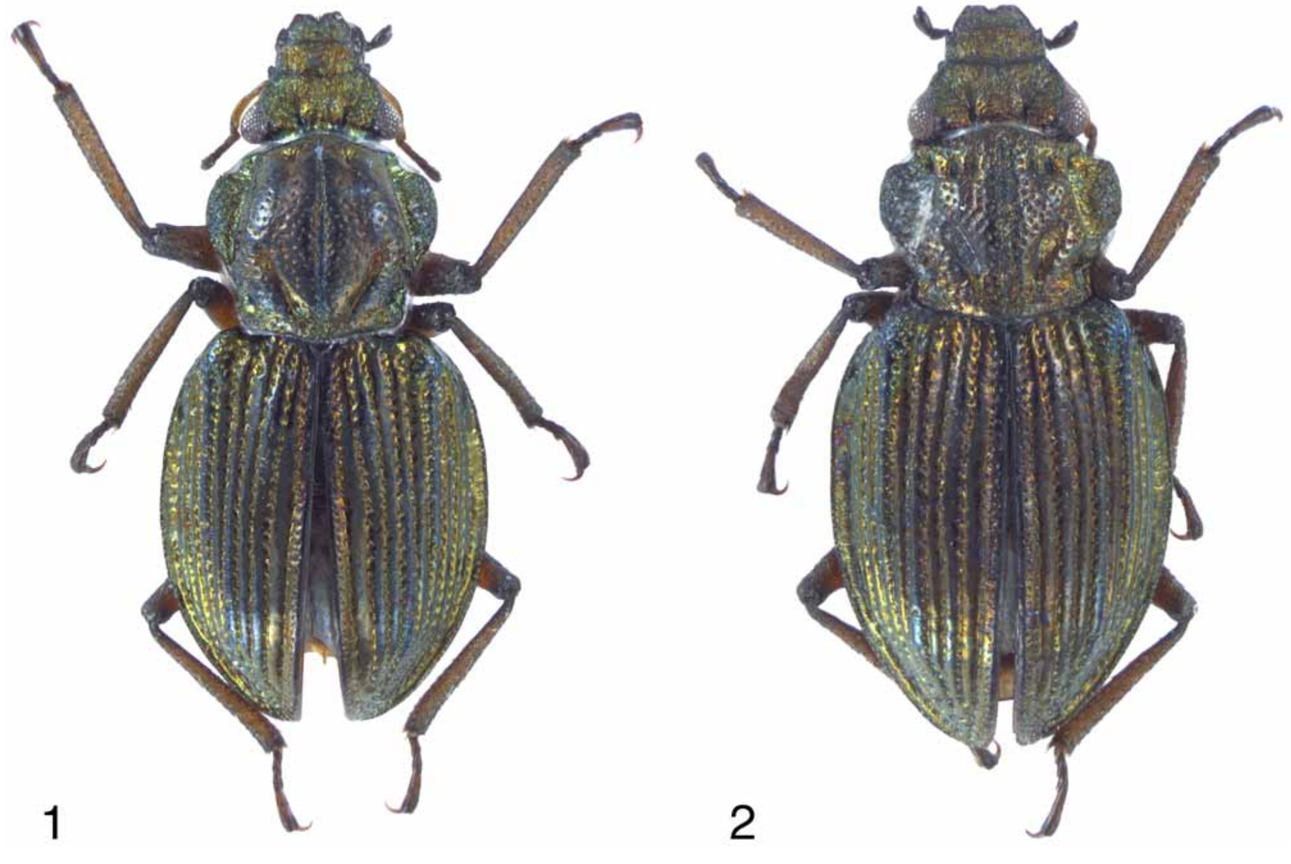
***Ochthebius* (*Enicocerus*) *aguilerai* sp.n.**

(Figs 1–4, 11–13)

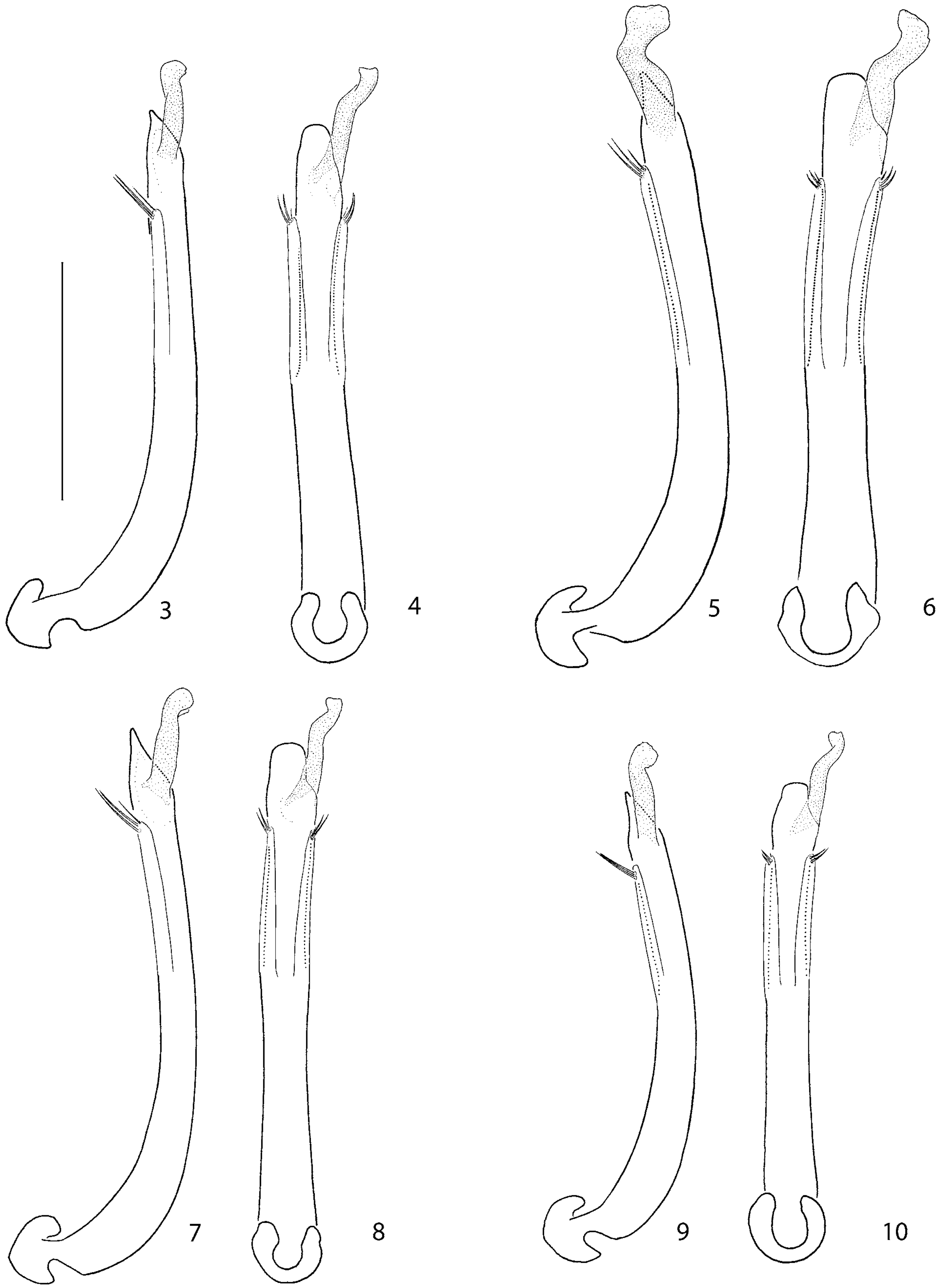
Type locality. Madrid, Puerto de los Cotos, Arroyo de la Laguna Grande de Peñalara, 1600 m, N40º49’59’’ W3º56’09’’ (Figs 18–19).

Type material. *Holotype* male (MNCN): “ES Madrid, Puerto de los Cotos \ Arryo. Laguna Grande de Peñalara \ 1600 m, N40º49’59’’ W3º56’09’’ \ rd M604 pk 38 20.7.2006 I. Ribera leg.” and holotype label (aedegus extracted and mounted on the same card). *Paratypes* (31 exx): 1 male and 1 female (NMW), 1 male (CVL) and 4 exx (IBE) labelled as holotype; 2 males (IBE) and 1 female (MNCN, DNA voucher MNCN-AI957) “2 ES Madrid Puerto de Cotos 1.7.2006 \ Arroyo de la Laguna Grande de Peñalara \ 1600m N40º49’59’’ W3º56’09’’ \ rd M604 pk 38 IR [I. Ribera] & AC [A. Cieslak] & BR [Bernard Ribera] leg”; 3 males 4 females (CDTB) “July 2008, Spain Madrid, Sierra de Guadarrama, Arroyo de la Laguna Grande de Peñalara below Puerto del Coto, D.T. Bilton leg.”; 1 male (RMNH) “ES: Rascafria, Rio Lozoya \ [WGS84] 30T VL257272 [= 30T 425.7 4527.2] M19W \ 5.vi.2007 \ leg. O.Vorst \ Bergriviertje”; 1 male, 1 female (COV) “ES: Rascafria, Rio Lozoya \ 30T VL253262 [=30T 425.3 4526.2] M6n \ 1.vi.2007 \ leg. O.Vorst \ Bergbeek”; 1 male (MNCN, DNA voucher MNCN-AI387) “10 SPAIN Avila, Arenas de S. Pedro \ r. Pelayo, rd. AV924 \ 500m N40º12’06” W5º06’42” \ I.Ribera leg. 15.5.2005”; 1 male (IBE) “2 ES Ávila, Sa. Gredos 280506 \ rv. Pelayo in Arenas de S. Pedro \ rd. AV924, ca. 2Km W Arenas \ 500m N40º12’5,6’’ W5º06’41,5’’ \ I. Ribera & A. Cieslak leg.”; 3 males (CAC, IBE, MNCN DNA voucher MNCN-AI648) and 1 female (IBE) “CÁCERES. Jarandilla \ Gta. De Jaranda \ 8.2002 A. Castro leg.”; 5 females (CDS, CJV, CVL) “ESPAÑA: Cáceres / Guijo de Sta. Bárbara / 26.06.1991 / leg. J.A. Régil & J.A. Díaz” [specimens recorded as *O. legionensis* in Garrido-González *et al*. (1994)].

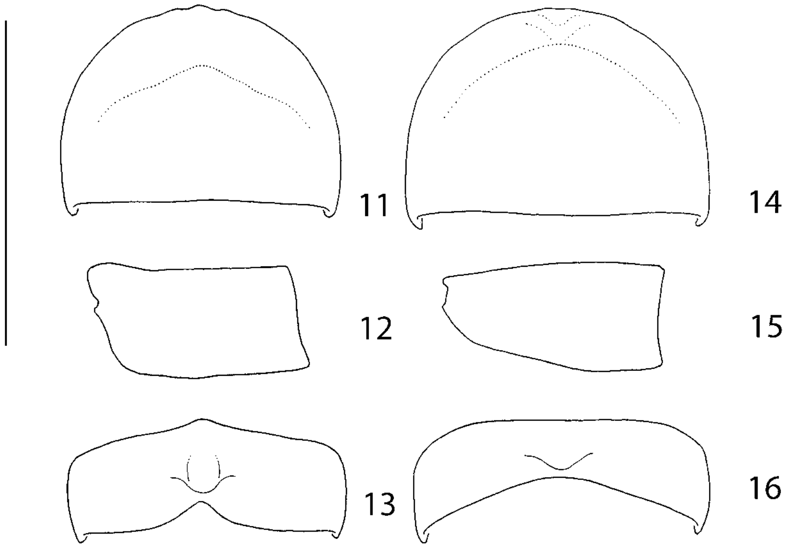
Aliquots of the DNA extractions are kept in the MNCN (DNA and tissue collection) and the IBE.



FIGURES 1–2. Habitus of *Ochthebius aguilerai* **sp.n.**, 1) male (paratype, NMW); 2) female (paratype, NMW).



FIGURES 3–10. Aedeagus of *O. aguilerai* **sp.n.** 3) lateral and 4) ventral view (Paratype from type locality); *O. legionensis* 5) lateral and 6) ventral view (voucher specimen MNCN-AI906); *O. exsculptus* (northern clade, voucher specimen MNCN-AI1069) 7) lateral and 8) ventral view; and *O. exsculptus* (southern clade, Granada, Río Genil) 9) lateral and 10) ventral view. See Table 1 for details of the localities. Scale bar, 0.25 mm.



FIGURES 11–16. Last abdominal tergites of *O. aguilerai* **sp.n.** 11) ventral, 12) lateral and 13) caudal views (paratype from type locality) and *O. legionensis* 14) ventral, 15) lateral and 16) caudal views (León, Rioscuro, 30.6.1985 L.F. Valladares leg.). Scale bar, 0.25 mm.

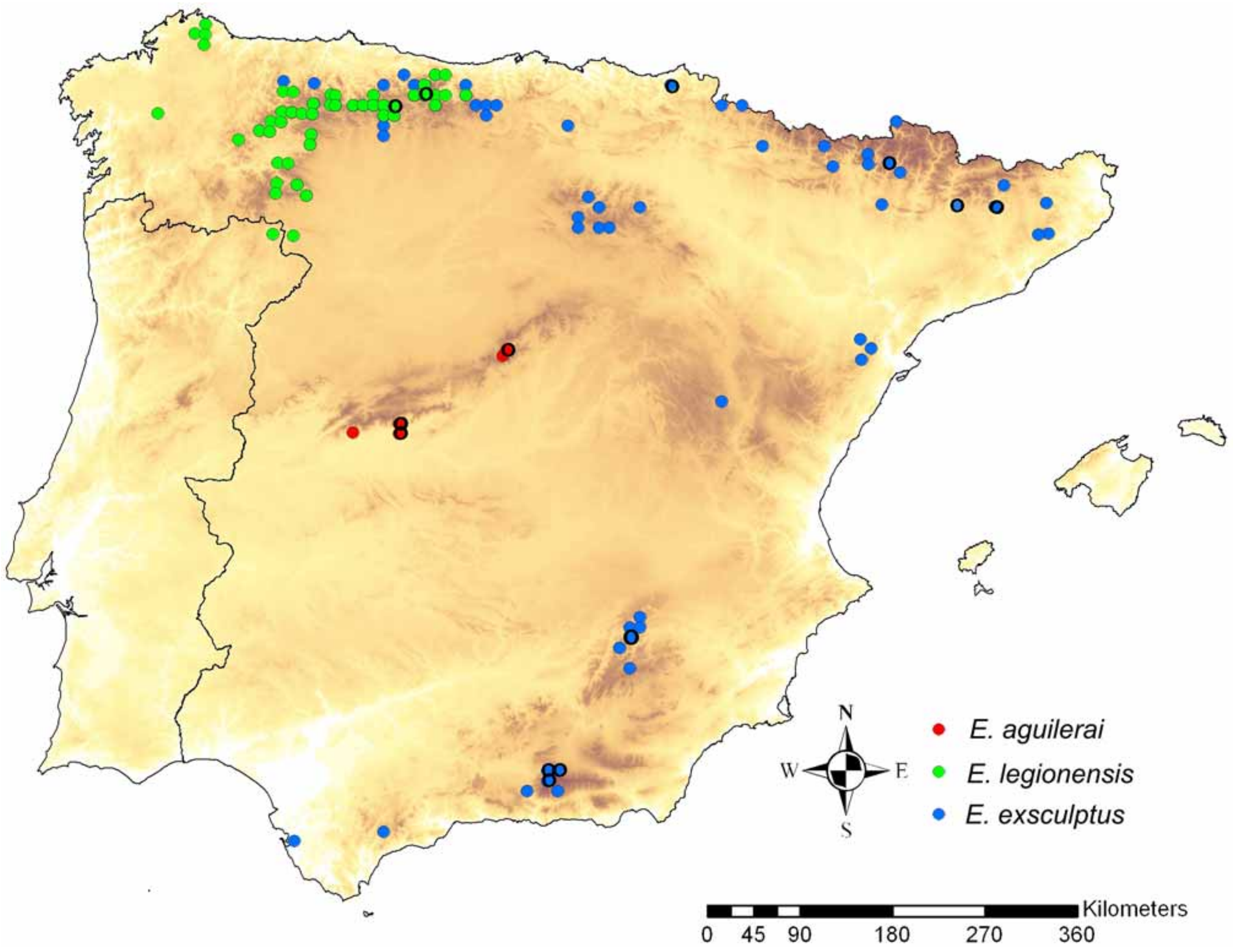
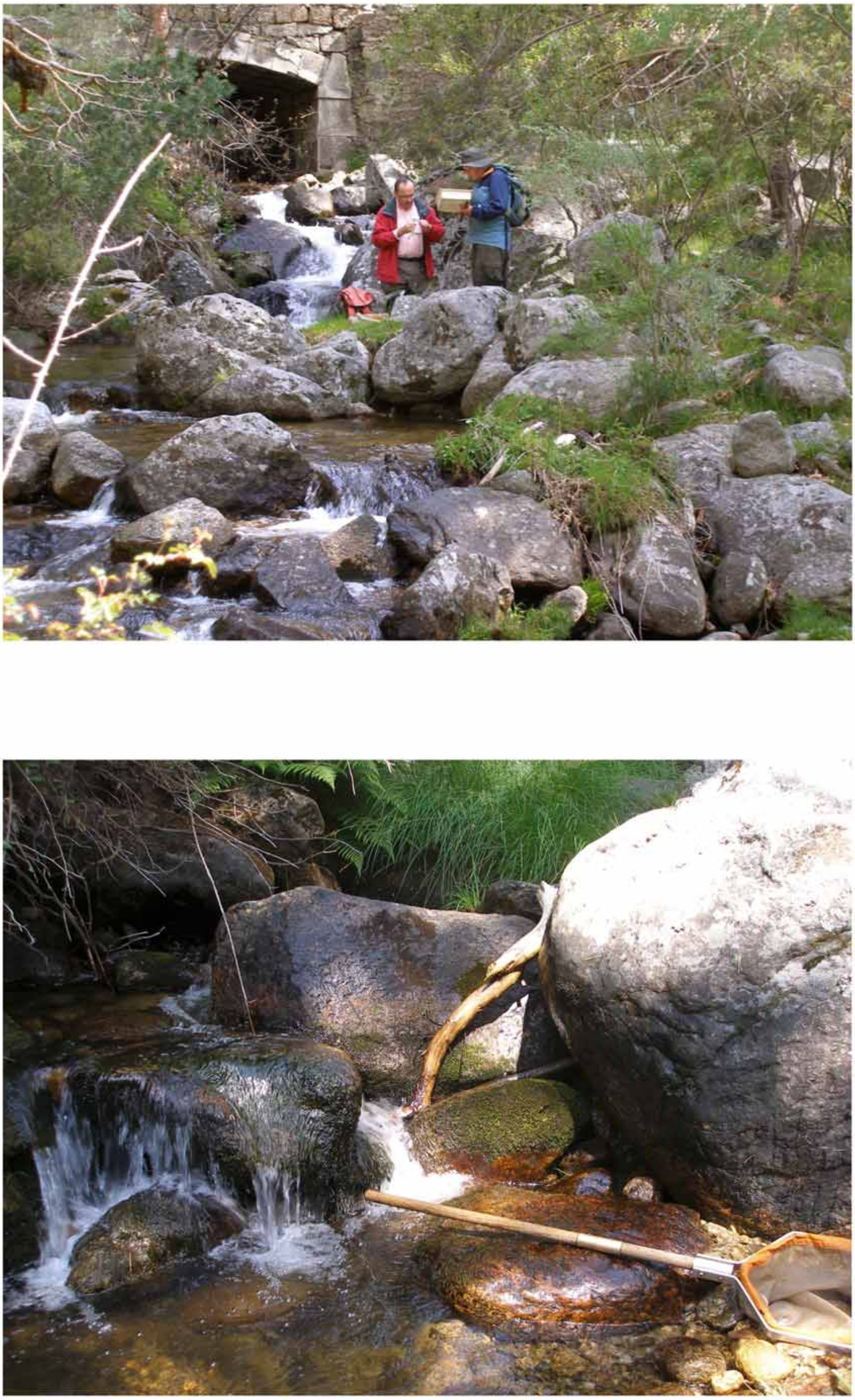


FIGURE 17. Distribution map of the Iberian species of the *Ochthebius* (*Enicocerus*) *exsculptus* group. With black circles, localities from which at least one specimen has been sequenced.



FIGURES 18–19. Type locality of *O. aguilerai* **sp.n.** (Arroyo de la Laguna Grande de Peñalara), 18) top, 2 June 2007, with D.T. Bilton and F. Bameul; 19) bottom, 20 July 2006, detail of the microhabitat (fotos, IR).

Additional material. 1 male (CVL): "Madrid (Norte), 1978 \ E. Roy leg." (not designated as paratype due to the imprecise location).

Description. Habitus as in Figs 1–2. Body length 1.9–2.1 mm. Colour black, with strong greenish metallic reflections. Body appendages yellowish brown; apical half of antennae, palpi, knees and tarsi darker. Upper surface very sparsely covered with short whitish adpressed pubescence. Anterior margin of the labrum emarginated; anterior lobes transverse, exterior angle frequently with a spine. Labrum and clypeus with large punctures; surface with a distinct microreticulation. Frons more densely punctate, with a shagreened appearance. Pronotum of female and some males not very convex, roughly trapezoidal, admedian fovea and sulcus well impressed, median sulcus reaching anterior and posterior margins; pronotal punctures strongly impressed, sparse, space between punctures usually smooth and glabrous. In some males pronotum strongly convex, with admedian foveas more elongated, surface with less impressed punctation (Fig. 1). Elytra oval, strongly convex, with ten strongly impressed regular striae; second interval flat and smooth, specially in male with convex pronotum; third interval strongly raised near the basis. Suture very depressed anterior to and strongly raised posterior to middle of elytra, so that in lateral view each elytron show a distinct median depression. Aedeagus as in Figs (3, 4), long and slender. Main piece, in ventral view, distinctly narrowed behind insertion of parameres, in lateral view, apex narrow and tapered; parameres short, adjoining median lobe. Female tergite X (Figs 11–13) with a small medial protuberance visible in lateral and caudal view.

Distribution. Mountains of the Spanish Sistema Central, from north-east of the province of Cáceres to Sierra de Gredos and Guadarrama (Fig. 17). The record of *O. legionensis* in Garrido-González *et al.* (1994) from NE Cáceres corresponds to this species (see Type Material). Although there are no genetic data available, according to the external morphology the populations of the north Iberian System (Burgos and Soria, Valladares *et al.* 2000) and Málaga (Jäch *et al.* 1999) (Fig. 17) would belong to *O. exsculptus* (J. Garrido, L.F. Valladares and M.A. Jäch, personal communication 2009). The records from Albarracín (Teruel) in d'Orchymont (1941) have been considered as *O. exsculptus* in Fig. 17, although we have not examined the specimens.

Etymology. Named after our friend and water-beetler Pedro Aguilera, who died in an accident in February 2009.

Ecology. The new species is found in fast flowing, clean mountain streams in granitic substratum, usually on the surface of large semi-submerged rocks (Figs 18–19). In the type locality (the one in which the species seems to be more abundant) it was collected in the company of an abundant and diverse fauna of Hydraenidae (*H. brachymera* d'Orchymont, *H. corinna* d'Orchymont, *H. exasperata* d'Orchymont, *H. iberica* d'Orchymont, *H. hispanica* Ganglbauer, *Hydraena sharpi* Rey, *Limnebius truncatellus* Thunberg, *Ochthebius heydeni* Kuwert), all of them Iberian endemics except *L. truncatellus* (Jäch 2004).

Comparative notes. *Ochthebius aguilerai* **sp.n.** is very similar to *O. legionensis* in its external morphology, but females of both species can easily distinguished by the shape of the last terguite, specially when observed in lateral and caudal view (Figs 11–16). We have not found any other constant external character to separate these two species, although *O. aguilerai* **sp.n.** tends to be slightly smaller and with a more strongly puncturated pronotum. The aedeagus of *O. legionensis* (Figs 5–6) differs from that of *O. aguilerai* **n.sp.** (Figs 3–4) by its more robust main piece, ventrally less narrowed behind insertions of parameres, and with a broadly truncate apex. The apical lobe of *O. aguilerai* **n.sp.** is more slender and with a less sharp bend that that of *O. legionensis* (Figs 3–6).

Ochthebius aguilerai n.sp. and *O. legionensis* can be easily distinguished from the other Iberian species of the *O. exsculptus* group by the presence of an elytral depression. The apex of the median lobe of the aedeagus of *O. aguilerai* **n.sp.** is less truncated in ventral view than that of *O. exsculptus*, and less pointed in lateral view (Figs 3–4, 7–10). The apical lobe of both species are very similar.

The size of the species of the *O. exsculptus* group is rather variable, and there is a significant correlation between body and aedeagus size (for Iberian specimens of the three species $r^2 = 0.74$, $p < 0.01$, $n = 13$). Specimens of *O. aguilerai* **sp.n.** and of the southern clade of *O. exsculptus* (see below) seem to have proportionally smaller aedeagus, but in any case, and due to the large overlap between species, these would be statistichal trends without diagnostic value.

Ochthebius aguilerai **sp.n.** seems to be in some characters closer to the Central European *O. halbherri*. Both species share an aedeagus with a dorsally constricted main piece, ending in a narrow apex, although both species can be easily distinguished by the shape of the apex of the median lobe (see Jäch 1992: Fig. 8). The female tergite X is also similar in both species (see Jäch 1992: Fig. 4), although the apical protuberance is distinctly less developed in *O. aguilerai* **sp.n.**

Molecular phylogeny of the species of the *Ochthebius* (*Enicocerus*) *exsculptus* group

Both reconstructing methods (Bayesian probabilities and ML genetic algorithms) produced the same topology, with good support for most nodes (Fig. 20). The *O.* (*Enicocerus*) *exsculptus* species group as defined here is monophyletic and sister (among the species included in the study) to *O. gibbosus*, with very strong support both for the combined and the nuclear datasets. *Ochthebius gibbosus* is a very distinct species, externally clearly differentiated from the species of the *O. exsculptus* group by its smaller size (1.2–1.6 mm) and non-metallic colouration (Jäch 1992). It does not have sexual dimorphism in the shape of the pronotum and the labrum is only slightly emarginated.

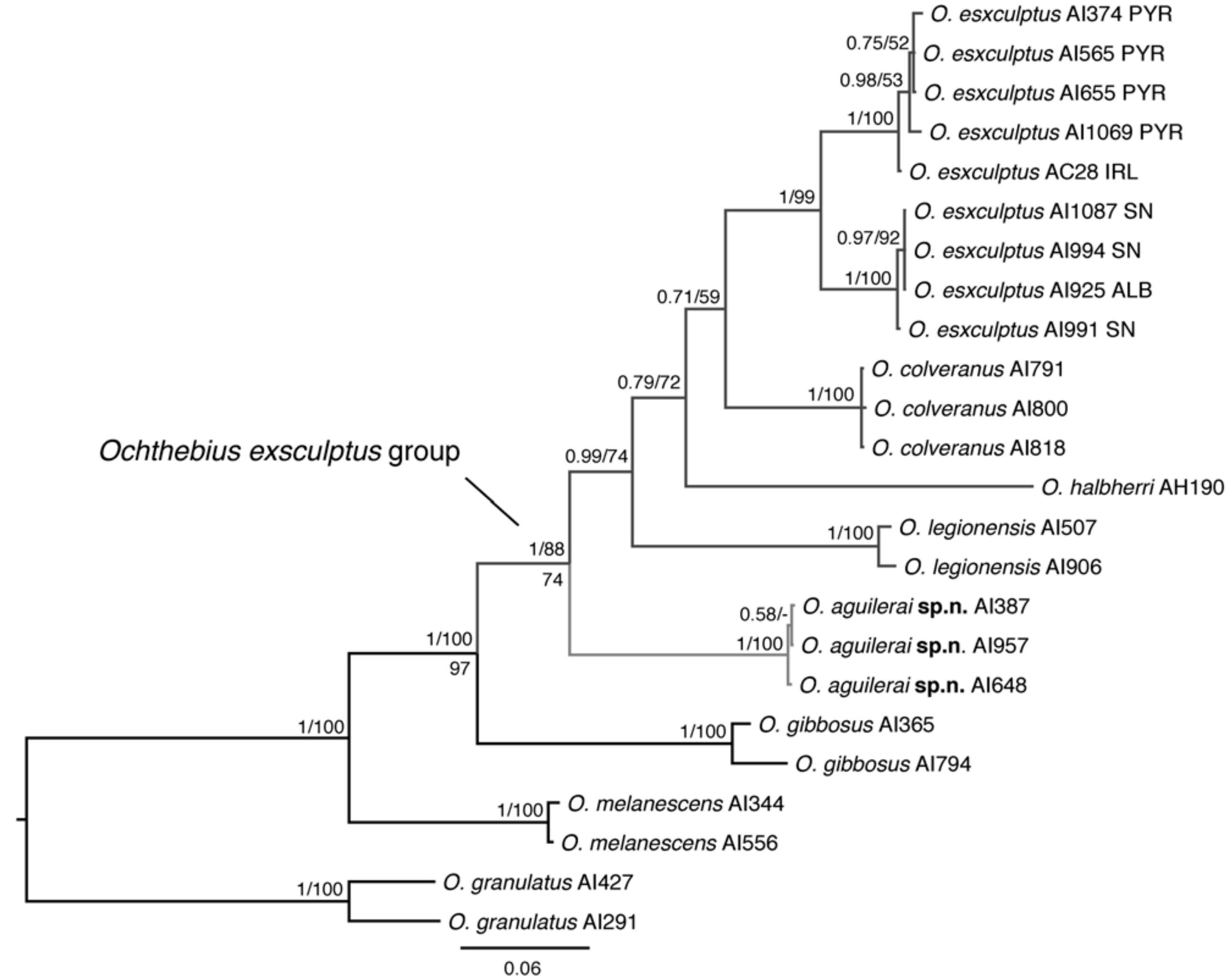


FIGURE 20. Phylogram obtained with MrBayes and the combined mitochondrial and nuclear data. Above nodes, Bayesian posterior probabilities / bootstrap support values in Garli. Below nodes, bootstrap support values for the analysis in Garli of the nuclear data only (when >50%). See Table 1 for the localities of the specimens (PYR, Pyrenees, IRL, Ireland; SN, Sierra Nevada; ALB, Albacete).

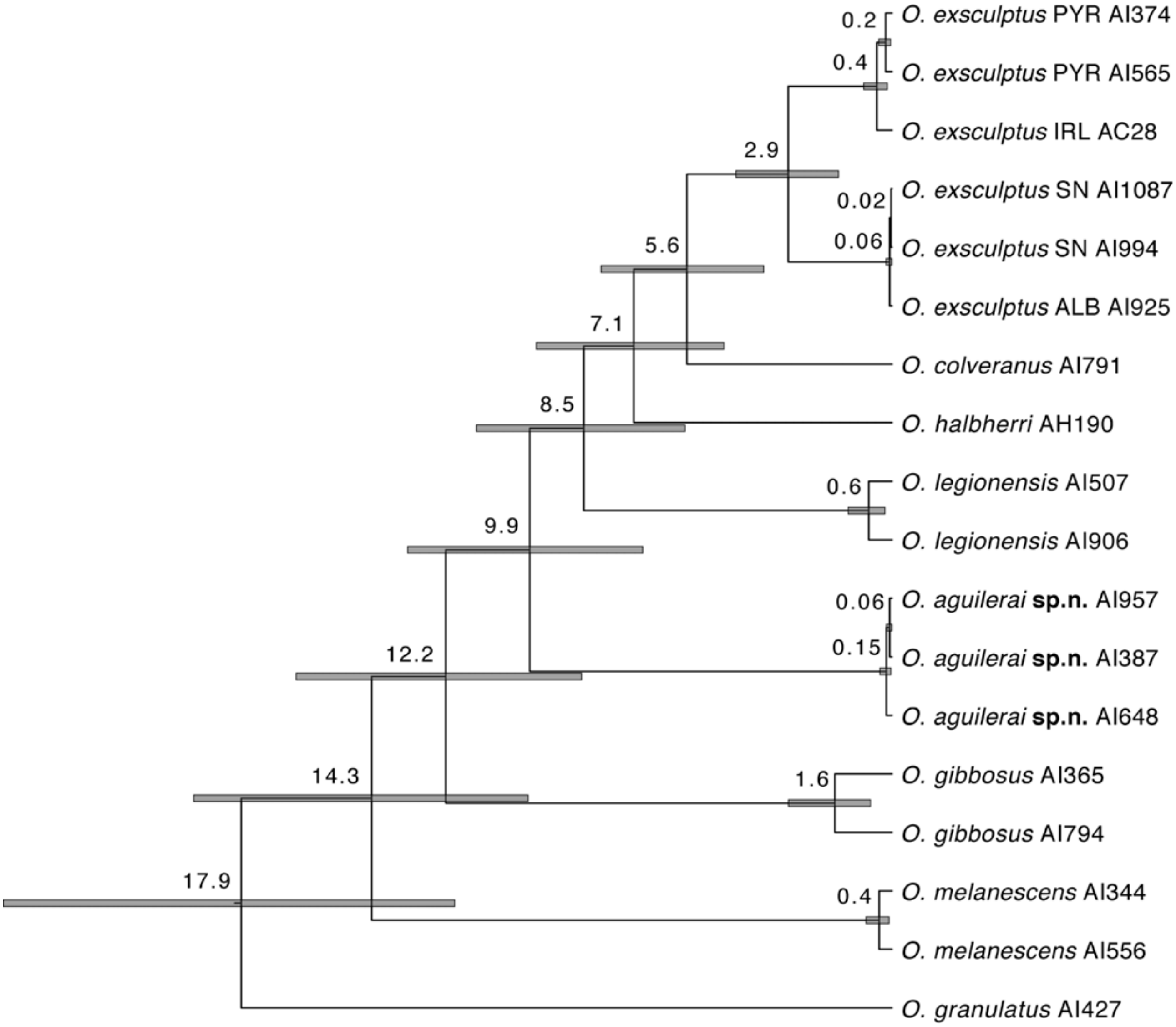


FIGURE 21. Ultrametric tree obtained with Beast and the mitochondrial sequences, with a molecular rate of 2% MY. The topology was constrained to be equal to that obtained with the combined dataset in MrBayes (Fig. 20). Number in nodes, estimated age (MY); blue bars in nodes, 95% confidence interval. See Table 1 for the localities of the specimens.

Within the *O. exsculptus* group the Iberian endemics *O. aguilerai* **sp.n**. and *O. legionensis* formed a paraphyletic series sister to the remaining species, followed by *O. halbherri* (Switzerland, Italy) and the species pair *O. exsculptus* (Western Europe) and *O. colveranus* (Eastern Europe from the Alps to Turkey) (Jäch 2004). It is interesting to note the non-monophyly of the two endemic Iberian species (*O. legionensis* and *O. aguilerai* **sp.n.**), which, if confirmed, would imply the Iberian origin of the *O. exsculptus* group, with a subsequent expansion to eastern Europe and Turkey.

There was a clear genetic separation between the south Iberian populations of *O. exsculptus* (Sierra Nevada and the mountains of Segura and Cazorla) and the populations north of the Ebro (Pyrenees and Ireland). We failed to find consistent morphological differences between these two clades, although the southern populations seem to have a smaller aedeagus, more curved dorsally and with a stronger preapical sinuation that in the northern populations (Figs 7–10). This lack of clear morphological differences, together with the lack of genetic data of intermediate populations in the Iberian system (d'Orchymont 1941, Valladares *et al*. 2000 and Teruel, Beceite, Riu Matarranya, 17.7.1997 and 18.4.1998, IR leg.) and south Spain west of Sierra Nevada (Rosenhauer 1859; Jäch *et al*. 1999) (Fig. 17), prevent us from naming the southern populations as a distinct taxa.

According to the estimations using the mitochondrial sequence and a molecular evolutionary rate of 2% per MY, the origin of the *O. exsculptus* group, and the split of *O. aguilerai* **sp.n**. from its sisters, dates back to ca. 10 MY, in the Tortonian. This is in sharp contrast with the recent, Pleistocene origin of most of the Iberian endemics in the family Dytiscidae (Ribera & Vogler 2004). In contrast, the genetic separation between the populations in Gredos and Guadarrama are very recent, not older that late Pleistocene (ca. 150,000 years). The separation between the southern and northern lineages within *O. exsculptus* date back to the Pliocene-Pleistocene transition (ca. 3 MY), while the divergence within each of them is again more recent, the oldest being the separation between the Irish and Pyrenean specimens at ca. 400KY. The lack of material from other areas from the distributional range of *O. exsculptus* prevents further interpretation of these differences. What seems clear from our data is the taxonomic and evolutionary distinctiveness *of O. aguilerai* **sp.n.**, the oldest of the extant species of the *O. exsculptus* group and likely to be one of the oldest and genetically most distinct endemic species of Iberian water beetles.

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