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Genetic and morphological studies of *Trichosirocalus* species introduced to North America, Australia and New Zealand for the biological control of thistles

A. De Biase[1]*, E. Colonnelli[2], S. Belvedere[1], A. La Marca[3], M. Cristofaro[4] and L. Smith[5]

[1]Dipartimento di Biologia e Biotecnologie 'Charles Darwin', Università di Roma La Sapienza, Viale dell'Università 32, 00185 Rome, Italy: [2]Via delle Giunchiglie 56, 00172 Rome, Italy: [3]BBCA-onlus, Via Angelo Signorelli 105, 00123 Rome, Italy: [4]ENEA C.R. Casaccia SSPT-BIOAG-PROBIO, Via Anguillarese 301, 00123 S. Maria di Galeria (Rome), Italy: [5]USDA-ARS, 810 Avenue du Campus Agropolis, 34980 Montferrier-sur-Lez, France

Abstract

Trichosirocalus horridus sensu lato has been used as a biological control agent of several invasive thistles (*Carduus* spp., *Cirsium* spp. and *Onopordum* spp.) since 1974. It has been recognized as a single species until 2002, when it was split into three species based on morphological characters: *T. horridus*, *Trichosirocalus briesei* and *Trichosirocalus mortadelo*, each purported to have different host plants. Because of this taxonomic change, uncertainty exists as to which species were released in various countries; furthermore, there appears to be some exceptions to the purported host plants of some of these species. To resolve these questions, we conducted an integrative taxonomic study of the *T. horridus* species complex using molecular genetic and morphological analyses of specimens from three continents. Both mitochondrial cytochrome c oxidase subunit I and nuclear elongation factor 1α markers clearly indicate that there are only two distinct species, *T. horridus* and *T. briesei*. Molecular evidence, morphological analysis and host plant associations support the synonymy of *T. horridus* (Panzer, 1801) and *T. mortadelo* Alonso-Zarazaga & Sánchez-Ruiz, 2002. We determine that *T. horridus* has been established in Canada, USA, New Zealand and Australia and that *T. briesei* is established in Australia. The former species was collected from *Carduus*, *Cirsium* and *Onopordum* spp. in the field, whereas the latter appears to be specific to *Onopordum*.

Keywords: biological control, molecular taxonomy, synonymy, thistles, *Trichosirocalus horridus* species complex, weevils

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Introduction

The genus *Trichosirocalus* Colonnelli, 1979 (Coleoptera, Curculionidae, Ceutorhynchinae) includes 17 Palaearctic species (Colonnelli, 2013), mainly feeding on Plantaginaceae and Asteraceae (Colonnelli, 2004). Weevils originating from Italy and Germany, identified at the time as *Trichosirocalus horridus* (Panzer, 1801), were released in Canada, USA, New Zealand

*Author for correspondence
A. De Biase
Phone: +39 0649914744
Fax: +39 064958259
E-mail: alessio.debiase@uniroma1.it

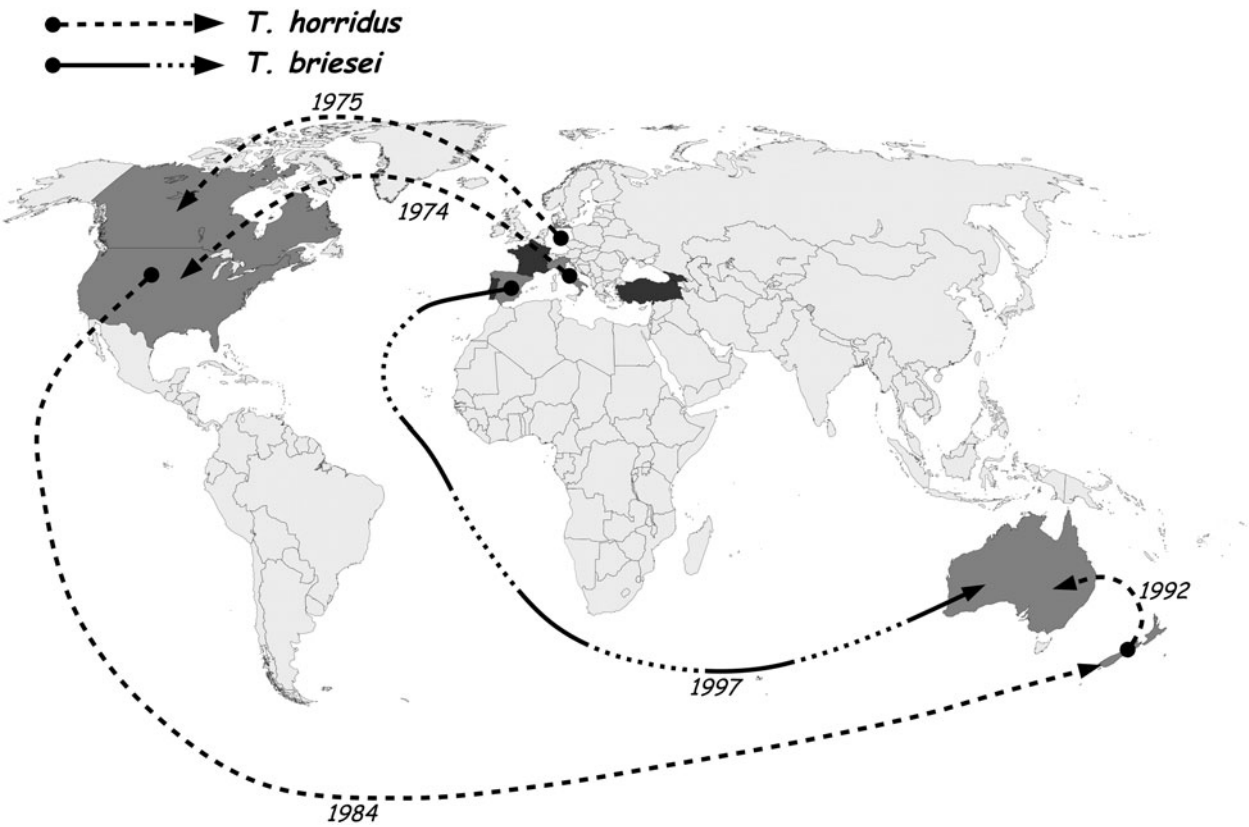

Fig. 1. Map showing the introductions of *Trichosirocalus horridus* sensu lato to Canada, USA, New Zealand and Australia. Medium grey countries were involved in translocation events as described in the text. Dark grey countries were included in the sampling to get more data on biological variation of the species complex. Both light and dark grey countries were sampled for the present study. Base Map from http://commons.wikimedia.org/wiki.

and Australia for classical biological control of Musk thistle (*Carduus nutans* L.) and its close relatives (Kok & Trumble, 1979; Harris, 1984; Jessep, 1989; Woodburn, 1997). In 1997, weevils associated with Scotch thistle (*Onopordum acanthium* L.) in Spain were introduced to Australia (Briese *et al*., 2002*a*; Briese, 2012). Until 2002, *T. horridus* (Panzer, 1801) was considered as single species, known to be associated in the larval and/or adult stages with several species of thistles of the tribe Cardueae (*Carduus* spp., *Cirsium* spp., *Onopordum* spp, *Silybum marianum* (L.) Gaertn and *Galactites tomentosa* Moench) (Zwölfer, 1965). Alonso-Zarazaga & Sánchez-Ruiz (2002), from a morphological study of samples collected by D. Briese in Australia on *Carduus* (*T. horridus* was released in Australia in 1985 as control agent against *Carduus* thistles), as well as samples from the Australian quarantine stock originally collected on *Onopordum* in Spain, concluded that *T. horridus* was a complex of three species, which differed in host range and geographical distribution. Accordingly, *T. horridus* sensu stricto, feeds on *Cirsium* spp. and occurs in Spain, France, Germany and Croatia, *Trichosirocalus mortadelo* Alonso-Zarazaga & Sánchez-Ruiz (2002), feeds on *Carduus* spp. and is known from Australia and Germany (Hannover) and *Trichosirocalus briesei* Alonso-Zarazaga & Sánchez-Ruiz (2002), feeds on *Onopordum* spp. and occurs in Austria, Morocco and Spain. Thus, the *T. horridus* complex was believed to encompass three very closely related species primarily distributed in South and Central Europe; however, it was not clear which species were introduced to North America, Australia and New Zealand.

Members of this species complex have been repeatedly translocated in biocontrol programmes against several thistles (fig. 1). First, colonies of *T. horridus* originating from *Carduus* in Italy were established to control *Carduus* thistles in the USA (Virginia, Kansas, Nebraska and Montana) in 1974 (Kok, 1978, 2001; Kok & Trumble, 1979). Weevils collected from Germany (Neuenburg) were introduced to Canada in 1975 to control *Carduus* spp. (Harris, 1984; De Clerck-Floate & Cárcamo, 2011). In 1984, specimens of *T. horridus* from the Canadian colony were introduced to New Zealand (Jessep, 1989), and in 1992 individuals from populations established in New Zealand were released in Australia to control *Carduus* spp. In the 1990s, Briese and colleagues carried out a field survey in Europe to seek prospective control agents to manage *Onopordum* thistles in Australia (Briese *et al*., 1994, 2002*a*). They discovered in Spain a population of the *T. horridus* complex apparently restricted to develop on *Onopordum* thistles, as suggested by the host range tests performed in an Australian quarantine laboratory (Briese *et al*., 2002*b*, *c*). These weevils were described as a new species (*T. briesei*) by Alonso-Zarazaga & Sánchez-Ruiz (2002) and released in Australia for the control of *O. acanthium* (Woodburn, 1997). Although the identity of insects in the latter introduction

was clear, it was uncertain whether *T. horridus* and/or *T. mortadelo* had previously been introduced in each of the above countries.

In North America, *T. horridus* has been reared from both *Carduus* and *Cirsium* species (McAvoy *et al.*, 1987; Takahashi *et al.*, 2009; Wiggins *et al.*, 2009), which suggests either the presence of two species of weevil (*T. horridus* on *Cirsium* and *T. mortadelo* on *Carduus*) or that at least one of the species is not as specific as Alonso-Zarazaga & Sánchez-Ruiz (2002) claimed. Furthermore, the weevils have been found on *Cirsium*, *Carduus* and *Onopordum* in New Zealand despite the introduction of only one population from Canada (Groenteman *et al.*, 2008). Because of concern about risk to non-target plant species and the need to better understand the specificity of biological control agents of weeds, it is important to clarify the taxonomic status of these species and their host specificity. Molecular genetic analysis has often contributed to the discovery of cryptic species that differ in important biological traits, including host specificity (Fumanal *et al.*, 2005; Madeira *et al.*, 2006; Mound *et al.*, 2010; Gaskin *et al.*, 2011). Furthermore, combining morphological, genetic and biological traits, known as integrative taxonomy, can provide a more robust and stable classification (Dayrat, 2005; Padial *et al.*, 2010).

The goal of this paper is to reassess the taxonomy of the species in the *T. horridus* complex using a combination of molecular genetic, morphological and host plant data.

Materials and methods

Sampling of investigated populations

Samples of adult weevils were collected during field trips carried out in Spain, Portugal, France, Italy, Turkey and Georgia between 2008 and 2013 to obtain specimens from several distinct localities. In addition, many adult specimens were kindly provided by several colleagues from Germany, the USA, Canada, Australia and New Zealand, and a few larvae from New Zealand were provided by R. Groenteman (Landcare Research, Lincoln, New Zealand). These larvae were of particular interest because they had been collected from all the currently recorded host plants (*Carduus*, *Cirsium*, *Onopordum*) of the *T. horridus* complex. A few specimens of *Trichosirocalus troglodytes* (Fabricius, 1787), used as an outgroup taxon in the statistical analyses, were collected during a field trip in Portugal in 2013 by one of us (Enzo Colonnelli). Figure 1 displays the countries in which the studied samples were collected, and Table S1 (see supplementary material) lists the details of the localities and host plants. To record the trophic range of the taxonomic entities under study, during the fieldwork, the host plant of each collected adult specimen was identified by the field collectors. Insect voucher specimens are preserved in the authors' collections.

Molecular genetic analysis

Following the procedure described in Cristofaro *et al.* (2013), the total genomic DNA was extracted and used as a template in polymerase chain reactions (PCR; Mullis *et al.*, 1986) to amplify a fragment of the mitochondrial genome coding for the cytochrome c oxidase subunit I (*cox1*) and a fragment of the nuclear gene coding for the elongation factor 1α (*ef1α*). Folmer's primers LC01490 and HC02198 (Folmer *et al.*, 1994) were used to amplify the 5′ upstream region of the *cox1* gene, or, when needed, the TY-J-1460 primer of Simon *et al.* (1994) as the forward one; few individuals not giving clean PCR results were amplified as described by Rector *et al.* (2010). The primers EF1-Bf and EF1-Br (Hernandez-Vera *et al.*, 2010), forward and reverse, respectively, were used to amplify the *ef1α* gene. We used several PCR thermal cyclers among those available at our laboratory (i.e., Perkin Elmer® GeneAmp PCR System 2400 thermal cycler; MWG® Biotech Primus 25, Biometra® Tpersonal 48), with the following amplification conditions: (a) *cox1*: 94°C denaturation (5 min), followed by 35 cycles of 95°C denaturation (1 min), 40°C annealing (1 min), and 72°C extension (1 min and 30 s), followed by a final 7 min elongation step at 72°C; (b) *ef1α*: touchdown PCR with 94°C denaturation (2 min), followed by 24 cycles of 94°C denaturation (30 s), 62–50°C annealing (1 min; decreasing 2°C every 2 cycles), and 72°C extension (1 min), followed by 2 cycles of 94°C denaturation (30 s), 48°C annealing (1 min), and 72°C extension (1 min), followed by a final 7 min elongation step at 72°C. Reactions were performed in 25 µl of cocktail containing $(NH_4)_2SO_4$ 16 mM, Tris–HCl 67 mM (pH 8.8 at 25°C), $MgCl_2$ 3 mM, Tween-20 0.01%, 1 mM of each deoxynucleotide, 0.8 pM of each primer, and 1.25 units of Taq DNA polymerase (Bioline Reagents Ltd, UK). Amplified products were purified by Exo-SAP enzymatic reactions and sequenced at the Macrogen Korea (Seoul, Korea) and Macrogen Europe (Amsterdam, The Netherlands) genomic centres, employing Applied Biosystems® 3730xl DNA Analysers and using the BigDye Terminator Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Sequencing primers were LC01490 and EF1-Bf for the mitochondrial and nuclear markers, respectively. When needed, the DNA of a few individuals was sequenced on both strands using the same reverse primers used during the PCR amplifications.

The acquired sequences were screened by a blast search of the GenBank nucleotide collection of the National Center for Biotechnology Information (NCBI) using the Mega BLAST procedure (Wheeler *et al.*, 2007) available at its website (http://www.ncbi.nlm.nih.gov/blast). The screening procedure was aimed at checking the assignment of the specimens to high-level categories (e.g., family and subfamily). Next, the sequences were edited and aligned using the Staden Package ver. 2006.1.7.0 software (Staden *et al.*, 1999). All peaks were checked for wrong base calls and noise and were cleaned when required. The two alignments were visually assessed without requiring any insertion–deletion (indel) typing for the *cox1* gene, whereas for the nuclear *ef1α* gene a little indel typing was needed within the encompassed intronic region. The latter sequences were also checked for heterozygous positions, and the gametic phases, where needed, were inferred with PHASE version 2.1 (Stephens *et al.*, 2001). Finally, both alignments were collapsed using FaBox tool (Villesen, 2007) to retain the scored haplotypes only.

Statistical analyses

Divergence analyses and Neighbour-Joining (NJ; Saitou & Nei, 1987) tree inference were performed for both markers by means of Molecular Evolutionary Genetics Analysis, version 5.2 (MEGA5), setting the *p* uncorrected model for the genetic distance values computation (Tamura *et al.*, 2007). Confidence at tree nodes was determined by bootstrapping 1000 times over the data. Genetic divergence was estimated as *p*

uncorrected distance computed as net averages among the groups scored on the inferred NJ topology.

Bayesian analyses were performed using Beast version 1.8.0 (Drummond *et al.*, 2012) under the substitution model (s) selected by AICc in jModelTest 2 for each marker/partition analysed (Darriba *et al.*, 2012). The HKY + I + G substitution model was selected for *cox1*, while the *ef1α* nuclear marker was modelled by setting three partitions, namely the coding and noncoding regions of the gene (exons and introns both with HKY substitution model with estimated base frequencies and no model for site heterogeneity) and the indels that were scored during the alignments of the sequences (stochastic Dollo model). We used the lognormal relaxed-clock model implemented in the software to take into account the variation of the substitution rate among lineages (prior distribution set as Exponential with initial value 1.0, mean 10.0 and offset 0.0). The tree prior was set using the Constant coalescent Kingman model (Kingman, 1982). The analysis was carried out using a random starting tree, running two Markov chains for 50×10^6 generations and sampling every 1000 generations. Finally the same analysis was performed sampling from priors only to evaluate the priors that we applied to the various parameters. Convergence was evaluated with Tracer version 1.6 (Rambaut *et al.*, 2013), and the two chains were combined with Logcombiner routine of Beast, discarding 12,500 burn-in trees each; the combined set of trees for each marker was summarized as a Maximum clade credibility tree with Beast's Treeannotator routine.

Degree of genetic divergence is related to the taxon under study, and there is no universal yardstick for unequivocally assigning a taxonomic rank to a scored value of genetic divergence (Blaxter, 2004; Moritz & Cicero, 2004). This is a key problem when studying species, or populations, closely related to each other or recently diverged. The issue is commonly referred to as the *species delimitation problem.* Authors adopting the barcoding approach argue that a certain amount of genetic divergence between groups, contrasted to the within-groups divergence (barcoding gap) should be used as a guideline for species delimitation (Hebert *et al.*, 2004). This approach has been enthusiastically supported or criticized owing to many issues related to sampling effort, incomplete lineage sorting and hybridization of recently diverged species and so on (Tautz *et al.*, 2003; Janzen, 2004; Moritz & Cicero, 2004; De Salle *et al.*, 2005; DeSalle, 2006). Recent years have seen many discussions and contributions on this issue, producing several noteworthy methods (Wiens, 2007; Ence & Carstens, 2011; Fujita *et al.*, 2012). We have adopted three models/methods to analyse our datasets: (a) a classical phylogenetic approach by using both the NJ algorithm and the Bayesian inference implemented in Beast (Drummond *et al.*, 2012); (b) the Automatic Barcoding Gap Discovery (ABGD) approach (Puillandre *et al.*, 2012), as a fast and simple method to discover partitions in our datasets; and (c) the General Mixed Yule Coalescent (GMYC) model (Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Monaghan *et al.*, 2009; Fujisawa & Barraclough, 2013), which helps in seeking the threshold that marks the transition between evolutionary dynamics within and among species, thus suggesting those clusters to be considered as distinct species on a phylogenetic tree.

The ABGD method (Puillandre *et al.*, 2012) stems from the barcoding methodology, which was originally focused on the identification of biological samples using a standard nucleotide sequence (a 5′ fragment of the mitochondrial *cox1* gene) compared with a reference dataset of previously characterized species. The method aims at defining partitions in a set of *cox1* sequences that must be considered as hypotheses of prospective distinct species to further investigate in an integrative framework. The partitions are defined by analysing the distribution of all pairwise distances between sequences in order to locate the most reliable 'barcode gap' between the intraspecific and interspecific divergence. After the initial partitions are defined, the algorithm is performed in a recursive way until no new partitions are defined. Our analyses were carried out on the alignment of all 165 *cox1* sequences of the analysed ingroup (*T. horridus* species complex) by using the ABGD method as available on the website http://wwwabi.snv.jussieu.fr/public/abgd/ (Puillandre *et al.*, 2012; last access 26/02/2015) with the following parameters: Pmin 0.001; Pmax 0.1; Steps 10; X (relative gap width) 1.5; Nb bins (for distance distribution) 20; Simple distance.

The GMYC method (Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Monaghan *et al.*, 2009; Fujisawa & Barraclough, 2013) is aimed at modelling in a probabilistic framework both the coalescence processes that occur within species at population level, as described by topology and length of the branches of a phylogenetic gene tree, and the speciation processes occurring at a certain level of divergence and identified as a threshold above which all nodes describe speciation events as defined by the Yule speciation model (Yule, 1925). This approach thus combines standard coalescent models that consider the diversification within populations (Hudson, 1991; Wakeley, 2008) with those models that describe the branching pattern of speciation events (Nee, 1994, 2001; Nee *et al.*, 1994). The method evaluates, by means of a likelihood test, alternative scenarios by assessing several thresholds as a boundary between intra- and inter-specific dynamics, and fitting the best one for delimiting the species encompassed by the gene tree under analysis. The analyses were performed on the *cox1* dataset by using the multiple-threshold version of the method (Monaghan *et al.*, 2009) implemented by the R package *splits* (Ezard *et al.*, 2009) version 1.0–18 with the following parameters: method = 'multiple', interval = c(0, 10).

Morphological analysis

In order to test the taxonomic pattern revealed by the molecular analyses, we performed a morphological analysis of the specimens used in molecular work. We also studied the *T. mortadelo* holotype, although we were not permitted to extract DNA from it for analysis. Morphological data collected by one of us (Enzo Colonnelli) over many years of observations from hundreds of specimens preserved in museums and private collections worldwide were also used. We found that the only available key (Alonso-Zarazaga & Sánchez-Ruiz, 2002) to these *Trichosirocalus* species was unreliable to identify the three purported species of the *T. horridus* complex. In the following section we list the few characters that permit identification of the valid species. The morphological characters of specimens were primarily studied using a Wild M5 microscope with up to 50× magnification. Among the measures reported by Alonso-Zarazaga & Sánchez-Ruiz (2002), we selected and reported here only the total body length, as all other ratios quoted in the above paper, and verified by us upon studied specimens, were variable to such an extent as to be generally useless to discriminate the species of this complex. Only the body length measures were diagnostic for species discrimination. Photographs of the holotype of *T. mortadelo* were taken with a Nikon D90 camera fitted with

an AF Micro Nikkor 60 mm lens and then enhanced using the programmes Helicon Focus and Adobe Photoshop PS4.

Results and discussion

Molecular genetic analyses

We obtained a fragment of nearly 650 bp from 168 individuals collected in the field for the *cox1* marker and a fragment of nearly 880 bp from 134 specimens for the *ef1α* nuclear marker. Only the *cox1* marker was successfully amplified from the outgroup taxon, *T. troglodytes*. The alignment was cut at the shortest aligned sequence, giving a final set of sequences each 621 bp long for *cox1* and 760 bp for *ef1α*. The two collapsed alignments consisted of 45 and 10 unique haplotypes, respectively, for *cox1* and *ef1α*, and Tables S2 and S3 (see supplementary material) list the distribution of the scored haplotypes for the two markers among all sequenced specimens and the accession numbers of the nucleotide sequences deposited in the NCBI/EMBL/DDBJ databanks.

The topologies obtained by NJ, that performed on the data of the *p* uncorrected distance pairwise matrix, are illustrated in fig. 2a, b. Both trees (*cox1* and *ef1α*) show two clear clusters in the analysed ingroup (*T. horridus* species complex) with good bootstrap support (100 on *cox1* tree; 79 on *ef1α*). In fig. 2a, b, the *p* uncorrected distance values among the scored groups are listed. The *p* values between the two clusters, representing *T. horridus* and *T. briesei*, are 0.109 and 0.016 for *cox1* and *ef1α*, respectively. The *T. horridus* cluster is somewhat variable (average within-*p* distance = 0.015 and 0.003 for *cox1* and *ef1α*, respectively), showing a structure grossly related to the sampled geographical areas (i.e., Spain, France, Germany [including Canada, Australia and New Zealand], Italy [including the USA], Turkey and Georgia). On the other hand, the *T. briesei* cluster is relatively homogeneous, with low *p* values (average within distance = 0.002 and 0.000 for *cox1* and *ef1α*, respectively), also mirroring the smaller extent of the geographical distribution of the sampled individuals.

Maximum clade credibility trees from the Bayesian analyses are depicted in figs 3 and 4; again both trees display two clusters in the ingroup, with high support values (e.g., posterior probability 1.0 for both *T. horridus* and *T. briesei cox1*). As for the NJ trees, one of the two clusters encompasses a larger amount of genetic variation, probably due to a wider geographical distribution of the sampled individuals. The NJ and Bayesian topologies obtained from the analysis of all haplotypes of both markers do not differ substantially (figs 2a, b, 3 and 4), strengthening our results. The trees show quite clearly that there are two taxa in our ingroup (i.e., *T. horridus* and *T. briesei*), both with high support values (100 as bootstrap value for NJ trees and 1.0 as posterior probability in the Bayesian trees). This is also confirmed by the *p* distance values on the NJ tree (fig. 2a, b) and the results of the ABGD analysis (fig. 5; see below). The cluster labelled as *T. horridus* includes adult specimens (see Table S2 and S3 for details) collected feeding on *Carduus* or *Cirsium* and identified on morphological characters as *T. horridus*. It also includes a few larvae assigned to *T. horridus* on their genetic characters. The *T. briesei* cluster includes all the specimens morphologically identified as *T. briesei* that were collected feeding on *Onopordum* spp. in Spain and Australia. It is noteworthy that the specimens collected in New Zealand as larvae on the three most important host plants (*C. nutans*, *Cirsium vulgare* (Savi) Ten. and *O. acanthium*) were all included in the *T. horridus* cluster. This is contrary to the assertion by Alonso-Zarazaga & Sánchez-Ruiz (2002) that each of these host plants should have only one associated weevil species, *T. mortadelo*, *T. horridus* and *T. briesei*, respectively. This supports the previously reported conclusion of a survey carried out by Groenteman *et al.* (2008) that currently only *T. horridus* occurs in New Zealand.

Results from the ABGD approach are depicted in fig. 5 and table 1. Figure 5a illustrates the distribution of the pairwise distances for the whole dataset, showing two modes that reflect intraspecific (low values) and interspecific (high values) distances, with a marked gap between them. The gap is mirrored in the steep slope in fig. 5b, in which the ranked pairwise distances are plotted. Table 1 lists the partitions found during the ten recursive steps in the ABGD analysis, lumping the *cox1* sequences in groups as first-species-partition hypotheses (Puillandre *et al.*, 2012). Partitions 1–5 ($P = 0.001000$–0.007743) were excluded for the oversplitting of the sequences in a high number of first-species-partition hypotheses and the consequent incongruence with the results of the phylogenetic analyses and the ecological and morphological evidence. The incongruence was always located in the sequences assigned to *T. horridus* in other statistical analyses. Partitions 6–10 ($P = 0.012915$–0.100000) perfectly match the phylogenetic, GMYC, morphological and ecological evidence. Overall the ABGD results clearly suggest the existence of a marked barcoding gap, further strengthening the hypothesis that there are only two species in the *T. horridus* species complex.

The analyses of the relationships among the haplotypes, as depicted in the Bayesian tree of fig. 3, by using the GMYC method with multiple thresholds, resulted in an oversplitting of our dataset into more than ten putative species (fig. 6; see the figure legend for more explanation) that clearly do not correspond to evolutionary units at specific rank. Many authors have recently discussed the oversplitting behaviour of the GMYC model when sequences, or haplotypes, span a large geographical extent, suggesting poor performance of the model when isolation by distance is manifest (Bergsten *et al.*, 2012; Talavera *et al.*, 2013). Our group # 1 encompasses *T. horridus*, which we sampled from the Iberian Peninsula to Georgia. This sampling scheme captured the natural genetic variation among geographically separated populations of this taxon. We then decided to limit the influence of the divergence likely due to isolation by distance, by analysing a subset of our dataset, only including the haplotypes scored for the specimens that were sampled within the Iberian Peninsula (that includes the ingroup and the outgroup). We ran the Beast analyses with the Iberian subset of haplotypes and then applied the GMYC model. The new results, shown in fig. 7, clearly suggest, once more, to split the ingroup (*T. horridus* species complex) into two distinct evolutionary lineages, that is two species, which we refer to as *T. horridus* and *T. briesei*.

Morphological analysis

Morphological characters of some 1550 adults were studied during a span of about 35 years, starting in the mid-1970s, when one of us (Enzo Colonnelli) was asked by the European Biological Control of Weeds Laboratory (EBCL) of the United States Department of Agriculture to identify specimens of *Trichosirocalus* reared chiefly from *C. nutans* and *C. macrocephalus* Desf. prior to the importation of adults weevils from Italy into the USA for biological control Musk Thistle

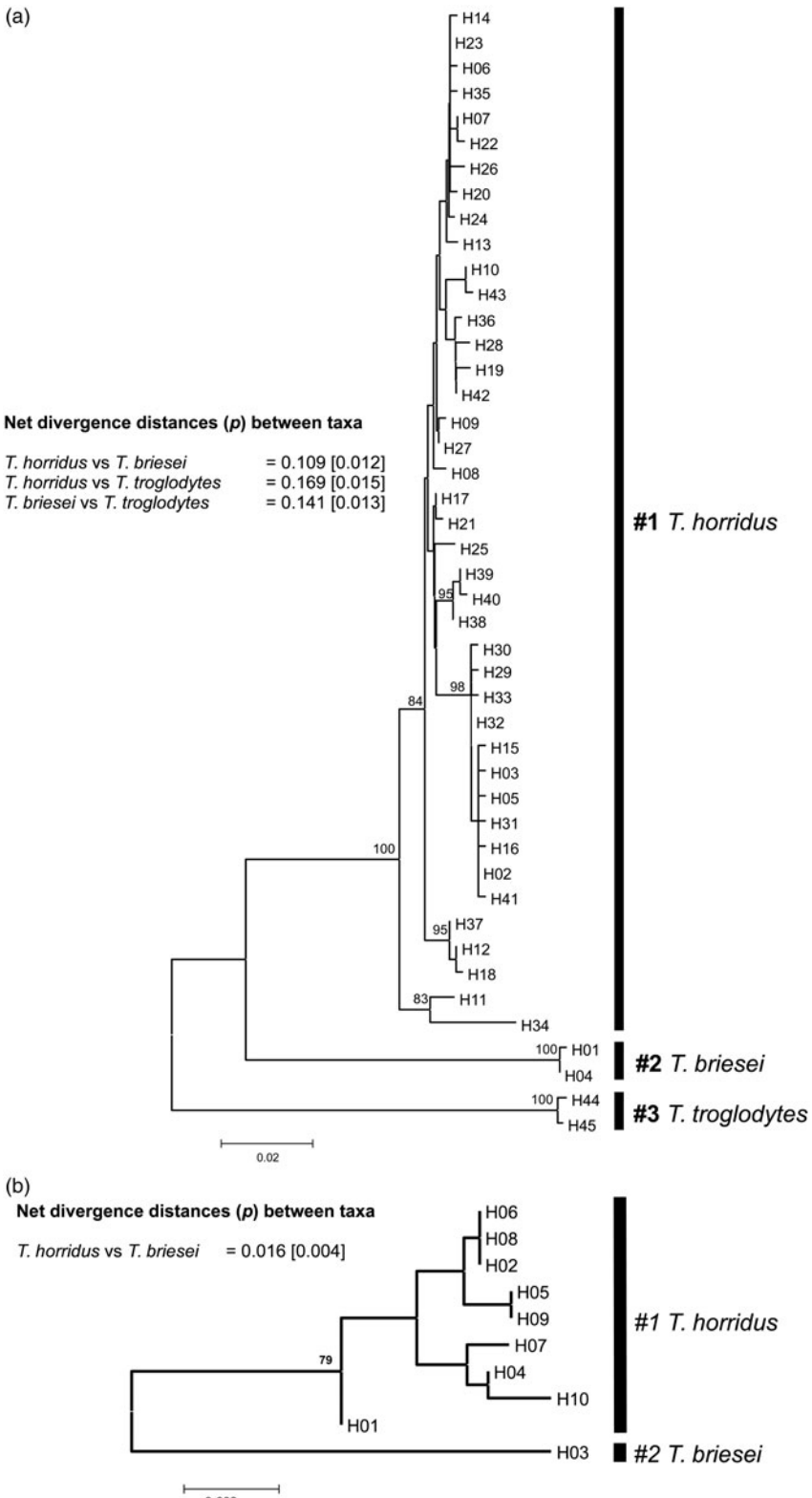

Fig. 2. NJ trees for (a) *cox1* and (b) *ef1a* genes. Net divergence distances (*p*) between *T. briesei*, *T. horridus* and *T. troglodytes*. Numbers at nodes are bootstrap values. Tip labels refer to haplotypes scored and listed in Table S2 for *cox1* and Table S3 for *ef1a*.

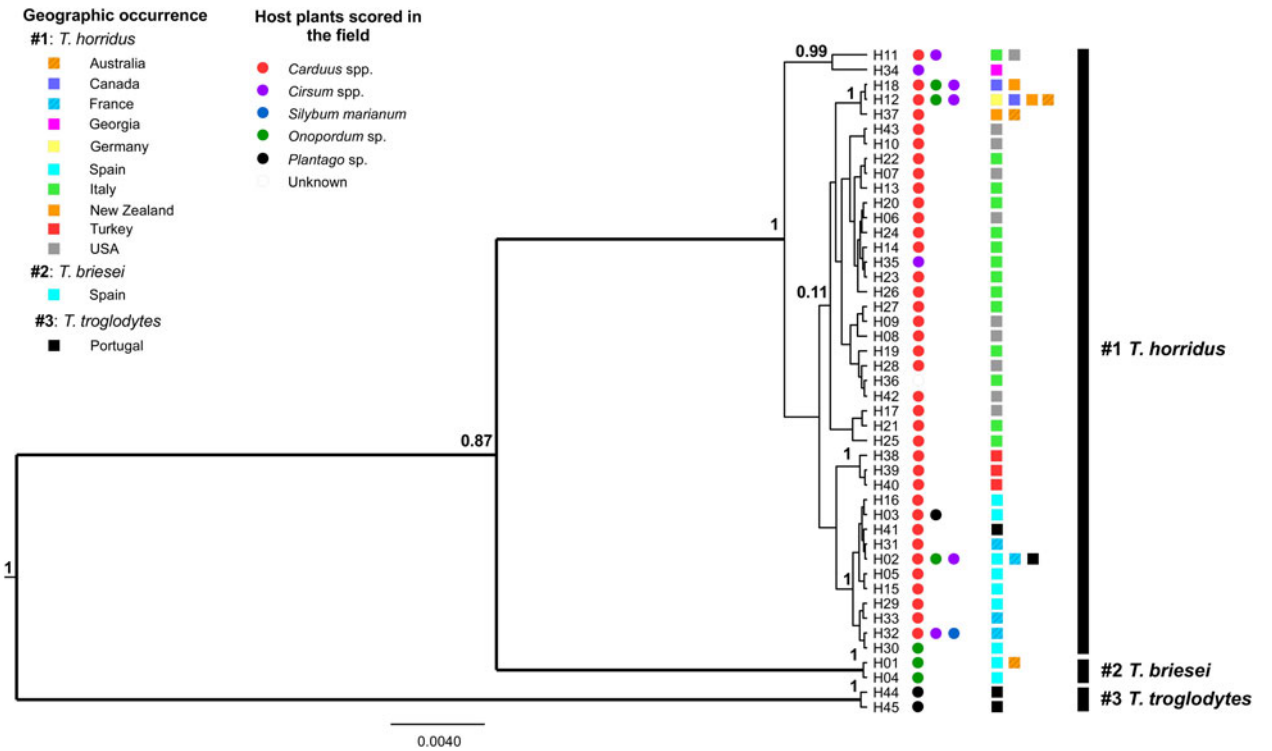

Fig. 3. Bayesian consensus tree (50% Majority Rule) for the scored haplotypes of the *cox1* gene; figures at nodes are posterior probabilities values; *T. troglodytes* was used as outgroup in statistical analyses; coloured squares refer to geographical distribution of haplotypes and coloured spots to the host plants on which adults were collected in the field (suggesting at least trophic association between the insect and the plant; no evaluation was made regarding the oviposition behaviour).

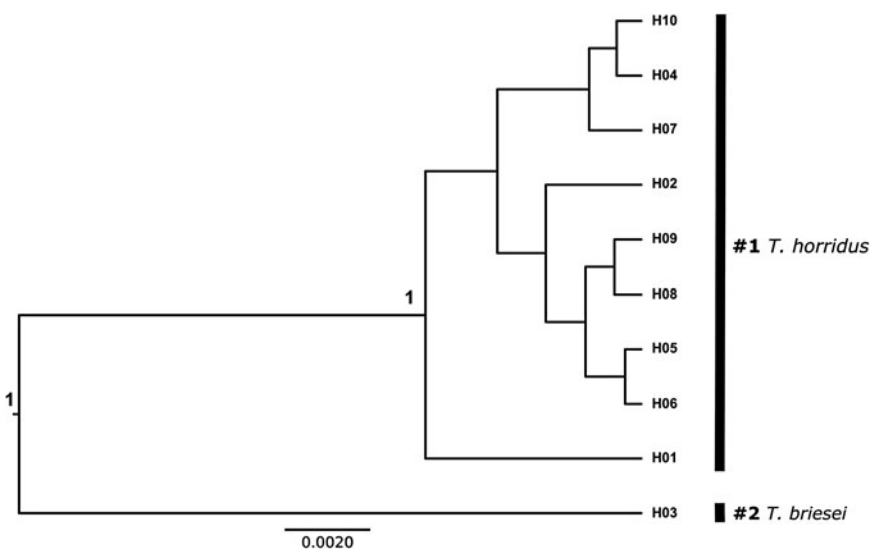

Fig. 4. Consensus tree (50% Majority Rule) obtained by Bayesian analysis of *ef1a* haplotypes; figures at nodes are posterior probabilities values.

(Kok, 2001). EBCL scientists provided also precise collecting and biological data for *Trichosirocalus* (Boldt & Campobasso, 1978, 1981; Boldt *et al.*, 1980), until then poorly known apart from scattered records of adults or larvae and the plants on which they were found (Perris, 1877; Kleine, 1910; Wagner, 1944; Hoffmann, 1955; Scherf, 1964; Dieckmann, 1972). The life history and host plants of the *T. horridus* complex were extensively studied before and after the release in the USA,

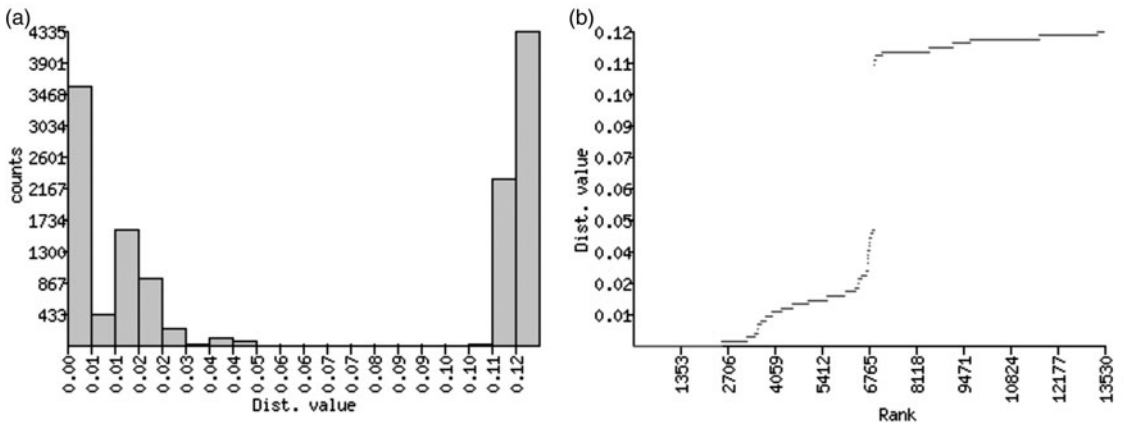

Fig. 5. Automatic Barcoding Gap Discovery (ABGD) web results using simple distance measure of distance for *cox1;* (a) distribution of pairwise distances among all sampled individuals shows two modes (low and high distance values) related to intraspecific and interspecific distances, respectively; (b) the same values plotted in ranked order showing a steep slope at the barcoding gap value.

Table 1. Results of the ABGD method (*P* value representing prior maximum divergence of intraspecific diversity; in our analysis it ranging between 0.001 and 0.1).

Partition id	Groups within partition	Prior maximal distance *P*
1	22	0.001000
2	9	0.001668
3	9	0.002783
4	9	0.004642
5	9	0.007743
6	2	0.012915
7	2	0.021544
8	2	0.035938
9	2	0.059948
10	2	0.100000

Canada, Australia and New Zealand (Ward *et al.*, 1974; Kok, 1975; Trumble & Kok, 1979; Boldt *et al.*, 1980; Boldt & Campobasso, 1981; Rizza & Spencer, 1981; Jessep, 1989; Kok & Mays, 1989; Briese *et al.*, 1994, 2002*c*; Woodburn, 1997). However, after the description of *T. briesei* and *T. mortadelo* by Alonso-Zarazaga & Sánchez-Ruiz (2002) there were very few records of either of these species, apart from the inclusion of their names in a world catalogue (Colonnelli, 2004) and in a Palaearctic catalogue (Colonnelli, 2013) of Ceutorhynchinae, and the mention by Groenteman *et al.* (2008) of their possible presence in New Zealand. *Trichosirocalus briesei* was added by Pelletier (2012) to the fauna of Morocco based on the study by Alonso-Zarazaga & Sánchez-Ruiz (2002) and recorded from some additional Spanish localities reported by Alonso-Zarazaga *et al.* (2006) and Alziar & Lemaire (2012), whereas *T. mortadelo* was just recorded from Germany after Alonso-Zarazaga & Sánchez-Ruiz (2002) by Rheinheimer & Hassler (2010), who also expressed, on page 782, some doubts about the distinctiveness of these two species. None of these reports was based on new material or a critical evaluation of the study by Alonso-Zarazaga & Sánchez-Ruiz (2002).

Our morphological study of the *T. horridus* complex revealed that some characters used by Alonso-Zarazaga & Sánchez-Ruiz (2002), including the different host plants, are either unreliable or erroneous, due to both the possibility of *T. horridus* developing on *Cirsium, Carduus* and *Onopordum* (Hoffmann, 1955; Scherf, 1964; Dieckmann, 1972; Groenteman *et al.*, 2008) and the morphological variability of this species across its wide geographical range (EC, personal observation). In addition, due to morphological variability, the shape of spermatheca is not diagnostic, and the softness of the ovipositor subjects it to easy deformation. Our examination of the aedeagi of numerous specimens indicates that the differences between *T. horridus* and *T. mortadelo*, as stated by Alonso-Zarazaga & Sánchez-Ruiz (2002), are due to their depiction from slightly different angles of view. Unfortunately the aedeagus of the holotype of *T. mortadelo* is embedded in Dimethyl hydantoin formaldehyd (DMHF) and we did not have permission to dissolve this mounting medium in order to better examine the aedeagus. However, in apical view the apex of the penis is sinuous like those of all other *T. horridus* males we studied (fig. 8e). In addition, the temones (the pair of basal apodemes of the penis) are not really as short as depicted in their figures (fig. 8c, d). We thus conclude that the shape of the penis of the *T. mortadelo* holotype falls within the variation of *T. horridus* (fig. 8e, f) as described in Alonso-Zarazaga & Sánchez-Ruiz (2002) and that genital differences illustrated by these authors appear to be due to improper mounting, which changes the angle of view of the curved penis (fig. 8c, e). Also, the appearance of the internal sac of *T. mortadelo*, as sketched in fig. 17 of Alonso-Zarazaga & Sánchez-Ruiz (2002), depends on how the triangular spiculum is placed inside the soft internal sac of the studied individual. Therefore this character is also unreliable.

In conclusion, our morphological and genetic analyses support the recently established synonymy by Pullen *et al.* (2014) of *T. mortadelo* Alonso-Zarazaga & Sánchez-Ruiz (2002) with *T. horridus* (Panzer, 1801). Our results also confirm the contention that only one species (*T. horridus*) was introduced to New Zealand (Groenteman *et al.*, 2008; Cullen & Sheppard, 2012; Sagliocco *et al.*, 2012). We conclude that the *T. horridus* complex includes only two species, *T. horridus* (Panzer, 1801) and *T. briesei* Alonso-Zarazaga & Sánchez-Ruiz (2002).

Ecological data from the literature also do not support the existence of more than two different species in the *T. horridus* complex. In fact, Ward *et al.* (1974) reported the possibility of

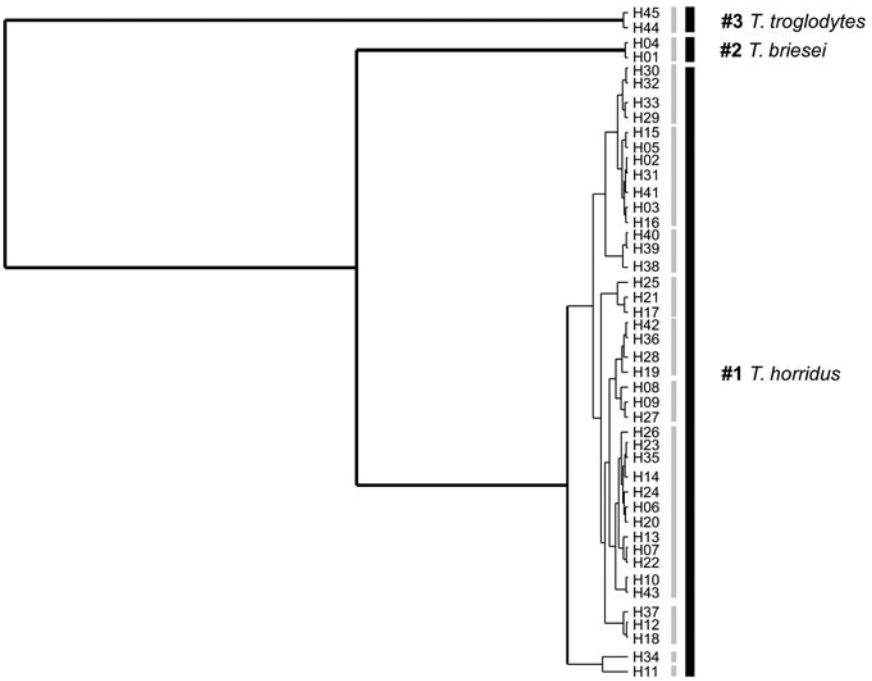

Fig. 6. General Mixed Yule Coalescent (GMYC) results obtained by analysing the tree in fig. 3 that depicts the phylogenetic relationships among all haplotypes found within our *cox1* dataset. Each light grey vertical bar groups haplotypes referring to putative species according to GMYC model. Likelihood values are: $L_0 = 395.4515$ for the null model; $L_{GMYCmultiple} = 403.1648$ for the GMYC model; Likelihood Ratio Test $P = 0.00045$***; number of ML clusters 10 (confidence interval 3–12); number of ML entities 12 (confidence interval 3–15). The black vertical bars with species names emphasize the oversplitting likely due to the genetic variation within the *T. horridus* dataset.

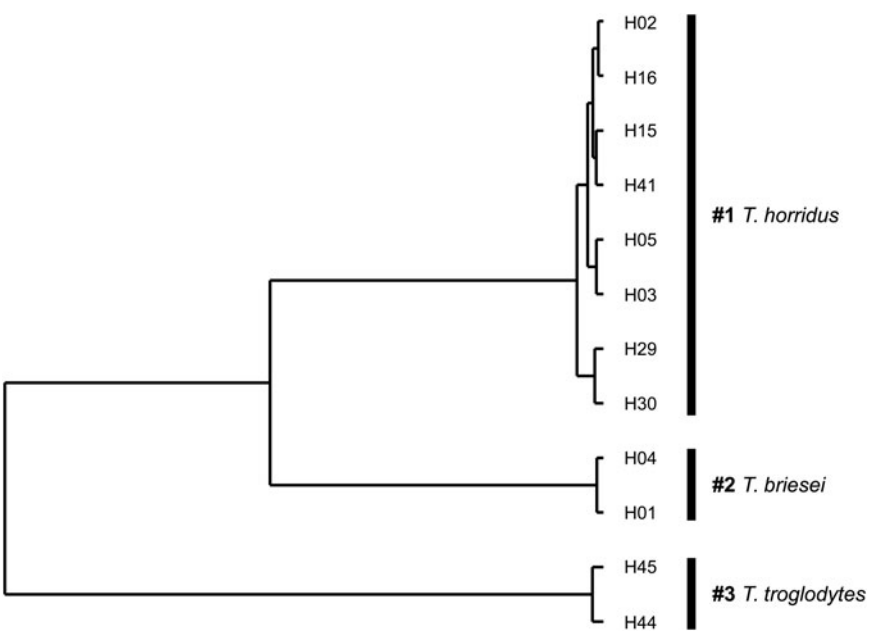

Fig. 7. General Mixed Yule Coalescent (GMYC) results obtained by analysing phylogenetic relationships among haplotypes scored only from the Iberian Peninsula, where *T. horridus* and *T. briesei* both occur. Black bars refer to putative species according to GMYC model. Likelihood values were: $L_0 = 79.44406$ for the null model; $L_{GMYCmultiple} = 84.23986$ for the GMYC model; Likelihood Ratio Test $P = 0.00826$**.

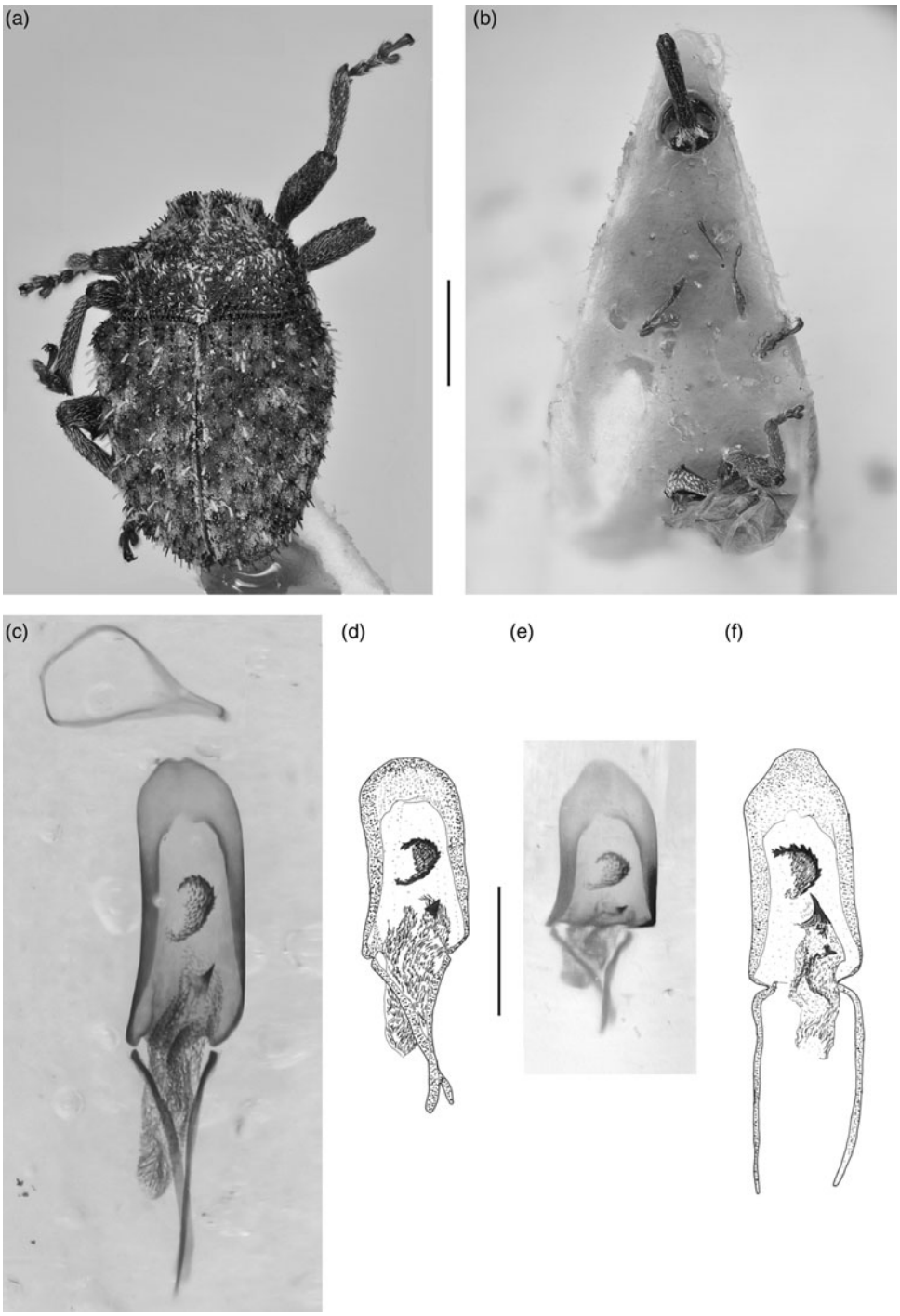

Fig. 8. Photos of holotype of *Trichosirocalus mortadelo* Alonso-Zarazaga & Sánchez-Ruiz (a and b). Aedeagus embedded in DMHF in dorsal (c) and dorsoapical (e) view. Drawing of penis in dorsal view (d) by Alonso-Zarazaga & Sánchez-Ruiz (2002). Drawing of penis in dorsal view (f) of *T. horridus* (Panzer) by Alonso-Zarazaga & Sánchez-Ruiz (2002).

neonate Italian *T. horridus* developing up to the third instar on *Carduus* and *Cirsium,* but not on *Cynara*, in larval transfer experiments. This scenario was described in previous experiments carried out by Zwölfer (1965). Boldt *et al.* (1980) dissected plants growing in the field in Italy and found most larvae in *C. nutans*, with small numbers in *C. pycnocephalus* and *Galactites tomentosa*, but none in *Onopordum, Silybum, Carthamus, Cynara* or *Sonchus*. May (1993) described the larva of *T. horridus* completing its development on *Carduus* in New Zealand. Woodburn & Swirepik (2002) were able to

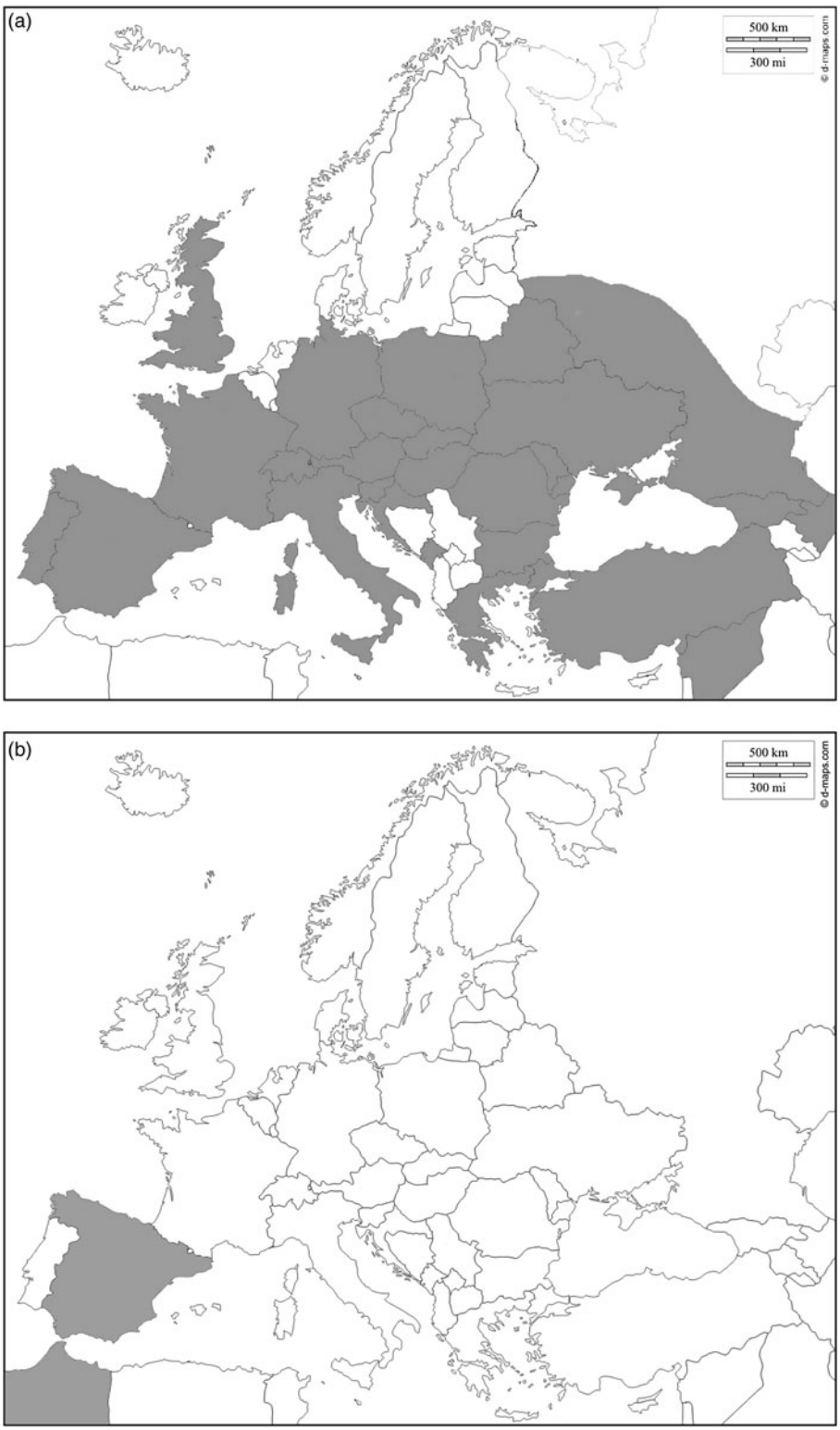

Fig. 9. Estimated native distribution of *T. horridus* (a) and *T. briesei* (b).

establish *T. horridus* on *Cirsium vulgare* in Western Australia but concluded this host to be less suitable than *C. nutans*. Groenteman *et al.* (2008) reported *T. horridus* from *C. nutans*, *C. vulgare* and *O. acanthium* in New Zealand, and the results of our genetic analyses confirm that all the specimens received from these three plants in that country are *T. horridus*. In the USA, *Trichosirocalus* weevils were released to control *Carduus* spp. but have been reported to attack also *Cirsium discolor* (Muhl. ex Willd.) Spreng. in Virginia (McAvoy *et al.*, 1987), *Cirsium altissimum* L. Hill in Nebraska (Takahashi *et al.*, 2009) and *C. altissimum*, *C. carolinianum* (Walt.) Fern & Schub., *C. discolor*, *C. horridulum* Michx. and *C. muticum* Michx. in Tennessee (Wiggins *et al.*, 2009). All specimens that we have morphologically and genetically analysed from the USA are *T. horridus*.

Finally, during our fieldwork adult specimens of *T. horridus* were collected on *Carduus* spp. and *Cirsium* spp. in complete syntopy (e.g., Perpignan, France [personal records]; Otago, New Zealand [Groentman, personal communications]), whereas *T. briesei* was only collected on *Onopordum* spp., often growing together in the same location with the other thistle species.

We agree with Alonso-Zarazaga & Sánchez-Ruiz (2002) that *T. horridus* is on average smaller (total body length 3.20–4.16 mm, almost all specimens less than 3.90 mm) than *T. briesei* (total body length 3.96–4.64 mm). Reliable morphological features that can be used to identify female specimens of *T. briesei* are the size, usually well above 4.10 mm (whereas we found only a few specimens of *T. horridus* larger than 4 mm), together with the longer second desmomere (funicular joint), at least five times longer than wide. Males of *T. briesei* are quite easy to identify using the external characters given by Alonso-Zarazaga & Sánchez-Ruiz (2002) in their key (see also their figures): protibial mucro not concealed by apical setae; sternite 2 tumid at middle and much more convex than on sides, hind margin prominent at middle over third sternite. However, the length of the second desmomere is quite variable in *T. horridus*, although all *T. horridus* specimens studied by us have this segment more or less distinctly shorter than it is in *T. briesei*.

Regarding the geographical distribution of *T. horridus* and *T. briesei*, current data support the presence of *T. briesei* only in Spain, from where we studied several adults, and Morocco (Alonso-Zarazaga & Sánchez-Ruiz, 2002). The single female from Austria cited by Alonso-Zarazaga & Sánchez-Ruiz (2002) was not seen by us but is probably a large individual of *T. horridus*. Of the latter species we studied material from Austria, Canada, France, Dagestan, Georgia, Germany, Italy, New Zealand, Portugal, Spain, Turkey and USA, and reliable literature records exist from Armenia, Azerbaijan, Belarus, Belgium, Bulgaria, Croatia, Czech Republic, Great Britain, Hungary, Moldavia, Montenegro, Poland, Romania, southern European Russia, Slovakia, Slovenia, Switzerland, Syria and Ukraine (Colonnelli, 2004, 2013). The distribution of *T. horridus* is shown in fig. 9a, that of *T. briesei* in fig. 9b.

Conclusions

Our results indicate that *T. horridus* is a widespread, genetically and morphologically variable species that is associated with several thistle species in the tribe Cardueae (*Carduus* spp., *Cirsium* spp. and *Onopordum* spp.) and that occurs from Spain to the Caucasus, whereas *T. briesei* is restricted to *Onopordum* and naturally occurs only in Spain and Morocco. We found no evidence of the existence of a third species that would correspond to *T. mortadelo*, and we confirm that this name is a synonym of that of *T. horridus*, as established by Pullen *et al.* (2014). The key of Alonzo-Zarazaga & Sánchez-Ruiz (2002) is suitable for distinguishing *T. horridus* and *T. briesei*. To date, specimens from North America and New Zealand correspond to only *T. horridus*, whereas both *T. horridus* and *T. briesei* are established in Australia. Given the diverse genetic structure of *T. horridus* (compared with the more restricted geographical distribution and genetically less differentiated *T. briesei*), it could be interesting to explore its population structure across the geographical distribution area in more depth, for biological control purposes. Finally, it would be interesting to investigate the possible evolution of host shifting in New Zealand populations of *T. horridus* associated with *Onopordum*.

Supplementary Material

The supplementary material for this article can be found at http://www.journals.cambridge.org/BER

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