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Introduction and Establishment of *Pissodes castaneus* (Coleoptera: Curculionidae) in the Andean Patagonia of Argentina

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Abstract

The pine weevils that occur in plantations of *Pinus* spp. in Andean Patagonia of Argentina belong to the species *Pissodes castaneus* (De Geer), a Eurasian endemic species, according to the identification based on molecular and morphological characters. Sequences of the mitochondrial Cytochrome oxidase subunit I and nuclear genes (28 S rDNA and ITS2) were obtained for individuals of 13 afforestations, covering the entire distribution area of the established populations in the Andean Patagonia of Argentina. Sequence comparison with representative species of the genus (European, North American, and Chinese species) shows that Patagonian specimens are conspecific to those of *P. castaneus* sequenced from Europe. Phylogenetic analyses indicate that all terminals from Patagonia form a monophyletic unit without evident subclades, eliminating the possibility of existence of more than one species of *Pissodes* Germar in this area, including cryptic ones. Moreover, the very low genetic divergence between the Patagonian populations suggests that it is plausible that *P. castaneus* was introduced into Patagonia from just one location. Mitochondrial DNA analysis shows that Patagonian terminals group together with a French haplotype and are clearly separated from other *P. castaneus* individuals represented in our sample, and reveal that established populations in Andean Patagonia originated via a limited introduction.

Resumen

Los gorgojos del pino presentes en plantaciones de *Pinus* spp. en la Patagonia andina de Argentina pertenecen a la especie *Pissodes castaneus* (De Geer), una especie endémica de Eurasia, de acuerdo a la identificación basada en caracteres moleculares y morfológicos. Se obtuvieron secuencias del gen mitocondrial Citocromo oxidasa subunidad I y de dos genes nucleares (28 S ADNr y ITS2), para individuos de 13 plantaciones, cubriendo toda el área de distribución de las poblaciones establecidas en la Patagonia andina de Argentina. La comparación de estas secuencias con aquellas de especies representativas del género (especies de Europa, América del Norte y China), muestra que los especímenes de la Patagonia son conespecíficos con los de *P. castaneus* de Europa. Los análisis filogenéticos muestran que todos los terminales de la Patagonia forman una unidad monofilética sin subclados evidentes, descartando la posibilidad de que exista más de una especie de *Pissodes* Germar en esta área, incluyendo especies crípticas. Más aún, la baja divergencia genética entre las poblaciones patagónicas sugiere que es posible que *P. castaneus* haya sido introducida en la Patagonia desde una única localidad. Los análisis con ADN mitocondrial muestran que los terminales de Patagonia se agrupan junto a un haplotipo francés y están claramente separados de otros individuos de *P. castaneus* representados

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en la muestra, y revelan que una introducción limitada dio origen a las poblaciones establecidas en la Patagonia andina.

Key words: *Pissodes castaneus*, South America, Patagonia, introduced species, DNA tracing

The species of *Pissodes* Germar have conifers as host plants and are naturally distributed in the Holarctic Region, about 20 of them from the Palearctic (Eurasia and North Africa) and 30 species from the Nearctic (North America with some range extended into Central America) (O'Brien 1989, Langor et al. 1992, Langor and Sperling 1994). The hosts of *Pissodes* are in the conifer genera *Pinus* L., *Abies* Milleri, *Picea* Link, *Larix* Milleri and *Pseudotsuga* Carrière (Family Pinaceae), and *Cedrus* Trew (Family Cupressaceae). Some *Pissodes* species are of high economic importance because they cause serious damage to conifer forests and plantations. Among the most harmful are *Pissodes strobi* (Peck) and *Pissodes terminalis* Hopping in North America, *Pissodes castaneus* (De Geer) in Europe and Asia, and *Pissodes nitidus* Roelofs and *Pissodes yunnanensis* Langor and Zhang in China (Langor 1991, Zhang et al. 2007). The growing importance of conifers for wood exploitation, ornamental use, and paper production has notoriously influenced the economical threat of *Pissodes* species as pests (O'Brien 1989), raising the risk of introduction of pine weevils in several countries.

Two *Pissodes* species were introduced into South America: *Pissodes radiatae* Hopkins (syn. of *P. notatus* sensu Trujillo, which was misidentified) and *P. castaneus* (syn. of *P. notatus* F.). The Monterrey pine weevil, *P. radiatae*, is native to North America (Monterrey peninsula) and was introduced into Brazil and Uruguay (Wibmer and O'Brien 1986), where it feeds mainly on *Pinus radiata* Don. On the other hand, the banded pine weevil, *P. castaneus*, is naturally distributed in Europe and North of Africa, also occurring in Siberia (Bichão et al. 2003) and Turkey (Panzavolta 2007), and it was introduced into Argentina (Lanteri et al. 2002), Uruguay (Morey and Porcile 1992), southern Brazil (Zaleski 2009, Iede et al. 2010), and more recently in southern Chile (SAG 2014).

In Europe, *P. castaneus* is considered to have reached its natural limits. Thus, it is not listed as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO). However, it represents a phytosanitary risk for other temperate regions where pine plantations are expanded. This species is listed by COSAVE as a quarantine pest (CABI 2014).

P. castaneus is associated with almost every pine species in its natural distribution range, preferring young plants of 4–15 yr (Day et al. 2004). Among the most frequent hosts are *Pinus* spp. (*Pinus contorta* Douglas ex Loudon, *Pinus pinaster* Aiton, *Pinus halepensis* Milleri, *Pinus taeda* L., *Pinus nigra* Arnold, *Pinus sylvestris* L., *Pinus elliottii* Engelmann, *Pinus canariensis* Sweet ex Spreng, *Pinus pinea* L., *Pin. radiata*, and *Pinus ponderosa* Lawson & Lawson) and occasionally *Abies* spp. and *Pseudotsuga menziesii* (Mirbel) Franco (Zaleski 2009, Gomez and Hartel 2010, Panzavolta and Tiberi 2010). In the Andean Patagonia of Argentina, *Pinus contorta* var. *murrayana* (Balfour) Engelmann is the most affected species followed by *Pin. radiata* and *Pin. ponderosa* (Gomez et al. 2013).

The sanitary condition of the pine afforestations in Argentinean Patagonia seems to have worsened since the detection of specimens identified as *P. castaneus* in 2005 (Gomez and Hartel 2010, Gomez et al. 2013). This may be explained by the presence of *Sirex noctilio* F. population outbreaks that may have stressed the plantations predisposing them to the attacks by *P. castaneus* (as a secondary, opportunistic pest), in a similar way as happened in South Africa with *Pissodes nemorensis* Germar (Gebeyehu and Wingfield 2003). Since its first-confirmed report in Chubut Province in 2006, this weevil has been detected in a wide distribution area affecting afforestations of *Pinus* spp. under different conditions (Gomez et al. 2013).

The taxonomy of the genus *Pissodes* is challenging due to the morphological similarity of its species, and other character sources are thus needed to be considered for their identification, like ecological and genetic features (Langor 1991). Problems of identification can result in impediments for accurate surveys, for studying population dynamics of the pests and for the implementation of management strategies. In this sense, molecular techniques are increasingly being used to study the population structure and species composition and to recognize complex of cryptic species (Gebeyehu and Wingfield 2003, Zhang et al. 2007). Indeed, molecular studies on *Pissodes* species have been undertaken to solve taxonomic problems, to explore phylogenetics, and to study aspects of population genetics within the *P. strobi* complex (Boyce et al. 1994, Langor and Sperling 1997, Lewis et al. 2002, Laffin et al. 2004) or within *Pissodes validirostris* (Gyllenhal) cone pine weevil (G.R., personal communication).

Despite the morphological evidence indicating that the weevil species associated with *Pinus* spp. plantations in Patagonia was *P. castaneus* (Fontana et al. 2006), some specialists have suggested that the presence of other *Pissodes* species cannot be discarded (R. Alfaro, personal communication). Moreover, different biotypes of the species could occur across the entire region.

In this study, we aim to confirm the taxonomic identification of the populations of pine weevils present in the Andean Patagonia of Argentina and to explore whether these introduced weevils originated from a single introduction or through multiple events. For these purposes, we have obtained sequences of the mitochondrial gene cytochrome oxidase subunit I (COI), the nuclear 28 S rDNA (regions D1–D3), and the internal transcribed spacer 2 (ITS2) from specimens of different localities across their distribution range in Patagonia and compared them with sequences from Europe, North America, and Asia. The molecular characterization has become a useful tool for analyzing intra and interspecific variation in agronomical important insects, to identify types associated with different hosts, and to test origin and dispersal hypotheses to implement effective management strategies (Scataglini et al. 2000, 2006; Guzmán et al. 2007). One of the most commonly used markers in the animal kingdom is the mitochondrial gene COI. This marker shows great variability at population level in insects and can be helpful for intercepting invasive species, especially of holometabolous insects in their egg, larval, or pupal stages, which can hardly be identified to species level using morphological characters (Hebert et al. 2003, Armstrong and Ball 2005, Lanteri 2007).

Materials and Methods

Study Area

Sampling and collection sites for weevils were selected according to the variability in environmental conditions across the entire distribution area of the weevil in Andean Patagonia, including Neuquén, Río Negro, and Chubut Provinces. The selection criteria were the typical east–west precipitation gradient in this region (Jobbágy et al.

Table 1. *Pissodes* species included in this study, with sampling locations and GenBank accession numbers for ITS2, 28S, and COI genes

Species	Country	Sampling sites	Host species	DNA extraction code	28S	ITS2	COI
P. castaneus	Argentina	Chubut, Esquel 42° 53′04.3″ S; 71° 20′20.5″ W	*Pin. radiata*	PFRC1	KR814426	KR814450	KT258997
P. castaneus	Argentina	Chubut, Esquel 42° 53′04.3″ S; 71° 20′20.5″ W	*Pin. radiata*	PFRC1B	KR814412	KR814451	KT259005
P. castaneus	Argentina	Chubut, Esquel 42° 53′04.3″ S; 71° 20′20.5″ W	*Pin. radiata*	PFRC1C	PCR failed	KR814452	KT259016
P. castaneus	Argentina	Chubut, Esquel 42° 55′15.7″ S; 71° 15′54.3″ W	*Pin. radiata*	PINC1	KR814420	KR814453	KT258998
P. castaneus	Argentina	Chubut, Esquel 42° 55′15.7″ S; 71° 15′54.3″ W	*Pin. radiata*	PINC1B	KR814429	KR814454	KT259006
P. castaneus	Argentina	Chubut, Esquel 42° 55′15.7″ S; 71° 15′54.3″ W	*Pin. radiata*	PINC1C	KR814427	KR814455	KT259017
P. castaneus	Argentina	Chubut, Esquel 42° 55′50.4″ S; 71° 22′29.7″ W	*Pin. radiata*	PMLZC1	KR814421	KR814458	KT259000
P. castaneus	Argentina	Chubut, Esquel 42° 55′50.4″ S; 71° 22′29.7″ W	*Pin. radiata*	PMLZC1B	KR814431	KR814459	KT259020
P. castaneus	Argentina	Chubut, Cholila 42° 27′17.7″ S; 71° 23′18.8″ W	*Pin. ponderosa*	PEGC1	KR814413	PCR failed	KT259007
P. castaneus	Argentina	Chubut, Cholila 42° 27′17.7″ S; 71° 23′18.8″ W	*Pin. ponderosa*	PEGC1B	KR814428	KR814456	KT259019
P. castaneus	Argentina	Chubut, Cholila 42° 27′17.7″ S; 71° 23′18.8″ W	*Pin. ponderosa*	PEGC1C	KR814430	KR814457	KT259029
P. castaneus	Argentina	Chubut, El Maitén 42° 00′31.3″ S; 71° 09′52.1″ W	*Pin. ponderosa*	PBEMC1	KR814422	KR814460	KT259003
P. castaneus	Argentina	Chubut, El Maitén 42° 00′31.3″ S; 71° 09′52.1″ W	*Pin. ponderosa*	PBEMC1B	KR814432	PCR failed	KT259012
P. castaneus	Argentina	Chubut, El Maitén 42° 00′31.3″ S; 71° 09′52.1″ W	*Pin. ponderosa*	PBEMC1C	KR814433	KR814461	KT259026
P. castaneus	Argentina	Chubut, Leleque 42° 29′40.3″ S; 71° 07′22.0″ W	*Pin. ponderosa*	PBLC1	KR814417	KR814462	KT259011
P. castaneus	Argentina	Chubut, Leleque 42° 29′40.3″ S; 71° 07′22.0″ W	*Pin. ponderosa*	PBLC1B	KR814434	KR814464	KT259023
P. castaneus	Argentina	Chubut, Leleque 42° 29′40.3″ S; 71° 07′22.0″ W	*Pin. ponderosa*	PBLC1C	KR814435	KR814463	KT259031
P. castaneus	Argentina	Chubut, Trevelin 43° 07′17.1″ S; 71° 33′19.5″ W	*Pin. radiata*	PEC1	KR814423	PCR failed	KT259004
P. castaneus	Argentina	Chubut, Trevelin 43° 07′17.1″ S; 71° 33′19.5″ W	*Pin. radiata*	PEC1B	KR814436	KR814465	PCR failed
P. castaneus	Argentina	Chubut, El Coihue 42° 09′44.1″ S; 71° 16′53.6″ W	*Pin. ponderosa*/*Pin. contorta* var. *murrayana*	PBECC1	KR814418	KR814466	KT259013
P. castaneus	Argentina	Chubut, El Coihue 42° 09′44.1″ S; 71° 16′53.6″ W	*Pin. ponderosa*/*Pin. contorta* var. *murrayana*	PBECC1B	KR814419	KR814467	KT259014
P. castaneus	Argentina	Chubut, El Coihue 42° 09′44.1″ S; 71° 16′53.6″ W	*Pin. ponderosa*/ *Pin. contorta* var. *murrayana*	PBECC1C	KR814437	KR814468	KT259027
P. castaneus	Argentina	Neuquén, Alicura 40° 41′00.3″ S; 71° 01′20.4″ W	*Pin. contorta* var. *murrayana*	PALN1	KR814445	KR814480	KT259015
P. castaneus	Argentina	Neuquén, Alicura 40° 41′00.3″ S; 71° 01′20.4″ W	*Pin. contorta* var. *murrayana*	PALN1B	KR814414	KR814481	KT259008
P. castaneus	Argentina	Neuquén, Alicura 40° 41′00.3″ S; 71° 01′20.4″ W	*Pin. contorta* var. *murrayana*	PALN1C	KR814446	KR814482	KT259024
P. castaneus	Argentina	Río Negro, Cerro Cdo. Rayhuao 41° 20′47.9″ S; 70° 35′29.8″ W	*Pin. contorta* var. *murrayana*	PRRN1	KR814415	KR814475	KT259009
P. castaneus	Argentina	Río Negro, Cerro Cdo. Rayhuao 41° 20′47.9″ S; 70° 35′29.8″ W	*Pin. contorta* var. *murrayana*	PRRN1B	KR814442	KR814476	KT259021
P. castaneus	Argentina	Río Negro, Cerro Cdo. Rayhuao 41° 20′47.9″ S; 70° 35′29.8″ W	*Pin. contorta* var. *murrayana*	PRRN1C	KR814443	KR814477	KT259030
P. castaneus	Argentina	Río Negro, Cuesta del Ternero 41° 52′32.6″ S; 71° 25′13.1″ W	*Pin. contorta* var. *murrayana*	PCTR1	KR814425	KR814478	KT259002
P. castaneus	Argentina	Río Negro, Cuesta del Ternero 41° 52′32.6″S; 71° 25′13.1″ W	*Pin. contorta* var. *murrayana*	PCTR1B	KR814444	KR814479	KT259022
P. castaneus	Argentina	Río Negro, Pilcaniyeu 41° 07′27.9″S; 70° 43′43.7″ W	*Pin. contorta* var. *murrayana*	PMPR1	KR814440	KR814472	KT259001
P. castaneus	Argentina	Río Negro, Pilcaniyeu 41° 07′27.9″S; 70° 43′43.7″ W	*Pin. contorta* var. *murrayana*	PMPR1B	KR814416	KR814473	KT259010
P. castaneus	Argentina	Río Negro, Pilcaniyeu 41° 07′27.9″ S;70° 43′43.7″ W	*Pin. contorta* var. *murrayana*	PMPR1C	KR814441	KR814474	KT259025
P. castaneus	Argentina	Río Negro, El Foyel 41° 41′39.9″ S; 71° 26′54.7″ W	*Pin. contorta* var. *murrayana*	PEFR1	KR814424	KR814469	KT258999
P. castaneus	Argentina	Río Negro, El Foyel 41° 41′39.9″ S; 71° 26′54.7″ W	*Pin. contorta* var. *murrayana*	PEFR1B	KR814438	KR814470	KT259018
P. castaneus	Argentina	Río Negro, El Foyel 41° 41′39.9″ S; 71° 26′54.7″ W	*Pin. contorta* var. *murrayana*	PEFR1C	KR814439	KR814471	KT259028
P. castaneus	France	Toulon	*Pinus* sp.	NA	KR814448	KR814484	KT259032
P. castaneus	France	Toulon	*Pinus* sp.	NA	NA	NA	KT259033
P. castaneus	Slovenia	Bratislava	*Pin. nigra*	NA	KR814449	KR814483	KT259038
P. castaneus	Italy	Framura	*Pin. halepensis*/*pinaster*	NA	KR814447	KR814485	KT259034
P. castaneus	Italy	Framura	*Pin. halepensis* /*pinaster*	NA	NA	NA	KT259035

(*continued*)

Table 1. Continued

Species	Country	Sampling sites	Host species	DNA extraction code	28S	ITS2	COI
P. castaneus	Italy	Framura	*Pin. halepensis /pinaster*	NA	NA	NA	KT259036
P. castaneus	Italy	Framura	*Pin. halepensis /pinaster*	NA	NA	NA	KT259037
P. strobi	Canada	Aberdeen	*Pin. strobus*	NA	KT799825	KT799819	KT799832
P. validirostris	France	Alps	*Pin. sylvestris*	NA	KT799822	KT799817	KT799829
P. pini	France	Col du Lautaret	*Pin. uncinata*	NA	KT799828	KT799820	KT799835
P. piceae	France	NA	*Abies* sp.	NA	KT799823	KT799821	KT799830
P. yunnanensis	China	Lijang	*Pin. yunnanensis*	NA	KT799827	NA	KT799834
P. punctatus	China	Midu	*Pin. armandii*	NA	KT799826	NA	KT799833
P. nemorensis[a]	USA	Syracuse, New York	Pine logs	NA	NA	NA	U77981
P. terminalis[a]	Canada	Hinton, Alberta 53°14′24″ N; 117°30′36″ W	*Pin. contorta* var. *latifolia*	NA	NA	NA	U77980
P. affinis[a]	Canada	McDowell, Saskatchewan 53°1′12″ N; 116°0′36″ W	*Picea glauca*	NA	NA	NA	U77982
P. schwarzi[a]	Canada	Ellis Creek, British Columbia 49°28′12″ N; 119°29′24″ W	*Pin. contorta* var. *latifolia*	NA	NA	NA	U77977

NA, not applicable or not available.
[a]From Langor and Sperling (1997).

1995), the forest species, plantations age, silvicultural management status, and phytosanitary status of *Sirex noctilio*, according to the surveys and monitoring conducted for the local Forest Service ("Subsecretaría de Bosques de Chubut") to control this pest. Every plantation selected was registered with a GPS system. All sampling sites are listed in Table 1.

Collection of Weevils for Morphological and Molecular Analyses

Adult weevils were collected from three attacked plants in each selected plantation. Two 80-cm-long logs were cut from each tree and transported to the laboratory where they were kept in brood cages outdoors. After emergence started, cages were checked every day and beetles removed. Adults were also collected in the field by checking the surface of infested trees. All adults were preserved in vials with absolute or 95° ethanol.

DNA Isolation, Polymerase Chain Reaction Amplification, and Sequencing

Total genomic DNA was extracted from ethanol-preserved adults (two or three individuals from each location) using an adapted "salting out" protocol based on that of Sunnuks and Hales (1996). The protocol is available at http://farrell.oeb.harvard.edu/techniques/dna_extraction.html.

Polymerase chain reaction (PCR) were performed in a 50 µl volume: 10 pmol for each primer, 0.8 mM dNTPs (Genbiotech SRL, Buenos Aires, Argentina), $MgCl_2$ 50 mM to a final concentration of 2–4 mM, 5 µl 10× Buffer, and 1.25 units of Taq DNA Polymerase (Invitrogen SA, Buenos Aires, Argentina). The primers used in both amplification and sequencing were LCO (5′GGTCAACAAAT CATAAAGATATTGG) and A3014 (5′TCCAATGCACTAATCTG CCATATTA) for the COI gene (Simon et al. 1994, McKenna et al. 2009), ITS2F (5′TGTGAACTGCAGGAACATG) and ITSR (5′AATGCTTAAATTYAGGGGGTA) for the ITS2 gene (Campbell et al. 1993), D1F (5′ ACCCGCTGAATTTAAGCATAT), and D3R (5′ TAGTTCACCATCTTTCGGGTC) for the 28 S gene (Lopez-Vaamonde et al. 2001). Temperature profiles consisted of 39 cycles: 5 cycles at 94°C for 30 s, 42°C for 30 s, 72°C for 1 min 30 s, followed by 34 cycles at 94°C for 1 min, 45°C for 30 s, 72°C for 1 min 30 s, with a final extension at 72°C for 5 min for the COI gene; a preheat cycle at 93°C for 3 min followed by 30 cycles of 98°C for 15 s, 50°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 3 min for the ITS2 gene; a preheat cycle at 93°C for 3 min followed by 35 cycles at 98°C for 15 s, 57°C for 30 s, 72°C for 40 s, and a final extension of 72°C for 3 min for the 28 S gene. Products obtained from PCR amplification were visualized on 1% agarose gels to verify fragment sizes and they were purified with the AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea). The purified PCR products were sent for sequencing to the internal sequencing service of INTA-Castelar in Buenos Aires, Argentina. All mitochondrial and nuclear sequences from Patagonia were edited with Proseq 2.91 (Filatov 2002) and then aligned together with published sequences belonging to different *Pissodes* species, including European populations of *P. castaneus*. The alignment was performed with Clustal W (Thompson et al. 1994) as implemented in SeaView (Gouy et al. 2010). All voucher specimens are held in Museo de La Plata, Buenos Aires, Argentina.

Data Analysis

The COI sequences of 1,257 bp obtained from Argentinean specimens were included in the data matrix together with additional

Pissodes sequences for a final alignment of 537 bp fragment corresponding to the internal part of the specific primers designed for *P. validirostris* (G. R., unpublished data). We have sequenced a longer fragment than the standard barcode considering that it could be phylogenetically more informative for further studies (Yoshitake et al. 2008). Because of numerous repeated motifs recovered in ITS2 sequences, all microsatellites were recoded so that each di- or trinucleotide repeat unit would be treated as a single mutational event, following Gómez-Zurita and Vogler (2003).

The final data set for the phylogenetic analyses comprised the sequences obtained from Patagonian specimens, plus sequences of *P. castaneus* from France, Italy, and Slovenia. In addition, mitochondrial and nuclear sequences for seven representative *Pissodes* species (G.R., personal communication) were added for comparison, i.e., the North American species *P. strobi* and *P. nemorensis*, the European species *P. validirostris*, *Pissodes pini* (L.), and *Pissodes piceae* (Illiger), and the Chinese ones *P. yunnanensis* and *Pissodes punctatus* Langor and Zhang. COI sequences from *P. nemorensis*, *P. terminalis*, *Pissodes schwarzi* Hopkins, and *Pissodes affinis* Randall, published by Langor and Sperling (1997), were also added to the final mt-DNA data set. The trees were rooted with the Chinese species for COI and 28 S genes, but for ITS2, due to the high amount of variability of the two Chinese species, they were rooted with the European species *P. pini* and *P. piceae*.

MtDNA pairwise genetic distances were computed using Mega 5.2.1 (Tamura et al. 2011) within and among *Pissodes* species. COI differences were used to split individuals into group memberships, using the Automatic Barcode Gap Discovery method (ABGD) developed by Puillandre et al. (2012), and implemented on a Web interface (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). Calculations were performed using the default settings search. Mitochondrial DNA haplotype network was performed using TCS 1.21 (Clement et al. 2000) for European *P. castaneus* and Patagonian specimens.

Mitochondrial and nuclear phylogenetic trees were inferred separately for each marker, using maximum likelihood (ML) method with the program PhyML version 3.0 (Guindon and Gascuel 2003, Guindon et al. 2010). The most appropriate model of nucleotide substitution was determined for each locus using the Akaike information criterion, as implemented in the program jModelTest 2.1.1 (Guindon and Gascuel 2003, Darriba et al. 2012): GTR+I+G for the three loci (Tavare 1986).

Morphological Assessment

Six specimens from different localities along Patagonian Provinces of Neuquén, Río Negro, and Chubut were sent to the weevil specialist Charles OBrien (Tucson, AZ), for confirming identification based on morphological characters. The adults were collected from the three cultivated pine species in the region: *Pin. radiata*, *Pin. contorta,* and *Pin. ponderosa* between 2007 and 2008.

Results

Sequences

Thirty-five sequences of the mitochondrial COI (1,257 bp), 35 of 28 S (707 bp), and 33 of ITS2 (342 bp) were obtained from the individuals of the 13 established populations, covering the entire distribution area of the insect in the Andean Patagonian Region of Argentina. New sequences are deposited in GenBank under accession numbers KR814426–KR814485 and KT258997–KT259038 (Table 1). Among these sequences, no polymorphic sites were observed between individuals, whatever the locus considered (COI, ITS2, and 28 S).

Table 2. Pairwise distances for COI, ITS2, and 28S genes, within and between *P. castaneus* and representative species of the genus

P. castaneus_Arg/	COI	ITS2	28S
P. castaneus _Arg	0	0	0
P. castaneus_Fr	0–0.004	0	0
P. castaneus_Slo	0.024	0	0
P. castaneus_It	0.028–0.030	0	0
P. validirostris	0.109–0.112	0.029	0.003
P. piceae	0.122–0,124	0.056	0.011
P. pini	0.099	0.062	0.009
P. strobi	0.108–0.114	0.041	0.006
P. punctatus	0.165	—	0.034
P. yunnanensis	0.163	—	0.041
P. nemorensis	0.124	—	—
P. terminalis	0.120	—	—
P. affinis	0.122	—	—
P. schwarzi	0.105	—	—

Arg, Argentina; Fr, France; It, Italy; Slo, Slovenia.

Data Analysis

Mitochondrial pairwise genetic distances among *Pissodes* species ranged from 0 to 0.165 (Table 2). The lowest genetic distances were observed between specimens from Patagonia and those belonging to *P. castaneus* population from France (0–0.004). COI genetic distances between Patagonian specimens and the other European or North American *Pissodes* species reached 0.124, and 0.165 when compared with Chinese specimens. Likewise, nuclear sequences (ITS2 and 28S) showed no difference with the European populations of *P. castaneus* (pairwise genetic distances of 0.000). Two deletions were observed within ITS2 locus (positions 349–421 and positions 427–480), matching to deletions observed for European *P. castaneus*.

Whatever the marker considered, the ML trees placed the Patagonian specimens together with the European populations of *P. castaneus*, the latter, clearly separated from the other representative species of the genus. In the ITS2 and 28 S ML trees (Figs. 1 and 2), all *P. castaneus* sequences analyzed cluster together. Among the six European mitochondrial haplotypes recovered within *P. castaneus*, all specimens from Argentina belong to the same haplotype (H1) shared with one French specimen of *P. castaneus*. Furthermore, the mitochondrial ML tree (Fig. 3) separates *P. castaneus* sequences in two subclades, i.e., specimens from Argentina and France and specimens from Italy and Slovenia, as confirmed in the haplotype network (Fig. 4).

The ABGD approach led to the split of the 53-sequence alignment set in 10 candidate species, grouping all specimens from Patagonia together with European specimens of *P. castaneus* in the same species group, at a priori genetic distance threshold of 0,0359 (Supp Fig. 1 [online only]).

The molecular evidence from three gene loci in our study supports that the weevils from Patagonia belong to the species *P. castaneus*, in agreement with the original identification made on morphological grounds by AAL and reconfirmed by Dr. Charles OBrien. Also, the COI data suggest that their populations originated via a limited introduction.

Discussion

The mitochondrial and nuclear phylogenetic analyses herein conducted clearly show that specimens sequenced from the Andean Patagonia are conspecific to those of European populations of

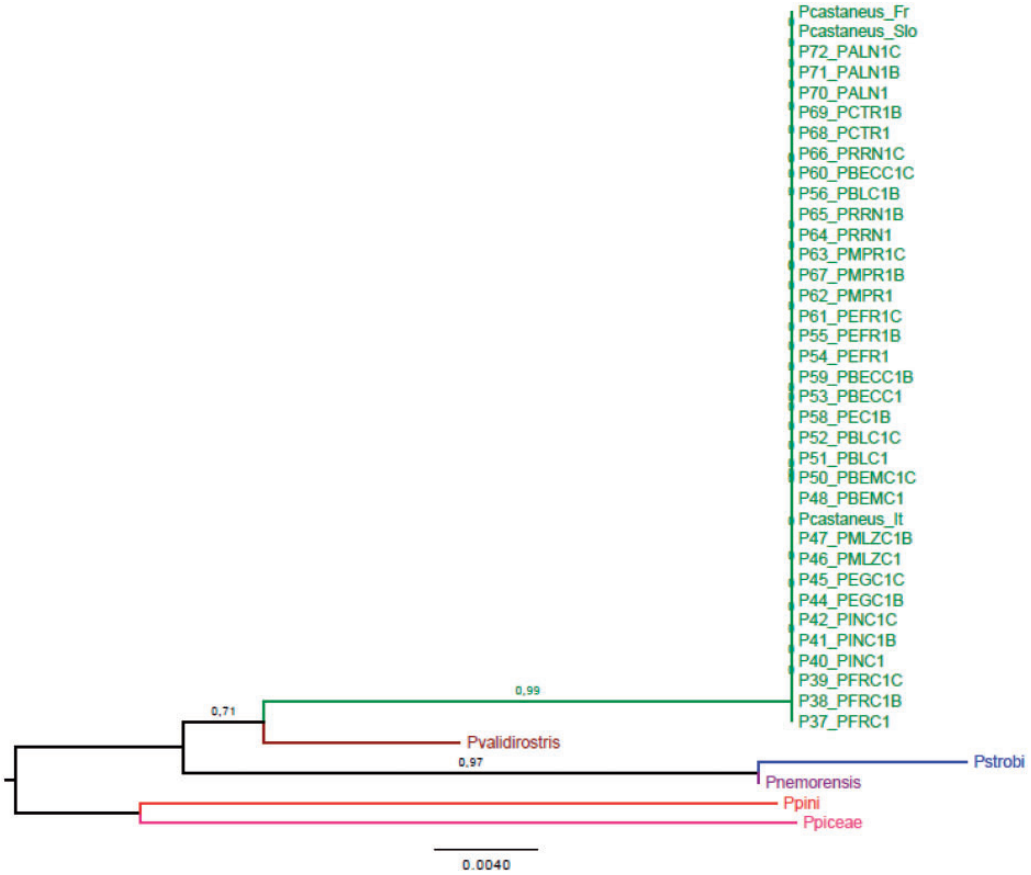

Fig. 1. ML tree resulting from analysis of ITS2 sequences. Numbers above branches indicate branch lengths; terminals from Patagonia are coded as P37-P72. Other *P. castaneus* terminals provenance is indicated as Fr, France; It, Italy; Slo, Slovenia.

P. castaneus, confirming the specific status of the pine weevil that occurs in this area of Argentina. This result refutes the statement that the species occurring in Argentina is *P. radiatae* (EPPO 2013). However, the possibility that *P. radiatae* occurs in other areas of Argentina besides Patagonia could not be discarded, since this Nearctic species occurs in Brazil and Uruguay (Wibmer and O'Brien 1986), and could have been introduced in some northeastern provinces through forest trade.

Although no sequences of *P. radiatae* were available for this study, congruent results allow us to unambiguously assign the individuals under study to *P. castaneus*. Both mitochondrial and nuclear genetic distances between individuals from Patagonia and European populations of *P. castaneus* were close to zero or did not exceed 3% when compared with COI sequences from Italy. This result was confirmed with the ABGD method for species delimitation, which grouped introduced and native specimens of *P. castaneus*. ML analysis showed that Patagonian sequences formed a monophyletic group with specimens from Europe whatever the locus considered. Furthermore, the fact that all Patagonian specimens shared the same mitochondrial haplotype (H1) or the same nuclear allele as European populations of *P. castaneus* further strengthened the confirmation of the identity of the species without any doubt. The main difference among the three ML trees obtained is that in the COI tree (Fig. 3), there are two subclades of *P. castaneus*, one including the sequences from Patagonia and France, and the other, the sequences from Italy (four different haplotypes) and Slovenia. This result shows that within its native area in the Palearctic region, there is genetic divergence within *P. castaneus* (several haplotypes and at least two haplotype groups) associated with geographical distribution. It is possible that a more extensive survey of *P. castaneus* throughout its native range shows a higher genetic variability as it was demonstrated for other *Pissodes* species (e.g., *P. strobi* in North America, *P. yunnanensis* in southeastern China, and *P. validirostris* in Europe) (Langor and Sperling 1997, Boyce et al. 1994, Zhang et al. 2007, G.R, personal communication). Laffin et al. (2004) sequenced a COI gene fragment for 130 *P. strobi* individuals in 11 locations of Canada and concluded that the high genetic variation found depends on host and geographical origin of the populations studied.

The ITS2 and 28S markers provide support for the identification of the species as *P. castaneus*; however, they do not allow discrimination between subclades within *P. castaneus*, being instead more useful to discriminate groups at higher levels. On the contrary, COI is very useful for the study of genetic variation within a single species or a group of closely related species, as well as to distinguish cryptic species (Scataglini et al. 2000, 2006; Kerdelhué et al. 2002; Guzmán et al. 2007, 2012; Mapondera et al. 2012; Winder et al. 2011; Koutroumpa et al. 2013).

The sampling size of Eurasian haplotypes in our study is not sufficient for a definitive conclusion regarding the probable origin of the introduced pest in the Andean Patagonia of Argentina. Although we found that the Patagonian COI haplotype is the same as one found in France, this could also occur throughout the vast Eurasian range of this species. Thus, the true origin of the Patagonian haplotype cannot be ascertained at this time until a more exhaustive survey of genetic variation through the native range is completed.

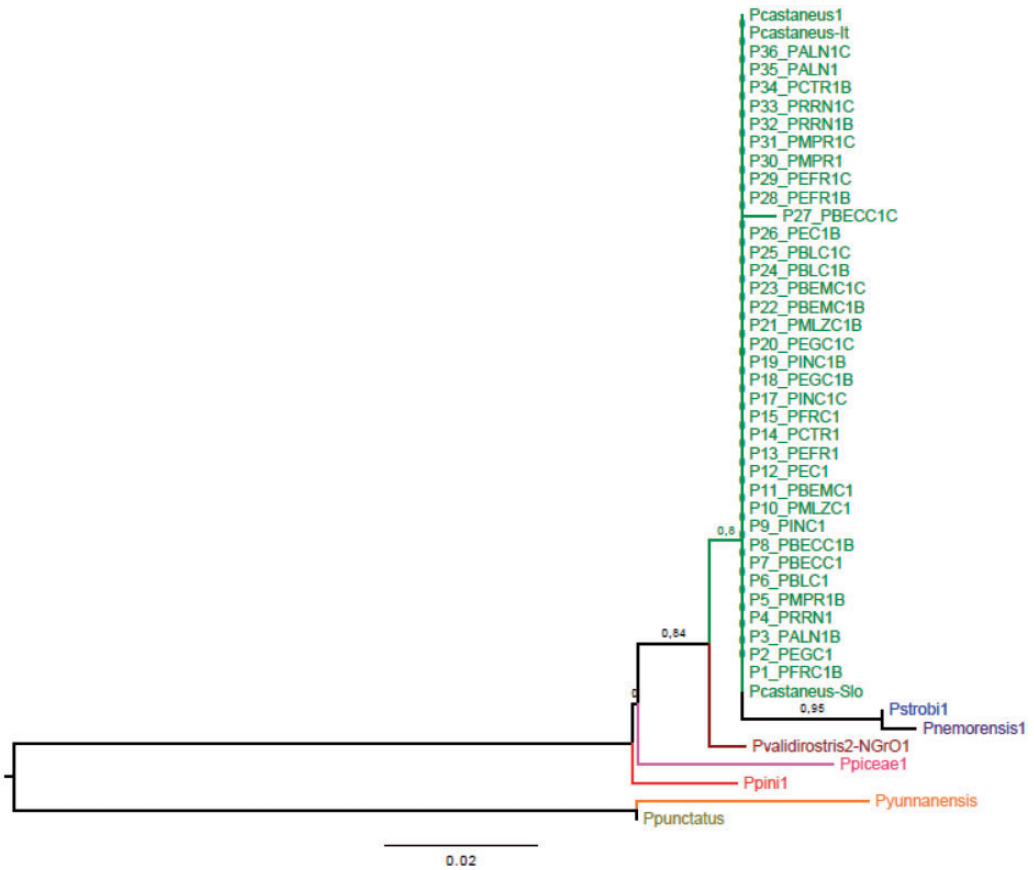

Fig. 2. ML tree resulting from analysis of 28S sequences. Numbers above branches indicate branch lengths; terminals from Patagonia are indicated as P1–P36. Other *P. castaneus* terminals provenance is indicated as It, Italy; Slo, Slovenia; and the French terminal is indicated as Pcastaneus1.

The null genetic variation of the Patagonian samples (a single haplotype) contrasts with the results obtained by Zaleski (2009) and Zaleski et al. (2013) based on specimens from Brazil. Through an AFLP analysis, these authors found that there were probably several founding events or multiple introductions of heterogeneous strains into this country. Moreover, they demonstrated that most Brazilian samples show genetic similarity with specimens from Italy. The findings of different lineages of *P. castaneus* in a given country where the species has been introduced have important implications in the development of control strategies, as insects of independent origins may respond differently to pest control management.

Dating the occurrence of *P. castaneus* in Argentina is difficult to assess based on available information. In Argentina, the first records of the species date back to 1947, according to a series of three exemplars, deposited in the Entomology Collection of the "Museo de La Plata" (Buenos Aires, Argentina), whose labels indicate that they were collected on *Pinus* sp. in Magdalena, Buenos Aires Province, and identified as *P. notatus* by the entomologist Juan M. Bosq. However, it is likely that the species was introduced before then, along with *Pinus* species imported for forest purposes.

Published records of *P. castaneus* in Argentina are available since the late 1990s, when damages were detected in Northern and Central areas of the country (Quintana et al. 1998, as *P. notatus*). In 2002, Lanteri et al. cited the species for Buenos Aires Province; in 2005, it was recorded in Southern Argentina, Andean Patagonian, in Neuquén Province, associated with plantations of *Pinus contorta* var. *murrayana* (Fontana et al. 2006); in 2006 and 2007, was found in Chubut Province, affecting pine afforestations in the Northwest of this province, and also in Rio Negro Province (Gomez et al. 2011).

In the Andean Patagonia, the introduction of exotic conifers began from about 1930, planted in small farms, public squares and gardens, and also immigrants and farmers brought seeds mainly from Europe and the United States. It is possible that *P. castaneus* was introduced in Argentina via *Pinus* seedlings, plant parts, and/or wood from the insect's natural distribution area in Europe. The plant parts most likely to carry the pest in trade/transport are stems (above ground), shoots, trunks, branches, and wood (CABI 2014), e.g., *P. castaneus* was imported in pit props from Germany and spread to Scotland in the United Kingdom (Gillanders 1908). Seedlings of *Pinus* were also shipped from the Netherlands infested with full-grown larvae of *P. castaneus* in stems (CABI 2014). The pest has also been intercepted at entry ports in the United States, usually as adults in various shipments (CABI 2014).

Between 1930 and 1950, the National Parks Administration from Argentina installed nurseries and large afforestations of several exotic species including *Pinus*, in Isla Victoria (Nahuel Haupi National Park), Pucará (Lanín National Park), and Villa Futalaufquen (Los Alerces National Park) (Enricci et al. 2000). In Isla Victoria specifically, 21 *Pinus* species were planted, e.g., *Pin. contorta*, *Pin. halepensis*, *Pinus jeffreyi* Greville & Balfour, *Pin. ponderosa,* and *Pin. sylvestris* (Relva and Nuñez 2014).

Currently, the transportation of propagules from one area to another has largely augmented because of the increasing movement of goods and people (Villacide et al. 2014). This process is reflected by the constantly rising number of exotic species deliberately or

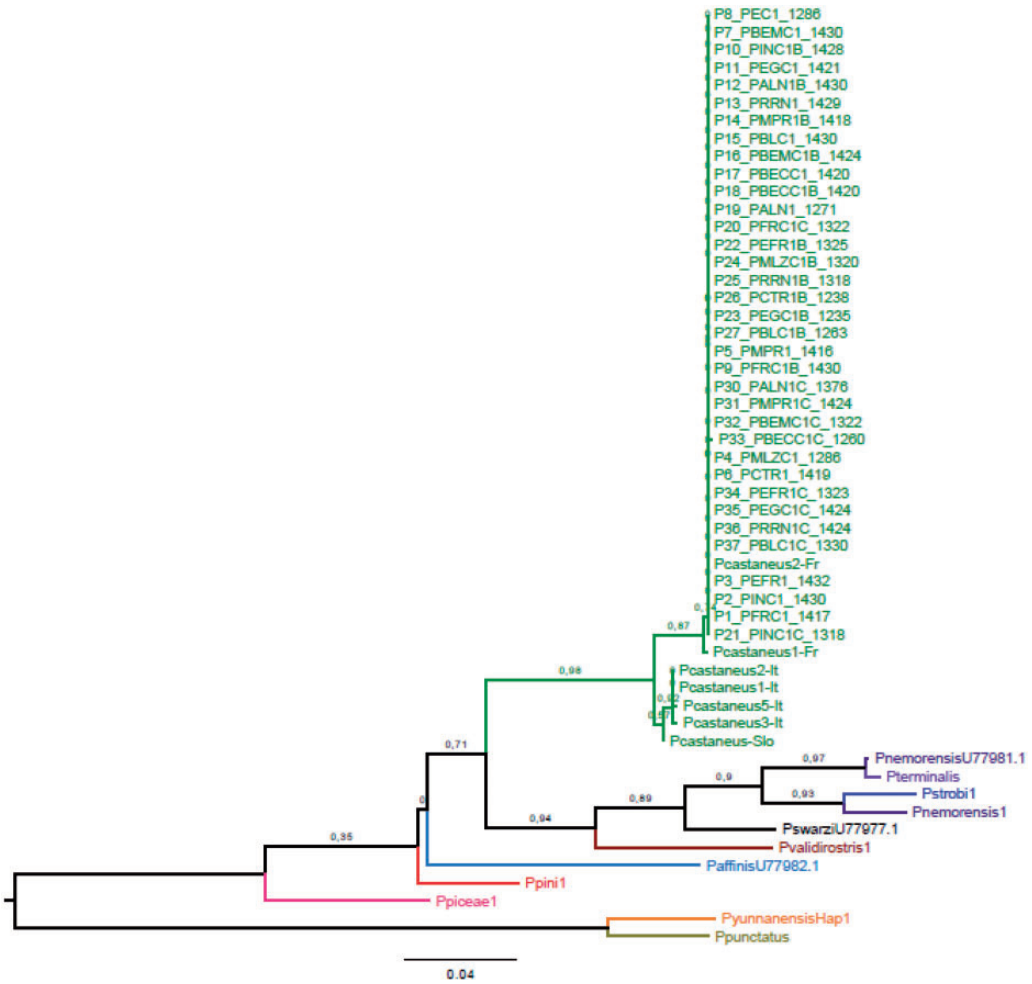

Fig. 3. ML tree resulting from analysis of COI sequences. Numbers above branches indicate branch lengths. Terminals from Patagonia are listed as P1–P37 and remaining *P. castaneus* terminals provenance is indicated as from France, Fr; from Italy, It; and from Slovenia, Slo.

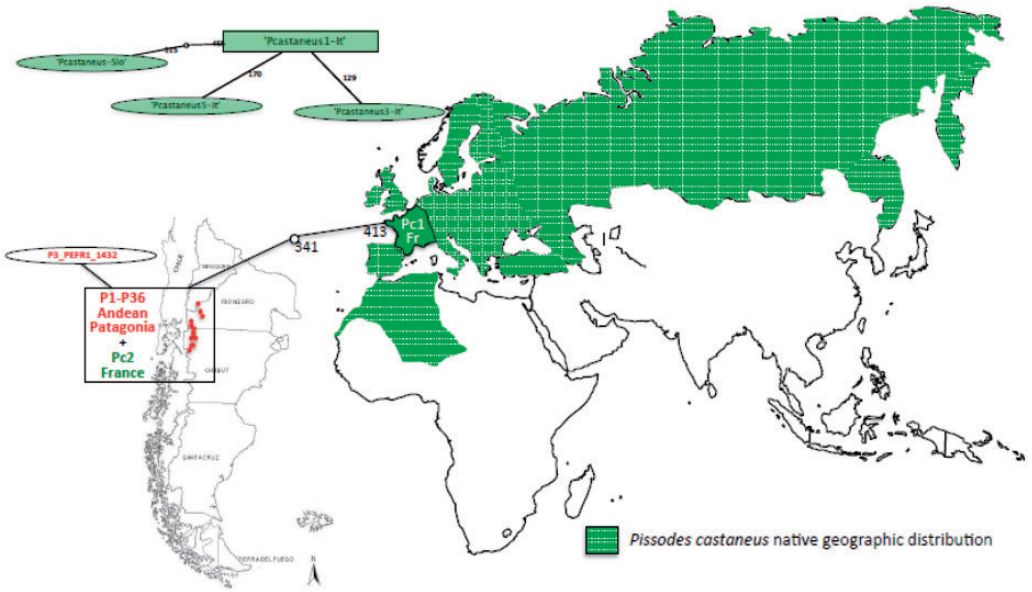

Fig. 4. Distribution map of native area of *P. castaneus* and haplotype network of corresponding mtDNA-COI sequences showing the origin of introduced Patagonian specimens (in red). On the haplotype network, each line corresponds to a mutational step and each empty circle to a missing intermediate.

accidentally introduced into new habitats (Hulme 2009). For Argentina, 433 exotic plant species have been so far reported to be introduced as ornamental, for honey production, and for wood production (Bentivegna et al. 2013).

Further studies on *P. castaneus* population genetics across the entire range of collection sites in Argentina, including northern and central areas, are necessary to know if there is another species of *Pissodes* occurring in the country or if there is a single species with several lineages as consequence of multiple introductions, that could be best adapted to different hosts and/or environmental conditions. It would be also interesting to do a microsatellite analysis to compare the Argentinean lineages with those of Brazil and Uruguay, because the forest trade among these countries is very frequent.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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