Identification and genetic diversity of two invasive *Pissodes* spp. Germar (Coleoptera: Curculionidae) in their introduced range in the southern hemisphere

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Abstract

During the first half of the twentieth century, two accidental cases of introduction of *Pissodes* weevils were recorded from the southern hemisphere. The weevils in South Africa were identified as the deodar weevil (*Pissodes nemorensis*) and those in South America as the small banded pine weevil (*Pissodes castaneus*). Wide distribution of the two species in their invasive range, general difficulty in identifying some *Pissodes* spp., and the varying feeding and breeding behaviours of the species in South Africa has necessitated better evidence of

species identity and genetic diversity of both species and population structure of the species in South Africa. Barcoding and the Jerry-to-Pat region of the COI gene were investigated. Morphometric data of the South African species was analysed. Our results confirmed the introduction of only one *Pissodes* species of North American origin to South Africa. However, this species is not *P. nemorensis*, but an unrecognized species of the *P. strobi* complex or a hybrid between *P. strobi* and *P. nemorensis*. Only *P. castaneus*, of European origin, was identified from South America. We identified ten mitochondrial DNA haplotypes from South Africa with evidence of moderate genetic structure among geographic populations. Terminal leader and bole-feeding weevils did not differ at the COI locus. A single haplotype was identified from populations of *P. castaneus* in South America. Results of the present study will have implications on quarantine, research and management of these insect species.

Keywords: bark weevils, invasive forest insects, *Pissodes castaneus*, *Pissodes nemorensis*, genetic diversity, population structure

Introduction

Insects are among the most dominant agents of biological invasions worldwide (Keller et al., 2011; McCullough et al., 2006). Forest ecosystems are increasingly threatened by invasive insect pests whose spread has been dramatically increased through the movement of wood products and packaging materials, as well as live plant material (Aukema et al., 2011; Tobin, 2015). It has also been shown that multiple introductions frequently increase genetic diversity of many introduced insects, including forest pests (Cognato et al., 2015; Garnas et al., 2016; Nadel et al., 2010). In addition, multiple introductions may allow unintended introductions of different lineages of the same species or cryptic species, as has been shown for the woodwasp, *Sirex noctilio* (Boissin et al., 2012), and the eucalyptus leaf weevil, *Gonipterus* spp. (Mapondera et al., 2012). Inabilities to recognize cryptic species and lack of knowledge of genetic diversity and population structure of invasive pests may lead to the use of uniform management approaches across populations of varying genetic background, which may potentially decrease the efficacy of management strategies. It can also have serious implications for the management of future spread, including quarantine (Garnas et al., 2012). Accurate identification of invasive insect pests and characterization of the genetic diversity and population structure are therefore key steps towards the development of successful management programs.

Members of the genus *Pissodes* Germar pose significant threats to their conifer hosts directly through feeding damage (Alfaro et al., 1994) and indirectly via their involvement in the transmission of forest pathogenic fungi (Jankowiak & Bilański, 2013). All members of this genus are native to the northern hemisphere (Zhang, 2007), with species such as *Pissodes strobi* (Peck), *P. terminalis* Hopping, *P. piniphilus* (Herbst), *P. yunnanensis* Langor and Zhang, *P. punctatus* Langor and Zhang, *P. nitidus* Roelofs, *P. nemorensis* Germar and *P. castaneus* (De Geer) causing significant economic loss (Alfaro & Ying, 1990; Craighead, 1950). The small banded pine weevil, *P. castaneus*, and the deodar weevil, *P. nemorensis*, were accidentally introduced outside their native ranges. The former has invaded South America (Uruguay, Argentina, Brazil and Chile)

(Abgrall et al., 1999; Anon, 1970; CABI, 2014; Pereyra et al., 2015b; Zaleski et al., 2013), while the later was reported to have spread to South Africa, Russia and Japan (EPPO, 1999; Zhang, 2007). These invasive species have now become pests of *Pinus* in non-native plantations in their introduced ranges in the southern hemisphere.

Pissodes nemorensis was first reported in South Africa in 1942 on *Pinus radiata* (EPPO, 1999; Webb, 1974; Zhang, 2007). *Pissodes nemorensis* belongs to the *P. strobi* complex (Boyce et al., 1994). Adults within this species complex are difficult to distinguish in part due to intraspecific variation in life history and morphology which confounds the designation of discrete characters uniquely and/or universally present to differentiate species (Phillips et al., 1987), due to variable host preference in different parts of their range (Smith & Sugden, 1969), and due to the fact that there is hybridization amongst some species (Boyce et al., 1994; Langor & Sperling, 1997; Langor, unpublished data). This confusion raises the possibility of misidentification of *Pissodes* spp. in South Africa and around the globe as well as possible undetected invasion of multiple cryptic species. As the identity of the *Pissodes* in South Africa is in question, we use *Pissodes* sp. in the manuscript. This weevil is now present throughout the major *Pinus* growing provinces of the country, infesting all major commercially grown *Pinus* species (authors' observation). The widespread distribution of *Pissodes* sp. in South Africa might be the result of the extensive movement of wood material within the country, in addition to natural spread via flight. Given how long the weevil has been known in the country, it is possible that multiple introductions have occurred, as has been observed in a number of other invasive insects in South Africa and elsewhere (Garnas et al., 2016).

In the Western Cape province of South Africa, *Pissodes* sp. has been observed feeding and breeding on previous year's terminal leaders of healthy *Pinus* trees and on the main trunks of dying or recently dead trees (M. Wondafrash, personal observation). Dieback of the terminal leader and the subsequent forking of the bole is relatively common in the provinces of Mpumalanga and KwaZulu-Natal, though the causal factors appear to be primarily abiotic; feeding and larval development within terminal shoots has only very rarely been observed outside of *P. radiata* in the Cape (J. Garnas, personal observation). The feeding and breeding behaviour on growing leaders is similar to that observed for *P. strobi* and *P. terminalis* (Langor & Sperling, 1995), while the feeding and breeding behaviour in the main trunk is similar to that of *P. nemorensis* and *P. schwarzi* in their native range (Atkinson et al., 1988; Fontaine et al., 1983; Langor & Sperling, 1997). However, in its native range, *P. nemorensis* is also known to sometimes infest the terminal leaders of healthy trees (Overgaard & Nachod, 1971; Phillips et al., 1984), thus making it difficult to distinguish damage caused by *P. terminalis* and *P. strobi*. Furthermore, hybrids of these two species have been detected in terminal leaders of *Pinus* (Boyce et al., 1994; Langor & Sperling, 1997). No published accounts are available that examine whether the observed variation and spatial partitioning of feeding and breeding behaviour in South Africa are the result of interactions with different hosts, environmental factors, or if they could arise from unique behaviour of distinct populations or cryptic species.

Pissodes castaneus was first recorded in South America in Uruguay in about 1919 and was subsequently recorded in Argentina, Brazil and Chile (Steve & Shaw, 1958; Zaleski et al., 2013). The weevil often prefers young, stressed *Pinus* trees (Gomez & Hartel, 2010; Iede et al., 2007). In its native range, the weevil almost exclusively prefers 2-15 year old *Pinus* trees and results in significant economic losses in

regenerating stands (CABI, 2014; Day et al., 2004; Panzavolta & Tiberi, 2010). In its invasive range in South America, *P. castaneus* causes significant damage in pine nurseries, young trees growing on marginal soils and mature plantations stressed by biotic and abiotic factors (Gomez & Hartel, 2010). It also attacks some species of *Abies* and *Pseudotsuga* in Uruguay (Panzavolta & Tiberi, 2010).

The aim of this study is firstly to assess the species identity of the two invasive *Pissodes* species in the southern hemisphere (South Africa and South America). In addition, we investigate the genetic diversity and structure of populations of the *Pissodes* in South Africa, specifically with respect to geographic distribution and feeding and breeding behaviours (i.e., terminal leader versus bole feeding). Finally, we compare the genetic diversity of the two invasive species in their respective invasive ranges.

Materials and methods

Specimen collection and preservation

Weevils were collected from 27 sites across three major *Pinus*-growing provinces of South Africa: Western Cape, Mpumalanga and KwaZulu-Natal, from July to September 2013. Three *Pinus* plantations were randomly selected from each province, and in each plantation three sites with active *Pissodes* infestation were selected for study. Insects were then collected from five randomly selected *Pissodes* infested trees per site, for a total of 135 trees (Online Resource 1). All specimens from Western Cape were collected from *P. radiata* and those from Mpumalanga and KwaZulu-Natal were from *P. patula*. Most of the collections were obtained from recently dead trees, though some collections were obtained from living trees in the Western Cape. A total of 675 insects (i.e five per tree and twenty five per site) were preserved in absolute ethanol and kept frozen at -20 ^{o}C until DNA extraction. Preserved (70% EtOH) *Pissodes* specimens were obtained from South America (Chile, Uruguay and Argentina), Europe (Spain and Czech Republic) and North America (USA) (Online Resource 2). As part of our effort to determine the identity of the *Pissodes* sp. in South Africa, we also obtained identified and frozen specimens of *P. nemorensis* from the USA (New York and Florida) and Canada (Ontario) (Online Resource 2).

DNA extraction and cleaning

Prior to DNA extraction, the preserved insect specimens were rinsed with sterilized distilled water and the whole insect body was ground with Retsch MM 301 Mixer Mill (Retsch GmbH, Rheinische, Germany) using metal beads. Total genomic DNA was extracted from 241 specimens (i.e. two insects per sampled tree) from South Africa (Online Resource 1) and from 34 specimens outside South Africa (Europe, North America and South America) (Online Resource 2) using a slightly modified phenol/chloroform extraction protocol (Goodwin et al., 1992). The genomic DNA was also cleaned following the phenol/chloroform DNA extraction protocol (Goodwin et al., 1992). The resulting DNA was suspended in 25 µl of ultrapure (SABAX) water. Concentration and quality of the DNA was determined by a NanoDrop 1000 Spectrophotometer (Thermo Fisher

Scientific Inc., Wilmington, U.S.A.). The quality was further evaluated through electrophoresis on 1% agarose gel. All extracted DNA was stored at -20 ^{o}C.

Amplification of mtDNA (COI)

The Jerry-to-Pat region of the mitochondrial cytochrome c oxidase I (COI) gene was amplified from 241 specimens from South Africa using the universal primers C1-J-2183 (Jerry): 5'-CAACATTTATTTTGATTTTTT-3' and TL2-N-3014 (Pat): 5'-TCCAATGCACTAATCTGCCATATTA-3' (Simon et al., 1994). The same gene region was amplified from specimens obtained from Europe, North America and South America. The COI barcoding region of all haplotypes from South Africa and the specimens from other countries was amplified using the primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Simon et al., 1994).

Amplification reactions were performed in a 25 µL reaction solution comprising 16.1 µL of ultrapure (SABAX) water, 3 µL of 10x concentrated PCR reaction buffer with 20 mM $MgCl_2$ (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 µL of deoxynucleotide triphosphate (dNTP) mix (10 mM; 2.5 mM each), 1 µL of each primer (10µM) (WhiteSci), 0.4 µL of FastStart *Taq* DNA polymerase (5U μL^{-1}) (Roche Diagnostics GmbH, Mannheim, Germany) and 1µL of cleaned insect genomic DNA (100ng μL^{-1}). The reactions were run in a Bio-Rad iCycler thermocycler (BIO-RAD, Hercules, CA, USA) at 95 ^{o}C for 3 min, followed by 35 cycles at 95 ^{o}C for 1 min, 51 ^{o}C for 1 min and 72 ^{o}C for 1.5 min and final extension at 72 ^{o}C for 8 min. The amplification reactions and thermocycling conditions for both primer sets remained the same, except an annealing temperature of 42 ^{o}C was used for amplifying the barcoding region of COI instead of the 51 ^{o}C used for the 3' end region of COI. Amplification of the target DNA fragments was checked by running electrophoresis of a mixture of 4 µL of the PCR aliquots and 2 µL of GelRed™ (Biotium, USA) along with 100bp DNA molecular weight marker (Thermo Scientific O'Gene Ruler™) on 2% agarose gel. Following this, the fragments were visualized under UV light and gel images were captured using Gel Doc™ EZ Imager (BIO-RAD).

Cleaning PCR products and sequencing PCR

The PCR products were cleaned by adding 8µL of ExoSAP-IT (USB Corporation, Cleveland, OH) and incubating the mixture at 37 ^{o}C and 80 ^{o}C; 15 min at each temperature point. The forward and reverse sequencing reactions were performed in a 12 µL reaction volume made of 6.4 µL of ultrapure (SABAX) water, 2.1 µL of sequencing buffer, 0.5 µL of BigDye™ Ready Reaction Mixture with Amplitaq DNA polymerase (Perkin-Elmer Applied Biosystems, Warrington, UK), 1µL of either of the primers (10 µM) and 2 µL of purified PCR product (50 ng μL^{-1}). The mixture was then incubated in a Bio-Rad iCycler thermocycler (BIO-RAD) for 35 cycles at 96 ^{o}C for 10 s, 51 ^{o}C for 5 s and 60 ^{o}C for 4 min. The sequencing products were cleaned and run and visualized on ABI PRISM™ 3500 automatic DNA Analyzer (Applied BioSystems, USA).

For some European and North American species (*P. strobi*, *P. terminalis*, *P. nemorensis*, *P. schwarzi*, *P. validirostris*, *P. pini*, *P. piniphilus*, *P. punctatus* and *P. harcyniae*), sequences of the barcoding and Jerry-to-Pat region of the COI gene were downloaded from the GenBank and the BOLD System

(http://www.boldsystems.org/). Jerry-to-Pat region sequences of three *P. castaneus* specimens from France, Italy and Slovenia (one from each) were included in our analyses.

Sequence edition and alignment

The raw sequence data were edited using CLC Main Workbench 6.0 (CLC Bio, Denmark) and Biological Sequence Alignment Editor (BioEdit) software (Hall, 1999) version 7.0.9. The edited sequences were aligned using an online Multiple Sequence Alignment Program (MAFFT) version 7 (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). The aligned sequences were further edited in BioEdit by comparing against sequencing trace files.

Morphometric measurements

Sixty-eight female and 72 male *Pissodes* specimens collected from nine sites across South Africa (Online Resource 1) were characterised by measuring 15 morphometric characters (Online Resource 5) used to discern members within the *P. strobi* complex (Williams & Langor, 2002). The specimens were measured using calibrated AxioCam ICc 5 Zeiss camera attached to a Carl Zeiss Stemi 2000 stereo microscope.

Data analysis

Phylogenetic analysis

Separate phylogenetic analyses were conducted on the Jerry-to-Pat and the barcoding region of the COI gene as sequences for both loci was not available for many of the specimens in publicly available databases. The analysis on the barcoding region was conducted with the aim of including some Eurasian *Pissodes* spp. (*P. validirostris*, *P. pini*, *P. piniphilus* and *P. harcyniae*) and *P. strobi*, whose specimens were missing in our collection, but their barcoding sequences were available in the Bold System. The analyses were done using sequences of *Pissodes* specimens from South Africa, USA, Canada, Argentina, Chile, Uruguay, Czech Republic, France, Italy, Slovenia, Spain and those from the GenBank and the BOLD System. GenBank sequences of *P. affinis* Randall and *Curculio salicivorus* Paykull were used as outgroups in phylogenetic analysis of the Jerry-to-Pat and barcoding region, respectively. Maximum Likelihood (ML) and Maximum Parsimony (MP) phylogenies were inferred separately for the Jerry-to-Pat and barcoding region of COI using PhyML version 3 (Guindon et al., 2010) and PAUP* 4.0 beta 10 (Swofford, 2000), respectively. Akaike information criterion in the program jModelTest 2.1.1 (Darriba et al., 2012) was used to determine the most appropriate nucleotide substitution model for each locus (TPM1uf+I+G for barcoding region and TrN+I for Jerry-to-Pat region). The bootstrap method (1000 iterations) was used to estimate the degree of support for internal nodes of the MP trees (Felsenstein, 1985). The net sequence divergence between and within species was computed using the Kimura 2-parameter model in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2011).

Analysis of genetic diversity and population structure was conducted only using sequences of the Jerry-to-Pat region of COI gene. The number of segregating sites (S), haplotype diversity (Hd), the average number of nucleotide differences (k) and nucleotide diversity (Pi) were calculated for each of the nine populations using

DNA Sequence Polymorphism (DnaSP) version 5.10.01 software (Librado & Rozas, 2009). An haplotype network was constructed using Network (Bandelt et al., 1999).

Genetic structure analysis

Analysis of molecular variance (AMOVA) and Mantel correlation test were conducted in GenAlex 6.501 (Peakall & Smouse, 2012) to examine the genetic structure of populations of *Pissodes* sp. across different *Pinus* growing provinces of South Africa. A separate AMOVA was done for the population in the Western Cape to investigate the genetic structure of the populations showing differential feeding and breeding behaviours (i.e., those in terminal leaders and those in boles). DNA Sequence Polymorphism (DnaSP) version 5.10.01 software (Librado & Rozas, 2009) was used to estimate the fixation indices (Fst). Due to differences in the number of specimens sequenced in South Africa and South America, diversity comparisons were made by resampling sequences of specimens from South Africa.

Morphometric data analysis

Morphometric data measured in the present study and those reported by Williams and Langor (2002) were compared by factor analysis (FA) in order to describe morphometric similarity/difference of the South African *Pissodes* specimens to members of the *P. strobi* complex (*P. strobi*, *P. terminalis*, *P. nemorensis* and *P. schwarzi*) in North America. All the morphometric characters used by Williams and Langor (2002) except "width of elytra at anterior margin of declivity (EA)" and "depth of mesothorax at deepest point (MD)" were used in our FA (i.e., 13 measured and three calculated ratio characters in total). Prior to FA, original datasets for North American species were simulated based on the published mean, standard error and range values (assuming normal distributions). The FA produced a set of variables (factors) that were linear combinations of the original variables. The new variables (factors) were independent of each other and ranked according to the amount of variation. After the initial factor extraction by the principal component method, an orthogonal normalised varimax rotation was used to estimate the factor loadings. Only factors with an eigenvalue greater than one were extracted. These analyses were performed in Statistica 12.0 (StatSoft, 2013).

Results

Identification of the Pissodes specimens introduced in South Africa

A 578 bp segment of the Jerry-to-Pat region of the COI gene was successfully sequenced for 241 specimens. There were 13 polymorphic sites (2.25% of the segment), resulting in 10 distinct haplotypes (Fig. 1) of an apparent single species. Consensus trees for both gene regions clearly showed that all representative haplotypes from South Africa grouped together in a strongly supported distinct clade (bootstrap value ≥ 95%, branch length ≥ 0.85; Figs. 2 and 3). For both COI regions, the South African clade was sister to GenBank sequences of *P. strobi*, also strongly supported as a distinct clade (bootstrap value ≥ 96%, branch length ≥ 0.94). Together *P. strobi*, *P. nemorensis* and the *Pissodes* sp. formed a well-supported clade, though *P. nemorensis*

does not share the most recent common ancestor of the other two taxa. The *Pissodes* species from South Africa has the least sequence divergence from *P. strobi* populations (0.031 - 0.035) compared to its divergence from other species, namely *P. nemorensis* (0.064 - 0.067), *P. terminalis* (0.070), *P. schwarzi* (0.082), *P. castaneus* (0.116 - 0.128), *P. pini* (0.119) and the outgroup *P. affinis* (0.159) (Online Resource 3). In general, the weevil in South Africa was more closely related to species native to North America than to those native to Europe. Similar results were obtained from phylogenetic and sequence divergence analyses based on sequences of the barcoding region of the COI gene (Online Resource 4).

Analysis of 13 morphometric measurements showed that both the females and males of the *Pissodes* sp. in South Africa grouped most closely to *P. nemorensis* and *P. schwarzi* than to *P. strobi*. More than 67% of the variance in the FA was explained by axis 1 (45.8%) and 2 (22.7%; Fig. 4). Measurements of all morphometric characters, except snout apex proportion (SA), were the highest in both females and males of the *Pissodes* sp. in South Africa (Online Resource 5) compared to published morphometries of other members of the *P. strobi* complex (Williams & Langor, 2002). The depth of pronotum along posterior margin (PD) and length of snout between apex and eye margin at mid-height (SL) were found remarkably longer in both the females and males of the *Pissodes* sp. in South Africa (Online Resource 5) than other members of the *P. strobi* complex (Williams & Langor, 2002).

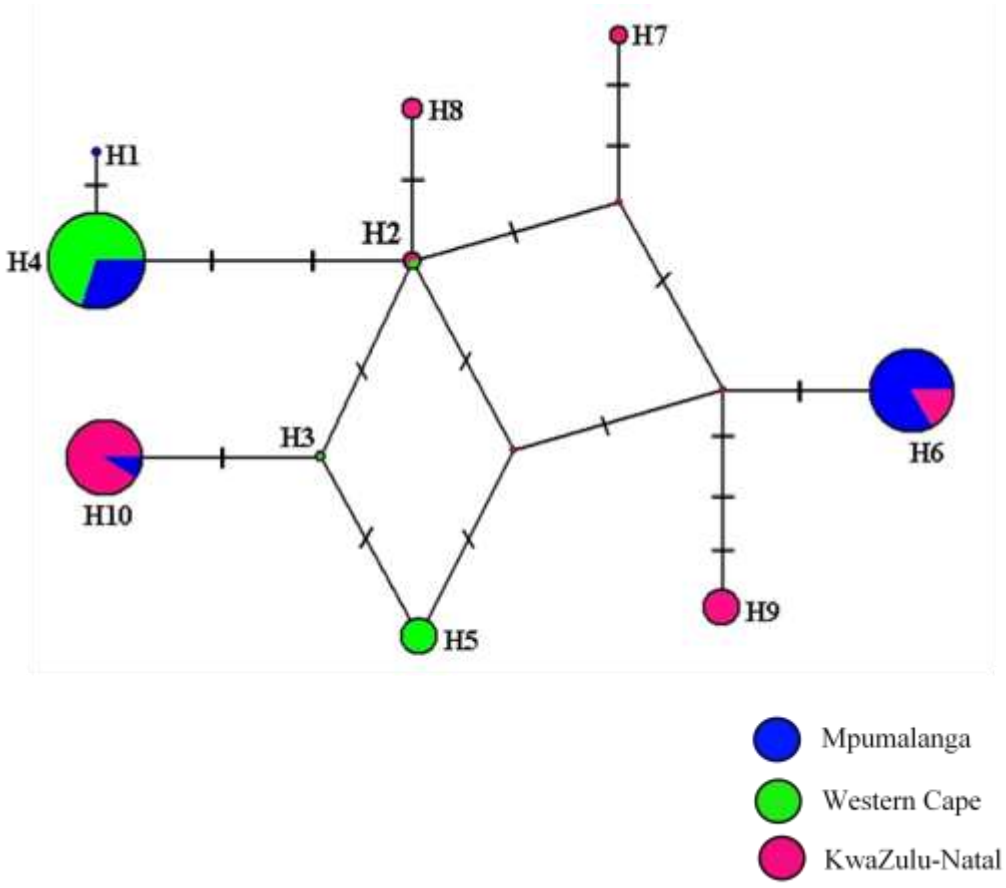

Fig. 1 Median-joining haplotype network of *Pissodes* sp. in South Africa based on 578 bp segment of the Jerry-to- Pat region of the COI gene**.** The size of the circle is proportionate to the abundance of the haplotype and *vice versa*

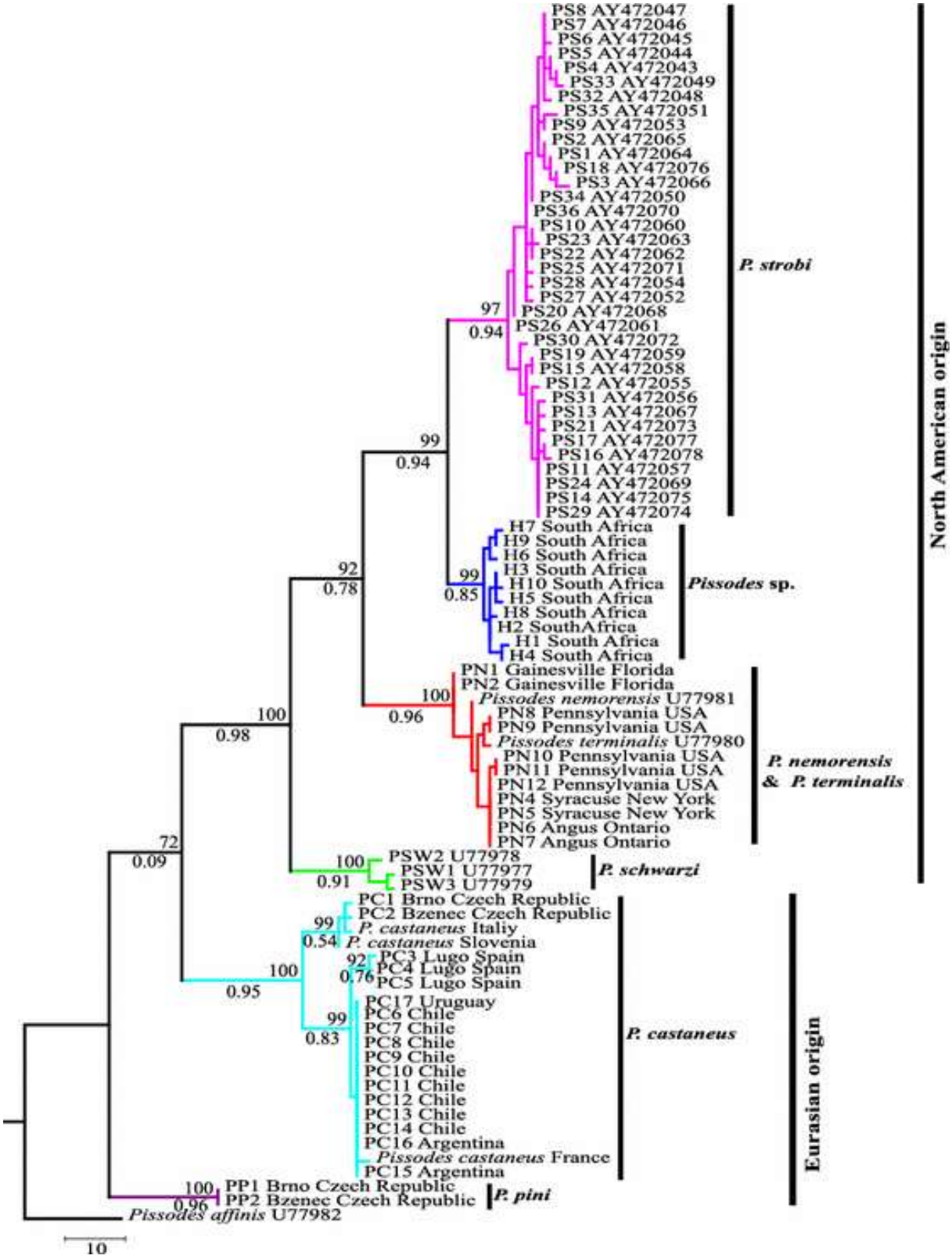

Fig. 2 Phylogenetic tree based on maximum parsimony and maximum likelihood analysis of a 578 bp segment of the Jerry-to-Pat region sequence of the COI gene. A sequence of *P. affinis* was used as an outgroup. GenBank accession numbers are indicated where available. The *numbersabove* and *below* branches indicate bootstrap value and branch length, respectively. Consistency index (CI) = 0.595; retention index (RI) = 0. 953; homoplasmy index (HI) = 0.405

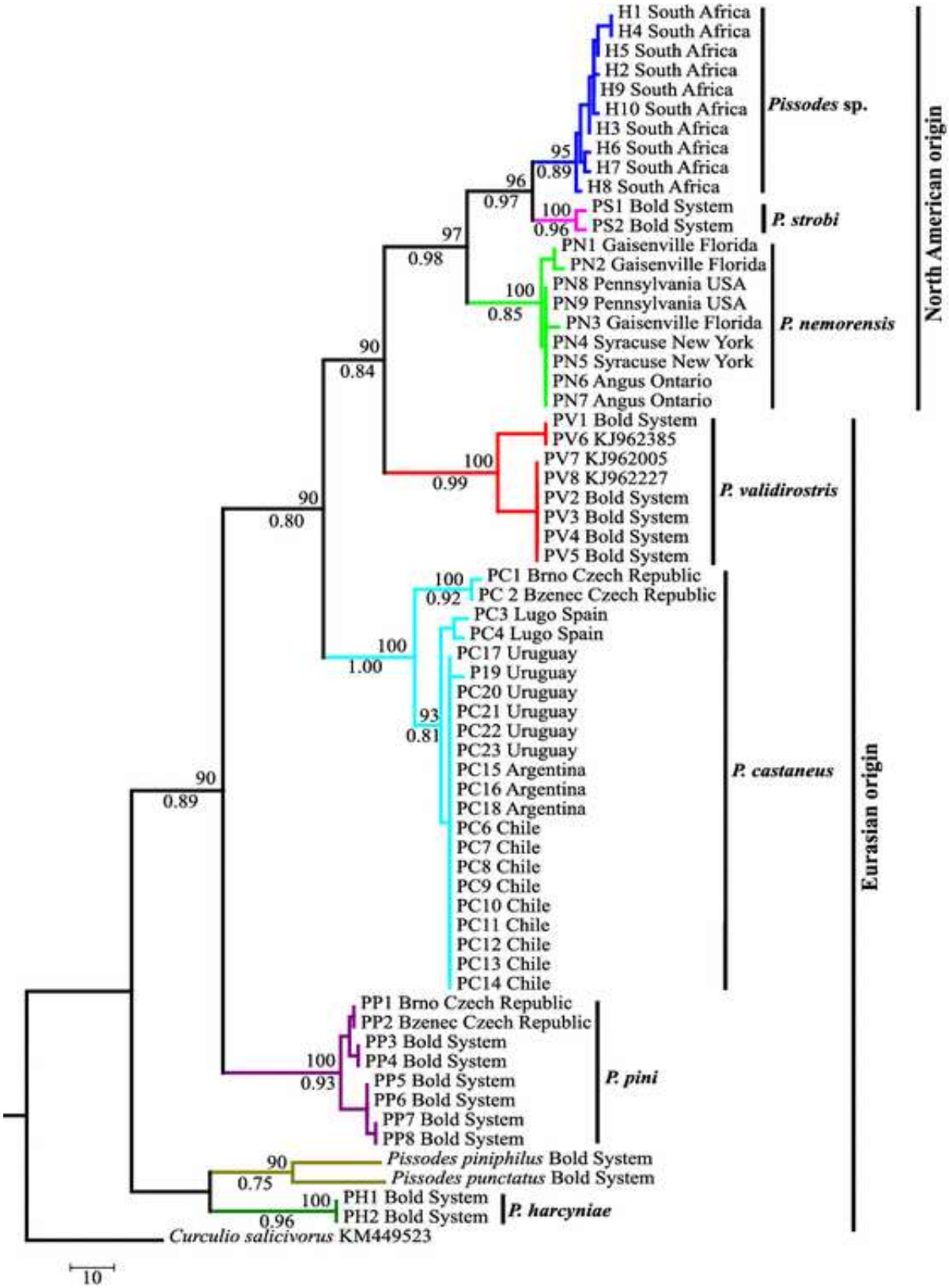

Fig. 3 Phylogenetic tree based on maximum parsimony and maximum likelihood analysis of a 582 bp sequence of the barcoding region of the COI gene for 8 species of *Pissodes*. A sequence of *Curculiosalicivorus* from GenBank was used as outgroup. GenBank accession numbers are indicated where available. The *numbersabove* and *below* branches indicate bootstrap value and branch length, respectively. Consistency index (CI) = 0.556; retention index (RI) = 0. 921; homoplasmy index (HI) = 0.444

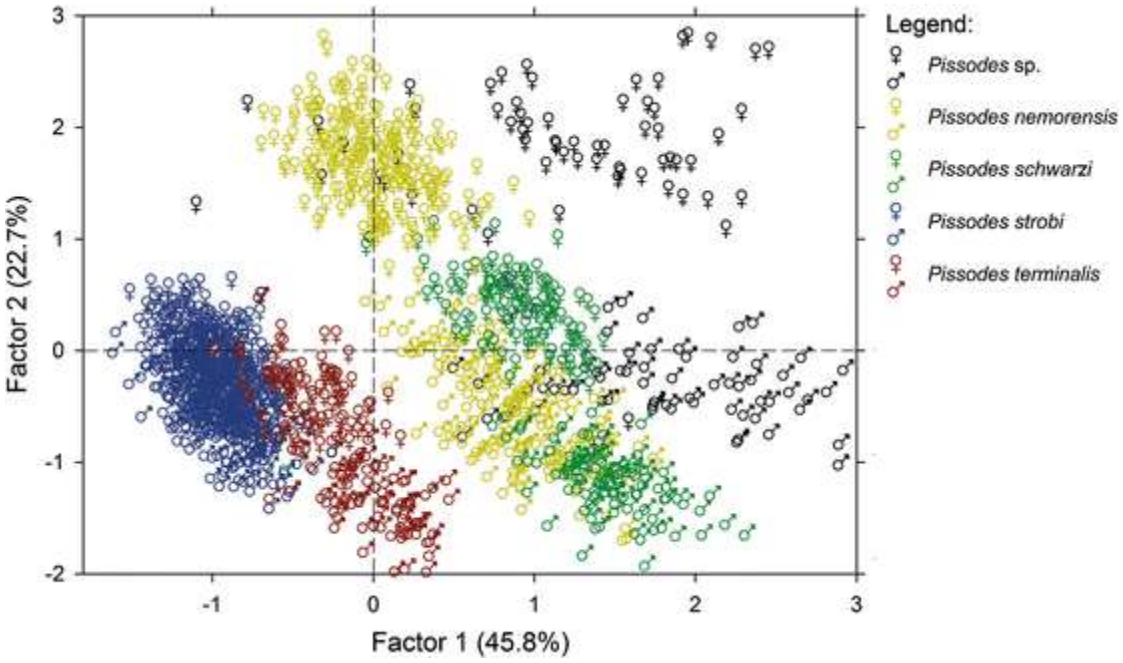

Fig. 4 Ordination plot based on factor analysis of female and male morphometric measurements of the *Pissodes* sp. in South Africa and members of the *Pissodes strobi* complex. Data for the four North American *Pissodes* spp. (*P. strobi*, *P. terminalis*, *P. schwarzi* and *P. nemorensis*) was obtained from Williams and Langor (2002)

Identification of the Pissodes species introduced in South America

In the present study, analysis of the barcoding region and the Jerry-to-Pat region of the COI gene grouped all specimens from South America (Argentina, Chile and Uruguay) and Spain and two specimens from the Czech Republic together with identified *P. castaneus* specimens from France, Italy and Slovenia, in a strongly supported clade (bootstrap value = 100; branch length = 1) (Fig. 2 and 3). The South American specimens grouped most closely with *P. castaneus* from France, compared to the specimens of the same species from Italy, Slovenia, Spain and Czech Republic. None of the specimens from South America grouped with the North American or the South African *Pissodes* species, suggesting its origin from Europe. The results supported the introduction of a single species, *P. castaneus*, in all three South American countries (Argentina, Chile and Uruguay).

Genetic diversity of Pissodes species in South Africa and P. castaneus in South America

Estimates of genetic diversity of the *Pissodes* species in South Africa was made based on sequences of the Jerry-to-Pat region of the COI gene (Table 1). The sequence divergence among the 10 haplotypes ranged from 0.2 to 1.4%. An estimated overall nucleotide diversity (Pi) of 0.006 (S = 13, h = 10, Hd = 0.750, K = 3.365) was obtained from the entire population in South Africa, with the highest nucleotide diversity of 0.005 (S = 13, h = 6, Hd = 0.597, K = 3.043) from KwaZulu-Natal and the lowest nucleotide diversity of 0.002 (S = 4, h = 4, Hd = 0.338, K = 1.260) from the Western Cape. At the population level, the lowest diversity detected for *Pissodes* in South Africa was in Ruigtevlei in the Western Cape and the highest from Underberg in KwaZulu-Natal (Table 1). In general, populations in the summer-rainfall regions of the country (where *P. patula* and other *Pinus* spp.

grow) tend to be more diverse than the populations in the winter-rainfall areas where *P. radiata* is predominantly grown. Analyses made on *P. castaneus* specimens from South America indicated that populations in Argentina, Chile and Uruguay consisted of only a single haplotype.

Table 1 Genetic diversity parameters of *Pissodes* sp. in nine populations in three provinces (Western Cape, Mpumalanga, and KwaZulu-Natal) of South Africa

Province	Site	n	S	h	Hd	K	Pi
Western Cape	Braken Hill	30	4	2	0.287	1.149	0.002
	Ruigtevlei	30	4	3	0.246	0.903	0.002
	Bergplaas	14	4	3	0.582	2.044	0.004
	Total	78	4	4	0.338	1.260	0.002
Mpumalanga	Helvetia	28	5	2	0.423	2.116	0.004
	Torburnlea	29	6	3	0.549	2.655	0.005
	Rooihoogte	29	7	3	0.493	2.414	0.004
	Total	86	8	4	0.509	2.50	0.004
KwaZulu-Natal	Shafton	26	9	5	0.625	3.428	0.006
	De Rust	29	9	4	0.414	2.153	0.004
	Underberg	26	10	6	0.738	3.474	0.006
	Total	81	13	6	0.597	3.043	0.005
Entire population	Grand total	241	13	10	0.750	3.365	0.006

[n] sample size

[S] number of segregating sites

[h] number of haplotypes

[Hd] haplotype diversity

[K] mean number of nucleotide differences

[Pi] nucleotide diversity

Genetic structure of the Pissodes species in South Africa

Of the 10 haplotypes of *Pissodes* sp. in South Africa, H4, H6 and H10 were the most common, representing 84.6% of all samples. Each of these haplotypes along with H2 were shared between at least two populations (Fig. 1). The remaining six haplotypes were unique to a single province. The shared haplotypes indicate a lack of strong population genetic structure. Genetic structure analysis for *P. castaneus* in South America was not determined, since all of the populations contained a single haplotype across its invasive range.

Analysis of molecular variance (AMOVA) was performed for the three provinces (Western Cape, Mpumalanga and KwaZulu-Natal) and nine populations; populations being represented by plantations (Braken Hill, Ruigtevlei, Bergplaas, Helvetia, Torbunlea, Rooihoogte, Shafton, De Rust and Underberg) in South Africa. The total molecular variance was partitioned into three different sources (among provinces, between populations in a province and within populations). A moderate level of structure was recorded among provincial populations (Fst = 0.41 and Nm = 0.7). The highest proportion of the total molecular variance was explained by the variation within populations (57%) followed by a 40% variation among provinces and 3% within populations in a province (Table 2). A Mantel correlation test resulted in a moderate level of correlation (R = 0.22) between genetic and geographic distance (Fig. 5).

Table 2 Hierarchical analysis of molecular variance (AMOVA) for nine geographically distinct populations of *Pissodes* sp. across three provinces (Western Cape, Mpumalanga and KwaZulu-Natal) in South Africa

Source of variation	Df	SS	MS	Est. Var.	%
Among provinces	2	130.01	65.00	0.78***	40%
Among populations within a province	6	14.86	2.48	0.05**	3%
Within populations	232	259.00	1.12	1.12***	57%
Overall Fst				0.42*	
Overall Nm				0.70	

* Significant at 0.05 level

** Significant at 0.01 level

*** Significant at 0.001 level

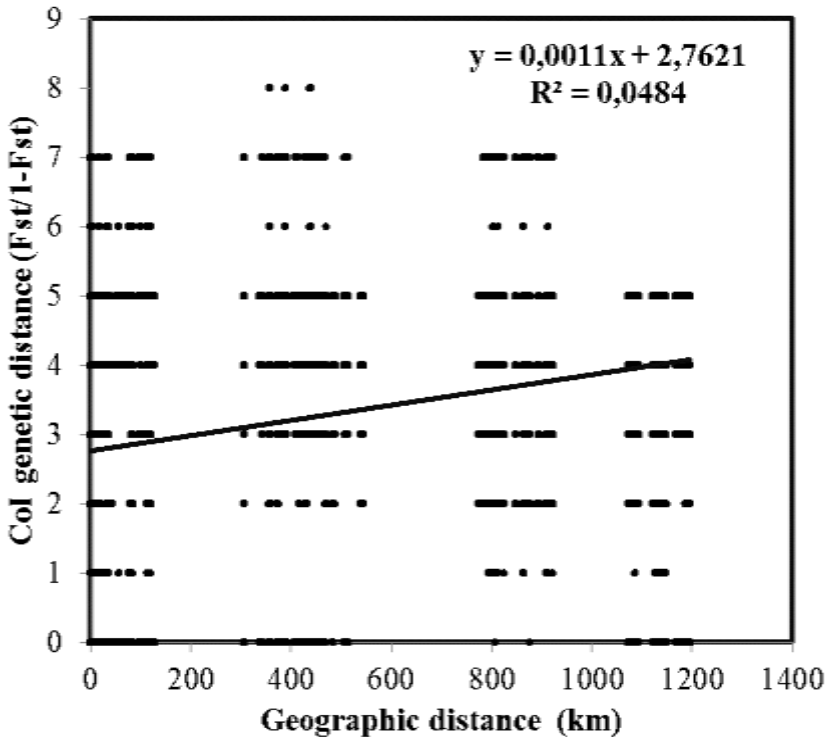

Fig. 5 Regression analysis of pairwise genetic distance versus pairwise geographic distance of populations of the *Pissodes* sp. in South Africa (Mantel R = 0.220, P = 0.0001)

AMOVA conducted on three populations of the Western Cape Province that showed different feeding and breeding behaviors (namely: those feeding on the terminals of healthy trees, the top trunk of dead trees and the bottom trunk of dead trees) resulted in a low between populations (16%) and a high within populations (84%) molecular variation. This was further supported by a Fst of 0.09 and Nm of 4.9 (Table 3).

Table 3 Hierarchical analysis of molecular variance (AMOVA) of *Pissodes* sp. exhibiting different feeding and breeding site preference: on the terminals of healthy trees, on the upper trunk of dead trees, and on the lower trunk of dead trees

Source of variation	Df	SS	MS	Est. Var.	%
Among populations	2	5.41	2.70	0.11*	16%
Within populations	71	40.58	0.57	0.57*	84%
Overall Fst				0.09*	
Overall Nm				4.90	

* Significant at 0.05 level

Discussion

In this study we examined the identity and genetic diversity of two invasive *Pissodes* spp. in the southern hemisphere and population structure of the *Pissodes* sp. in South Africa. Sequence analysis of both the barcoding and the Jerry-to-Pat region of the COI gene revealed the introduction of only one *Pissodes* species into South Africa. Our results strongly suggest that the species introduced into South Africa and identified as *P. nemorensis* in 1942 (Webb 1974) is very likely not *P. nemorensis*. Based on current sampling and available GenBank resources, we are not currently able to ascertain the identity of the *Pissodes* species present in South Africa. The close grouping with the North American species and apparent sister species status with *P. strobi* strongly suggests a North American origin for this weevil, as well as membership in the *P. strobi* complex. However, this weevil is clearly distinct from the four described members of the *P. strobi* complex (*P. strobi*, *P. nemorensis*, *P. terminalis* and *P. schwarzi*). We, therefore, hypothesize the possible existence of an additional, an unrecognized member of this complex or a hybrid between *P. nemorensis* and *P. strobi* that has become invasive in South Africa.

It now appears clear that two distinct *Pissodes* species, very likely of different continental origins and among the most damaging *Pissodes* species, have been introduced into the southern hemisphere. Our study confirmed the identity of the species in South America (Chile, Uruguay and Argentina) as the small banded pine weevil (*P. castaneus*), in agreement with the recent confirmation of the species in Argentina (Pereyra et al., 2015b). This species is of Eurasian origin. We detected only a single haplotype in Chile, Argentina and Uruguay, suggesting a single, relatively recent introduction.

Although the *Pissodes* sp. in South Africa is phylogenetically closest to *P. strobi*, this relationship was not congruent with the analysis of morphometric data and the observed breeding and feeding behavior. Adults of *P. strobi* lay their eggs on the upper half of the previous year's terminal shoot and the hatching larvae feed gregariously, moving downwards (Belyea & Sullivan, 1956; Langor et al., 1992). However, the weevil in South Africa often prefers the main trunk of dead or dying *Pinus* trees and rarely seen feeding and breeding on the terminal leaders (authors' observation). This observed feeding and breeding behavior of the *Pissodes* sp. in South Africa is more similar to that of *P. nemorensis* in its native range (Atkinson et al., 1988). *Pissodes nemorensis* shows two types of feeding behaviors in its native range in North America (Ollieu, 1971; Overgaard & Nachod, 1971). In the first case, it attacks terminal leaders of young healthy trees. In the second case, and

most commonly, the weevil attacks the main trunk and roots of declining or recently dead trees. *Pissodes castaneus* has similar plasticity in resource utilization. It attacks pine nursery seedlings, young trees growing on marginal soils and mature plantations stressed by biotic and abiotic factors (Gomez & Hartel, 2010). Similarly, results from our analysis using morphometric data grouped the *Pissodes* sp. in South Africa closer to *P. nemorensis* and *P. schwarzi* than to *P. strobi*, although still a distinct group.

Cross breeding experiments conducted under laboratory condition have confirmed that some members of the *P. strobi* complex can interbreed to produce viable offspring (Godwin & Odell, 1967; Smith & Takenouchi, 1969). Indications of natural hybridization and mitochondrial introgression between *P. strobi* and *P. nemorensis* were also reported from their native range (Boyce et al., 1994; Williams & Langor, 2002). This suggests the possibility that the species in South Africa may be a hybrid, possibly of *P. strobi* and *P. nemorensis* which could explain why the South African *Pissodes* sp. is phylogenetically closer to *P. strobi* based on mitochondrial sequence data, but closer to *P. nemorensis* behaviorally, ecologically and morphologically. Alternatively, it could represent an unrecognized species, probably from North America, though this seems unlikely given considerable study on *Pissodes* in this region (Langor & Sperling, 1995; Langor & Sperling, 1997; Williams & Langor, 2002).

The possible misidentification of the *Pissodes* sp. in South Africa is not unique among invasive pest insects. For example, the eucalyptus leaf weevil (formerly known as *Gonipterus scutellatus*) introduced into Africa, North and South America, Europe and New Zealand, was for many decades considered to be one species (Mapondera et al., 2012). However, recent molecular studies revealed that there are eight cryptic species within the *G. scutellatus* complex, with *G. platensis* introduced to New Zealand, North and South America and western Europe, *G. pulverulentus* to South America and an undescribed species introduced to Africa and southern Europe. Failure to recognize cryptic species may lead to additional damage and implementation of inappropriate regulatory and management programs. Our ability to identify cryptic and invasive species has significantly increased with the development of molecular approaches such as barcoding techniques.

We found 10 haplotypes of *Pissodes* present along a 578 bp COI sequence segment from 241 individuals from 9 localities in South Africa. This is less than, though comparable to, the 36 haplotypes of *P. strobi* in its native range in North America (826 bp segment of 130 individuals from 11 localities) (Laffin et al., 2004) and 21 haplotypes of *P. yunnanensis* in its native range in China (761 bp segment of 60 individuals from 7 localities) (Zhang et al., 2007). The 0.2 - 1.4% sequence divergence found among the 10 South African haplotypes falls within the range of the sequence divergence for the above-mentioned studies of *Pissodes* in its native range (0.001 - 2.1% for *P. yunnanensis* and 0.12 - 2.54% for *P. strobi;* (Laffin et al., 2004; Zhang et al., 2007). Similarly, the nucleotide diversity (Pi) in South Africa (0.002 to 0.006) is comparable to that of *P. yunnanensis* (0.00088 to 0.0033) (Zhang et al., 2007). This considerable amount of mitochondrial genetic diversity for an exotic insect is likely to have resulted either from a single introduction of a large number of individuals, or from multiple introductions over time, both of which occur with some regularity in invasive organisms (Garnas et al., 2016; Nadel et al., 2010).

A moderate level of genetic differentiation was detected among populations of the *Pissodes* sp. in different provinces of South Africa. The shared haplotypes between provinces in South Africa and the moderate level of genetic isolation by geographic distance could be the result of historically unregulated plant and wood material movement within the country and natural spread of the weevil. With the natural and human-mediated dispersal of the weevil, it is likely to experience migration and gene flow between populations, even though *Pinus* plantations in the country are spatially disjunct. A similar pattern of haplotype sharing between populations of an exotic fungus gnat, *Bradysia difformitis* was reported in four different South African forestry nurseries. Movement of pine nursery plants and bark medium were suspected as possible pathways of movement of the fungus gnat among nurseries (Hurley et al., 2010). The pattern in these invasive populations is, however, in contrast to studies on *Pissodes* species in their native ranges that have shown population genetic structure based on geographic location (Glaubitz, 2001; Laffin et al., 2004; Zhang et al., 2007). There was no evidence supporting genetic differentiation among terminal leader and bole-feeding individuals in the Western Cape.

The identity of the *Pissodes* sp. in South America remained unconfirmed for many years (Abgrall et al., 1999; Anon, 1970; O'Brien, 1989). Zaleski et al. (2013) and Marques et al. (2011) mentioned that the *Pissodes* sp. in Brazil is *P. castaneus*. Recent CABI report stated that *P. castaneus* is absent from Argentina and Uruguay, but present in Brazil and Chile (CABI, 2014). However, a very recent study supported with mitochondrial and nuclear sequence data reported the *Pissodes* species in Argentina as *P. castaneus* (Pereyra et al., 2015b). Our study confirmed the presence of the European species, *P. castaneus*, in South America (Argentina, Chile and Uruguay). Zaleski et al. (2013) reported considerable genetic diversity of *P. castaneus* based on a study conducted on 19 populations across its introduced range in Brazil using PCR-AFLP data. This substantial amount of diversity suggested the establishment from multiple introduction events as *P. castaneus* was only detected in Brazil in 2001 (Zaleski et al., 2013). However, our analysis of sequences of the COI gene on three populations from Argentina, Chile and Uruguay (one population from each country) has shown the spread of only a single haplotype throughout these countries, suggesting possible establishment from a limited introduction from single origin. This invasive haplotype matches a haplotype from France and is in complete agreement with Pereyra et al. (2015a) which showed matching of an Argentine haplotype with the one from France. A previous study suggested Uruguay and Argentina as possible sources of the *P. castaneus* in Brazil (Abgrall et al., 1999), but the link between these populations remains to be studied.

Based on the current trend on the spread of invasive insect pests of forestry trees (Brockerhoff et al., 2006a; Tobin, 2015) further introductions of *Pissodes* spp. could occur into South Africa and South America, and to other *Pinus* growing countries. Strict adherence to regulations on the movement of wood products, such as the International Standard for Phytosanitory Measures No. 15 (ISPM15) (Haack et al., 2014) should assist in decreasing the rate of spread of these insects, although it is unlikely to completely stop such movement (Brockerhoff et al., 2006b). Though most *Pissodes* spp. are pests of secondary importance in their native ranges, there are some species, including *P. strobi*, that cause significant primary damage to conifer plantations. The threat posed by these weevils increases substantially when considering the known association of some species with tree diseases such as *Leptographium procerum*, *Fusarium circinatum* (=*Fusarium moniliforme* var. *subglutinans*), *Cronaritium flaccidum* and several species of *Ophiostoma*, and their possible role in transmitting

these diseases (Blakeslee et al., 1981; Coutinho et al., 2007; Jankowiak & Bilański, 2013; Lewis & Alexander, 1986). The establishment of the pitch canker fungus, *F. circinatum*, in nurseries, established and mature *Pinus* plantations in South Africa (Coutinho et al., 2007; Mitchell et al., 2011; Wingfield et al., 2008) and its presence in South America (Wingfield et al., 2008), undoubtedly increases the importance of these two invasive *Pissodes* spp. It is therefore worthwhile investigating the role of *Pissodes* sp. and *P. castaneus* in the transmission and spread of tree pathogenic fungi such as *F. circinatum*.

The present study demonstrated the importance of using a combination of morphological and molecular approaches to ascertain species identity of invasive pest species. Results of this study will have implications on quarantine, research and management of these two invasive *Pissodes* species. Countries in the southern hemisphere which were already infested by either of the two invasive *Pissodes* species should avoid further introductions of the same species or other *Pissodes* species in order to prevent introduction of multiple genotypes of same species or cryptic forms which may then complicate pest problem via admixture and hybridization. Growers in the northern hemisphere (native range of *Pissodes*) should be aware of the possibilities for back introductions of *P. castaneus* and *Pissodes* sp. from their invasive range, which may lead to complex pest problems via admixture. The presence of a single haplotype of *P. castaneus* in its introduced range in South America and lack of strong genetic structure among geographic populations of the *Pissodes* sp. in South Africa suggests the possibility of using the same or similar management approaches across the invasive ranges of each of these *Pissodes* species. Further studies are required in order to determine whether the *Pissodes* sp. in South Africa is an unrecognized member of the *P. strobi* complex or a hybrid/introgression between *P. strobi* and *P. nemorensis* using a combination of classical taxonomic approaches, breeding (hybridization) experiments and molecular approaches.

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Supplementary material

ESM 1 Sampling areas, GPS coordinates and number of specimens used for genetic and morphometric studies of the *Pissodes* sp. in South Africa

Province	Plantation (population)	Site	Latitude	Longitude	Number of insects		
					n1	n2	n3
Western Cape	Braken Hill	S14	S 34° 00.785'	E23° 06.868'	10	2	3
		S30	S 34° 01.246'	E 23° 07.271'	10	4	4
		S23	S 34° 01.621'	E 23° 06.749'	10	3	4
	Ruigtevlei	F16	S 33° 59.644'	E 22° 53.261'	10	7	5
		G14	S 33° 59.358'	E 22° 52.412'	10	2	2
		G20	S 33° 59.542'	E 22° 53.006'	10	4	4
	Bergplaas	K14	S 33° 51.889'	E 22° 41.017'	2	0	0
		E12	S 33° 55.656'	E 22° 40.748'	8	4	8
		A2	S 33° 55.849'	E 22° 46.094'	4	2	1
Mpumalanga	Helvetia	C43a	S 25° 30.000'	E 30° 22.026'	8	0	0
		E31	S 25° 33.011'	E 30° 18.040'	10	1	1
		C32	S 25° 32. 002'	E 30° 23.017'	10	3	3
	Torbumlea	T13A	S 26 °11.986'	E 30 °35.118'	10	1	2
		T7A	S 26 °12.209'	E 30 °35.054'	9	2	2
		T17	S 26 °10.174'	E 30 °32.156'	10	4	4
	Rooihoogte	N56	S 26 °38.031'	E 30 °20.046'	10	2	2
		N12A	S 26 °00.004'	E 30 °17.031'	9	2	3
		V32	S 26 °02.028'	E 30 °15.050'	10	0	2
KwaZulu-Natal	Shafton	C5B	S 29 ° 23.128'	E 30 ° 14.489'	6	1	1
		7B-1	S 29 ° 23.363'	E 30 ° 14.304'	10	2	1
		7B-2	S 29 ° 23.363'	E 30 ° 14.304'	10	3	3
	De Rust	A32	S 28 ° 97.816'	E 30 ° 60.200'	10	4	4
		B49B	S 29 ° 39.144'	E 30 ° 63.659 '	10	2	2
		C17A	S 28 ° 99.958'	E 30 ° 63.664'	9	7	5
	Underberg	M24A	S 29 ° 83.235'	E 29 ° 69.000'	8	4	3
		D2	S 29 ° 65.661'	E 29 ° 611.69'	10	0	0
		B16	S 29 ° 67.353'	E 29 ° 71.086'	8	2	3

[n1] number specimens from which the Jerry-to-Pat region of COI gene was sequenced

[n2] number of female insects used for morphometric study

[n3] number of male insects used for morphometric study

EMS 2 Species, number, collection locality and country information of the non-South African specimens of which the Jerry-to-Pat and barcoding region of COI gene was sequenced

Pissodes **species**	Locality	Country	No. insects sequenced	
			Jerry-to-Pat	Barcoding
P. nemorensis	Angus, Ontario	Canada	2	2
P. nemorensis	Syracuse, New York	USA	2	2
P. nemorensis	Gainesville, Florida	USA	2	3
Undetermined	Pennsylvania	USA	5	2
Undetermined	Begonte, Lugo	Spain	3	2
Undetermined	Brno, Southern Moravia	Czech Republic	2	2
Undetermined	Bzenec, Southern Moravia	Czech Republic	2	2
Undetermined	Futalelfu, Los Lagos	Chile	9	9
Undetermined	-	Uruguay	1	6
Undetermined	Esquel, Chubut	Argentina	2	3
P. castaneus	Toulon	France	1	0
P. castaneus	Framura	Italy	1	0
P. castaneus	Bratislava	Slovakia	1	0

ESM 3 Estimates of net sequence divergence between different *Pissodes* spp. based on sequences of the Jerry-to-Pat region of the COI gene

Sequences of *Pissodes* spp.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1. *Pissodes* sp., South Africa	0.000																		
2. *P. strobi*, across Canada (GenBank)	0.032																		
3. *P. strobi*, East Canada (GenBank)	0.031	0.000																	
4. *P. strobi*, Central Canada (GenBank)	0.031	0.002	0.001																
5. *P. strobi*, West Canada (GenBank)	0.035	0.000	0.001	0.005															
6. *P. nemorensis* (GenBank)	0.067	0.080	0.079	0.076	0.083														
7. *P. nemorensis*, USA and Canada	0.064	0.078	0.076	0.076	0.081	0.004													
8. *P. terminalis* (GenBank)	0.070	0.077	0.075	0.076	0.080	0.008	0.004												
9. *P. schwarzi* (GenBank)	0.082	0.086	0.086	0.086	0.088	0.075	0.071	0.080											
10. *P. castaneus*, Argentina	0.125	0.119	0.119	0.126	0.118	0.133	0.128	0.128	0.105										
11. *P. castaneus*, Uruguay	0.125	0.119	0.119	0.126	0.118	0.133	0.128	0.128	0.105	0.000									
12. *P. castaneus*, Chile	0.125	0.119	0.119	0.126	0.118	0.133	0.128	0.128	0.105	0.000	0.000								
13. *P. castaneus*, Spain	0.125	0.122	0.122	0.129	0.121	0.137	0.132	0.132	0.103	0.004	0.004	0.004							
14. *P. pini*, Czech Republic	0.119	0.120	0.120	0.127	0.120	0.121	0.112	0.112	0.105	0.027	0.027	0.027	0.025						
15. *P. castaneus*, Czech Republic	0.116	0.117	0.115	0.121	0.117	0.114	0.105	0.105	0.109	0.112	0.112	0.112	0.111	0.105					
16. *P. castaneus*, France	0.128	0.124	0.123	0.131	0.123	0.139	0.129	0.128	0.110	0.004	0.004	0.004	0.008	0.027	0.112				
17. *P. castaneus* Italy	0.124	0.124	0.123	0.131	0.123	0.123	0.114	0.114	0.110	0.029	0.029	0.029	0.029	0.002	0.107	0.029			
18. *P. castaneus*, Slovenia	0.119	0.119	0.118	0.126	0.118	0.118	0.110	0.109	0.105	0.025	0.025	0.025	0.025	0.002	0.102	0.025	0.004		
19. *P. affinis* (GenBank)	0.159	0.163	0.162	0.165	0.164	0.156	0.146	0.146	0.135	0.134	0.134	0.134	0.133	0.125	0.110	0.129	0.124	0.124	0.000

ESM 4 Estimates of net sequence divergence between different *Pissodes* spp. based on sequences of the barcoding region of the COI gene

Sequences of *Pissodes* spp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Pissodes* sp., South Africa	0.000														
2. *P. strobi* (BOLD System)	0.037														
3. *P. nemorensis*, USA and Canada	0.074	0.081													
4. *P. castaneus*, Argentina	0.117	0.120	0.101												
5. *P. castaneus*, Uruguay	0.117	0.120	0.101	0.000											
6. *P. castaneus*, Chile	0.117	0.120	0.101	0.000											
7. *P. castaneus*, Spain	0.117	0.128	0.101	0.010	0.010	0.010									
8. *P. validirostris* (BOLD System)	0.114	0.102	0.109	0.108	0.108	0.108	0.115								
9. *P. pini* (BOLD System)	0.138	0.139	0.126	0.133	0.133	0.133	0.132	0.124							
10. *P. piniphilus* (BOLD System)	0.186	0.193	0.181	0.150	0.149	0.150	0.149	0.147	0.160						
11. *P. harcyniae* (BOLD System)	0.188	0.196	0.184	0.174	0.174	0.174	0.180	0.167	0.169	0.115					
12. *P. punctatus* (BOLD System)	0.185	0.190	0.180	0.166	0.166	0.166	0.172	0.156	0.170	0.078	0.120				
13. *P. pini*, Czech Republic	0.132	0.135	0.126	0.135	0.136	0.135	0.135	0.122	0.007	0.161	0.166	0.180			
14. *P. castaneus*, Czech Republic	0.121	0.130	0.105	0.027	0.027	0.027	0.024	0.126	0.135	0.152	0.179	0.179	0.138		
15. *Curculio slicivorus* (GenBank)	0.190	0.200	0.197	0.212	0.211	0.212	0.212	0.213	0.232	0.196	0.209	0.188	0.228	0.000	

EMS 5 Mean ± SE and range of morphometric characters of adults of the *Pissodes* sp. in South Africa

Character code	**Character**	**Female (n = 68)**	**Male (n = 72)**
MW	Width of snout at apex	0.49 ± 0.0051 (0.38 – 0.58)	0.49 ± 0.0043 (0.36 – 0.56)
NP	Width of snout at narrowest point	0.33 ± 0.0038 (0.21 – 0.37)	0.37 ± 0.0033 (0.27 – 0.42)
AW	Width of snout at anterior insertion	0.38 ± 0.0042 (0.29 – 0.44)	0.40 ± 0.0036 (0.29 – 0.45)
SL	Length of snout between apex and eye margin at mid-height	2.39 ± 0.0349 (1.56 – 3.03)	2.06 ± 0.0226 (1.41 – 2.43)
MA	Length of snout between antennal insertion and apex	1.22 ± 0.0205 (0.77 – 1.60)	0.95 ± 0.0113 (0.63 – 1.17)
SD	Depth of snout at antennal insertion	0.33 ± 0.0037 (0.24 – 0.42)	0.33 ± 0.0033 (0.24 - 0.41)
PL	Length of pronotum on midline	2.01 ± 0.0261 (1.46 – 2.54)	1.98 ± 0.221 (1.35 – 2.35)
PW	Width of pronotum at widest point	2.37 ± 0.0309 (1.68 – 2.87)	2.33 ± 0.0288 (1.48 – 2.87)
EL	Length of elytra along midline	4.69 ± 0.0628 (3.04 – 5.63)	4.48 ± 0.0498 (3.08 – 5.25)
EB	Width of elytra at base	2.69 ± 0.0342 (1.86 – 3.23)	2.63 ± 0.0290 (1.70 – 3.09)
PD	Depth of pronotum along posterior margin	1.83 ± 0.0240 (1.30 – 2.17)	1.78 ± 0.0204 (1.14 – 2.08)
FL	Length of fore femur	1.98 ± 0.0253 (1.38 – 2.35)	1.96 ± 0.0221 (1.31 – 2.26)
FW	Width of fore femur at widest point	0.60 ± 0.0076 (0.43 – 0.72)	0,59 ± 0,0064 (0.38 – 0.68)
SN	Snout narrowness, defined as the ratio of SL/NP	7.29 ± 0.0694 (5.62 – 8.74)	5.57 ± 0.0407 (4.44 – 6.72)
SA	Snout apex proportion, defined as the ratio of MA/SL	0.51 ± 0.0023 (0.44 – 0.55)	0.46 ± 0.0021 (0.43 – 0.50)
SS	Relative snout length, defined as the ratio of SL/EL	0.51 ± 0.0022 (0.45 – 0.55)	0.46 ± 0.0027 (0.41 – 0.55)