Parasitoid assemblage associated with a North American pine weevil in South Africa

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Running title: Parasitoid assemblage on a pine weevil

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Abstract

1. The weevil *Pissodes* sp. was first reported as an introduced pest on exotic *Pinus* spp. in South Africa in 1942. It is only recently that the native wasp *Pycnetron pix* Prinsloo was described from South Africa as a parasitoid of this weevil.

2. We estimated the frequency and distribution of the association between *P. pix* and *Pissodes* sp., and the occurrence of possible other natural enemies. Parasitoids were reared from *Pissodes*-infested *Pinus radiata* D. Don and *P. patula* Schiede ex Schltdl. & Cham logs collected from major *Pinus*-growing regions.

3. The identity of parasitoids was confirmed using morphological and molecular techniques. Parasitism was confirmed by analysing gut content sequences of parasitoids.

4. *Pycnetron pix* was found parasitizing *Pissodes* sp. throughout major *Pinus*-growing provinces of the country. Another native parasitoid, *Cratocnema* sp., is reported for the first time as a parasitoid of *Pissodes* sp. *Rhopalicus tutela* (Walker), a known parasitoid of *Pissodes* spp. in their native range, was also detected and confirmed to be of European origin.

5. Though characterized by erratic distribution and low parasitism rate, an accruing suite of natural enemies was documented, suggesting that there is potential for augmentative biological control of *Pissodes* sp. in South Africa.

Keywords: *Cratocnema*; host associations; parasitoids; *Pissodes*; *Pycnetron pix*; *Rhopalicus tutela*

Introduction

According to the enemy release hypothesis, the absence of natural enemies in an introduced range can be a major cause for the success of invasive species (Keane & Crawley, 2002; Torchin et al., 2002; Mitchell & Power, 2003; Roy et al., 2011). Natural enemy richness may increase over time due to adoption of invasive species as hosts by native natural enemies or via intentional or accidental introductions (Cornell & Hawkins, 1993; Grabenweger et al., 2010; Roy et al., 2011; Hajek et al., 2016; Kenis et al, 2017). Given the high, sustained rate of insect invasion (Aukema et al., 2010; Garnas et al. 2012), new associations between invasive insects and native natural enemies as well as accidental introductions of natural enemies are becoming increasingly common (Cornell & Hawkins, 1993; Bush et al. 2016; Kenis et al., 2017). Some of these new parasitoid-host associations are detected rapidly after the report of non-native insects (Zappala et al., 2012; Matošević & Melika, 201; Zappala et al., 2013; Duan et al., 2015; Francati et al., 2015; Kos et al., 2015) while other associations are only reported decades after the introduction of host insects (Cornell & Hawkins, 1993; Prinsloo, 2005). In some cases, successful control of damaging invasives by adapted native natural enemies have been reported (Kenis et al., 2017). However, in most cases, parasitism rates remain low and result in poor or no control of the invasive hosts (Cornell & Hawkins, 1993).

Surveys on natural enemies of invasive insects in their new environment provide valuable information on the natural enemy complexes of invasive insects, including their local abundance and spatial and temporal dynamics. In the case of internally feeding herbivores, such surveys are commonly conducted by sampling infested plant parts coupled with rearing and identifying the emerging natural enemies (Francati et al., 2015; Miller-Pierce et al., 2015). Identifications of natural enemies are often done morphologically using taxonomic

keys and voucher specimens and sometimes using a combination of morphological and molecular tools (Panzavolta et al., 2013). Results from natural enemy surveys can also assist to inform management strategies of invasive insects, including the consideration of conservation or augmentative biological control, or possibly the need to introduce further classical biological control agents.

Parasitism of an introduced *Pissodes* sp. by the native pteromalid parasitoid, *Pycnetron pix* Prinsloo, in South Africa is an example of a new host-parasitoid association between a native parasitoid species and an introduced insect host (Prinsloo, 2005). *Pissodes* sp. was detected in South Africa in 1942 (Webb, 1974) on pines and gradually spread throughout the major *Pinus*-growing regions of the country on all commercial *Pinus* species (Gebeyehu & Wingfield, 2003). Though the weevil was originally identified as *P. nemorensis*, a recent study by Wondafrash et al. (2016) showed that it could be an unrecognized species of the *P. strobi* complex or a hybrid between *P. strobi* and *P. nemorensis*, and of North American origin. This weevil often attacks declining or recently dead trees of commercially grown *Pinus* species (Webb, 1974; Gebeyehu & Wingfield, 2003) and as such is often considered a pest of minor importance. However, it is a potential pest of *Pinus* trees in plantations of South Africa because of its emerging maturation feeding damage on healthy trees and likely involvement in the transmission of tree diseases such as the pitch canker caused by the pathogen *Fusarium circinatum* Nirenberg & O'Donnell (Coutinho et al., 2007). Similar to other members of the genus *Pissodes*, *Pissodes* sp. undergoes complete metamorphosis. All the immature stages develop under the bark of infested trees, the larvae feeding on the cambium and phloem and pupation taking place in chambers excavated in xylem and covered with wood fibres. After emergence, the adults move to host trees and undergo feeding and mating (Hopkins, 1911, O'Brien, 1989; Gebeyehu and Wingfield, 2003). Despite the long

history of *Pissodes* sp. in South Africa, it was only in 2005 that *P. pix* was reported parasitizing this weevil (Prinsloo, 2005). All reports of *P. pix* were from the Mpumalanga province. Though there is no detailed study of the biology of this parasitoid, late larvae or pupae are indicated as likely stages of attack (Gebeyehu & Wingfield, 2003; Prinsloo, 2005). There is no current information available on the distribution and frequency of this association or its role in top down weevil control.

In this study we investigated the frequency of the novel association between a native parasitoid, *P. pix*, and an invasive pest, *Pissodes* sp., in major *Pinus*-growing areas in South Africa. We also investigated the presence of possible other new parasitoid-host associations with *Pissodes* sp. We combined taxonomic characteristics and DNA sequence data to identify parasitoids. Since parasitoids were collected from log emergence containers and not directly from insects, sequence analysis of DNA from gut contents of a subset of parasitoids using *Pissodes* sp.-specific primers were used to confirm trophic relationships. This technique has been successfully used to confirm parasitoid-host relationship in some other insects (Rougerie et al., 2011; Gariepy et al., 2014; Foelker et al., 2015) and is suggested to be useful in studying parasitism in cryptic insects feeding under the bark and deep in the xylem tissue of trees.

Materials and methods

Sampling strategy

The search for natural enemies was conducted in three major *Pinus*-growing provinces of South Africa, namely the Western Cape, Mpumalanga and KwaZulu-Natal, between 2013 and 2015 (Fig. 1). Three *Pinus* plantations, with three to seven sites per plantation, were selected per province based on the existence of infestations in preceding years (Table 1).

Table 1. Sites and seasons in which surveys on natural enemies of *Pissodes* sp. were conducted.

Province	Plantation	Site	Sampling season	Latitude	Longitude
Western Cape	Braken Hill	S14	July 2013	S 34° 00.785'	E 23° 06.868'
		S30	July 2013, November 2015	S 34° 01.246'	E 23° 07.271'
		S23	July 2013	S 34° 01.621'	E 23° 06.749'
		S7a	November 2015	S 34° 00.660'	E 23° 08.640'
	Ruigtevlei	F16	July 2013	S 33° 59.644'	E 22° 53.261'
		G14	July 2013	S 33° 59.358'	E 22° 52.412'
		G20	July 2013	S 33° 59.542'	E 22° 53.006'
		H2	July 2013	S 33° 19.955'	E 22° 49.708'
		F18	November 2015	S 33° 59.580'	E 22° 53.520'
		F1	November 2015	S 33° 57.780'	E 22° 53.220'
		F4	November 2015	S 33° 58.320'	E 22° 52.920'
	Bergplaas	E12	July 2013	S 33° 55.656'	E 22° 40.748'
		A2	July 2013, November 2015	S 33° 55.849'	E 22° 46.094'
		A11	November 2015	S 33° 55.443'	E 22° 40.406'
		E7	November 2015	S 33° 55.180'	E 22° 44.291'
		A10	November 2015	S 33° 55.234'	E 22° 44.382'
		A6a	November 2015	S 33° 55.321'	E 22° 40.406'
Mpumalanga	Rooihoogte	N56	August 2013, April 2015	S 26° 38.031'	E 30° 20.046'
		N12A	August 2013	S 26° 00.004'	E 30° 17.031'
		V32	August 2013	S 26° 02.028'	E 30° 15.050'
		C3	April 2015	S 26° 44.005'	E 30° 18.041'
	Torburnlea	T13A	August 2013	S 26° 11.986'	E 30° 35.118'
		T7A	August 2013	S 26° 12.209'	E 30° 35.054'
		T17	August 2013	S 26° 10.174'	E 30° 32.156'
		T11	April 2015	S 25° 43.921'	E 30° 21.175'
		T12	April 2015	S 25° 42.491'	E 30° 21.613'
		T6a	April 2015	S 26° 12.215'	E 30° 35.054'
	Lothair	U13	August 2013	S 26° 22.715'	E 30° 37.172'
		U14	August 2013	S 26° 22.715'	E 30° 37.172'
		U19	August 2013	S 26° 22.867'	E 30° 37.214'
		W55	April 2015	S 26° 24.611'	E 30° 38.526'
		W17	April 2015	S 26° 25.521'	E 30° 37.628'
KwaZulu-Natal	Shafton	C5B	September 2013	S 29° 23.128'	E 30° 14.489'
		7B-1	September 2013	S 29° 23.363'	E 30° 14.304'
		7B-2	September 2013	S 29° 23.363'	E 30° 14.304'
	De Rust	A32	September 2013	S 29° 37.816'	E 31° 00.200'
		B49B	September 2013	S 29° 39.144'	E 31° 03.659'
		C17A	September 2013	S 29° 39.958'	E 31° 03.664'
	Underberg	M24A	September 2013	S 30° 23.235'	E 30° 09.000'
		D2	September 2013	S 30° 05.661'	E 30° 01.169'
		B16	September 2013, April 2015	S 30° 07.353'	E 30° 11.086'

Surveys were conducted in Monterey pine *Pinus radiata* D. Don plantations in the Western Cape and Mexican weeping pine *P. patula* Schiede ex Schltdl. & Cham in Mpumalanga and KwaZulu-Natal. A total of 41 sites were used in the study. Sampling was conducted in winter (August 2013) and autumn (April 2015) in Mpumalanga, in early spring (September 2013) and autumn (April 2015) in KwaZulu-Natal and in winter (July 2013) and summer (November 2015) in the Western Cape. Sampling dates were chosen to capture apparent variation in the phenology of weevil across the country, particularly across the summer versus winter rainfall areas (i.e., inland versus parts of the Eastern and Western Cape provinces respectively). No published studies exists on the weevil's phenology in South Africa. Two to five *Pissodes*-infested trees were felled per site depending on availability. Immediately after felling, two logs (each 0.8 m long) were cut and stored in separate insect emergence cages at 23 °C and monitored twice a week in an insectary located at the Experimental Farm of the University of Pretoria. Emerged adult parasitoids and *Pissodes* sp. were collected and counted. The parasitoids were preserved in 96 % ethanol at -20 °C until DNA extraction.

Morphological identification of parasitoids

The collected parasitoids were grouped into morphospecies based on their external characteristics as viewed under a Nikon SMZ745/745T stereomicroscope at a magnification of 40x. Body size, colour, the shape and surface structure of wings, ovipositors, antennae and legs were some of the important morphological characters used in grouping. Representatives of each of the morphospecies were sent to the National Insect Collection at the Agricultural Research Council (ARC), South Africa for morphological identification. Identifications were possible to family, genus and sometimes species by Gerhard Prinsloo of ARC. Specimens of an unknown braconid wasp were sent to Simon van Noorten (Iziko Museums, Cape Town,

South Africa) and identified as *Cratocnema* sp. Specimens which were identified as *Rhopalicus* sp. by taxonomists at the ARC were sent to Hannes Baur at the Department of Invertebrates, Natural History Museum, Bern, Switzerland for identification to species level.

Molecular identification of parasitoids

The barcoding region of the cytochrome c oxidase subunit I (COI) gene was sequenced from a total of 80 specimens from South Africa (25 *P. pix*, 15 *Cratocnema* sp. and 40 *Rhopalicus* sp.) and two identified *Rhopalicus tutela* (Walker) specimens from Europe. The two European *R. tutela* specimens were collected in July 2013 from dead logs of spruce, *Picea abies*, from Bălan in Romania (46.68º N, 25.71º E, 1238 m.a.s.l) by Hannes Baur (National History Museum, Bern, Switzerland) and were kept frozen. Analysis of sequences was done with the objectives of confirming the presence or absence of cryptic parasitoid species and comparing the *Rhopalicus* specimens from South Africa and Europe. The primers LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ (Folmer et al., 1994) and C1-N-2191: 5′-CCCGGTAAAATTAAAATATAAACTTC-3′ (Simon et al., 1994) were used for both PCR and sequencing PCR.

The following procedures were applied in DNA extraction, PCR and sequencing. Prior to DNA extraction, the preserved insect specimens were rinsed with sterilized distilled water and the head, thorax, legs and wings of each parasitoid were ground with Retsch MM 301 Mixer Mill (Retsch GmbH, Rheinische, Germany) using metal beads. Total genomic DNA was extracted and cleaned using a slightly modified phenol/chloroform DNA extraction protocol (Goodwin et al., 1992). The resulting DNA was suspended in 25 µl of ultrapure (SABAX) water and the concentration was determined by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). Genomic DNA quality was checked on a 1 % agarose gel and stored at -20 °C until further use. All

amplification reactions were performed following the procedures in Hurley (2010), except a 50 $°$C annealing temperature was used instead of 52 $°$C. PCR products were cleaned by adding 8 µL of ExoSAP-IT (USB Corporation, Cleveland, OH) and incubating the mixture at 37 $°$C and 80 $°$C; 15 min at each temperature. Sequencing reactions were performed according to standard protocols and products were cleaned following sodium acetate precipitation method using 3 M NaOAc (pH 4.6) and 96 and 70 % ethanol. The ABI PRISM^{TM} 3500 automatic DNA Analyzer (Applied BioSystems, USA) was used for sequencing.

Confirmation of parasitism

Molecular analysis of gut contents of parasitoids was conducted to confirm that the collected parasitoids had utilised *Pissodes* sp. as a host. This is an emerging DNA-based technique used to confirm parasitism via analysis of DNA of gut contents of parasitoids (Rougerie et al., 2011). *Pissodes* sp.-specific primers (Psp F1: 5′-TTTGTAGTTTGAGCCCACCA-3′ and Psp R: 5′-TTGTGAAA-GCATCGGGATAA-3′, Jerry-to-Pat region of COI gene) were developed using Primer3web version 4.0.0 (Koressaar & Remm, 2007; Untergasser et al., 2012). The structure and annealing temperatures of these primers were analysed using the same software. The abdominal region of three insects of each parasitoid species were rinsed with distilled water and DNA was extracted following a slightly modified phenol/chloroform DNA extraction protocol (Goodwin et al., 1992). We then amplified and sequenced a part of the COI gene from the gut contents of the parasitoids using the *Pissodes* sp.-specific primers. Similar PCR and sequencing PCR procedures were followed as in the above, except an annealing temperature of 56 $°$C was used.

Data analysis

The number of sites and trees where parasitoids were detected were calculated for each parasitoid species across all plantations in the surveyed provinces. Tree level parasitism rates

were calculated for each parasitoid species. The number of parasitoids was divided by the sum of the number of parasitoids and *Pissodes* sp., and multiplied by 100. The tree level parasitism rates were then averaged to get the site rates and the site rates were averaged to get the plantation rates across all the surveyed provinces. The male-to-female sex ratios of all the parasitoid species were also calculated.

The raw sequence data were edited using Biological Sequence Alignment Editor (BioEdit) 7.0.9 (Hall, 1999) and sequences of each of the three morphologically identified parasitoid species were aligned separately 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 Biological Sequence Alignment Editor (BioEdit) 7.0.9 (Hall, 1999) and the number and location of polymorphic sites were viewed in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2011). Similarity or divergence between sequences of the *Rhopalicus* species in South Africa and sequences of the two *R. tutela* specimens from Europe (the Romanian Carpathians) was analysed using MEGA with the objective of confirming the identity and origin of the South African *Rhopalicus* species. Gut content sequences of the three morphologically identified parasitoid species were compared with GenBank sequences of the Jerry-to-Pat region of the COI gene from *Pissodes* sp. from a previous study (Wondafrash et al., 2016).

Results

Parasitoid complex of Pissodes sp.

We identified three species of parasitoids of *Pissodes* sp. using morphological and DNA barcoding methods. Two of these parasitoids are native to South Africa while the third species was accidentally introduced. The native parasitoids were identified as *Pycnetron pix* (Hymenoptera: Pteromalidae) and a *Cratocnema* sp. (Hymenoptera: Braconidae). The

introduced parasitoid species was identified as *Rhopalicus tutela* (Walker) (Hymenoptera: Pteromalidae). This is the first report of *Cratocnema* sp. from *Pissodes* weevils and *R. tutela* from South Africa. Sequence diversity analysis for each parasitoid species did not indicate the presence of cryptic diversity within these species, cryptic diversity being defined as the presence of deeply diverged lineages within a single species (Espindola et al, 2016) or the resolution of morphologically indistinct species using molecular tools (Hebert 2004). Only a single haplotype of *R. tutela* was detected in South Africa and was identical to sequences of *R. tutela* specimens from the Romanian Carpathians (Europe).

Gut content sequences of the parasitoid species matched with GenBank sequences of the different haplotypes of *Pissodes* sp. from a previous study (Wondafrash et al., 2016). Gut content sequences of three *R. tutela*, three *P. pix* and one *Cratocnema* sp. specimens were identical with one haplotype of *Pissodes* sp. (accession number: KX581013) and gut content sequences of the other two *Cratocnema* sp. specimens were identical to another haplotype of *Pissodes* sp. (accession number: KX581019).
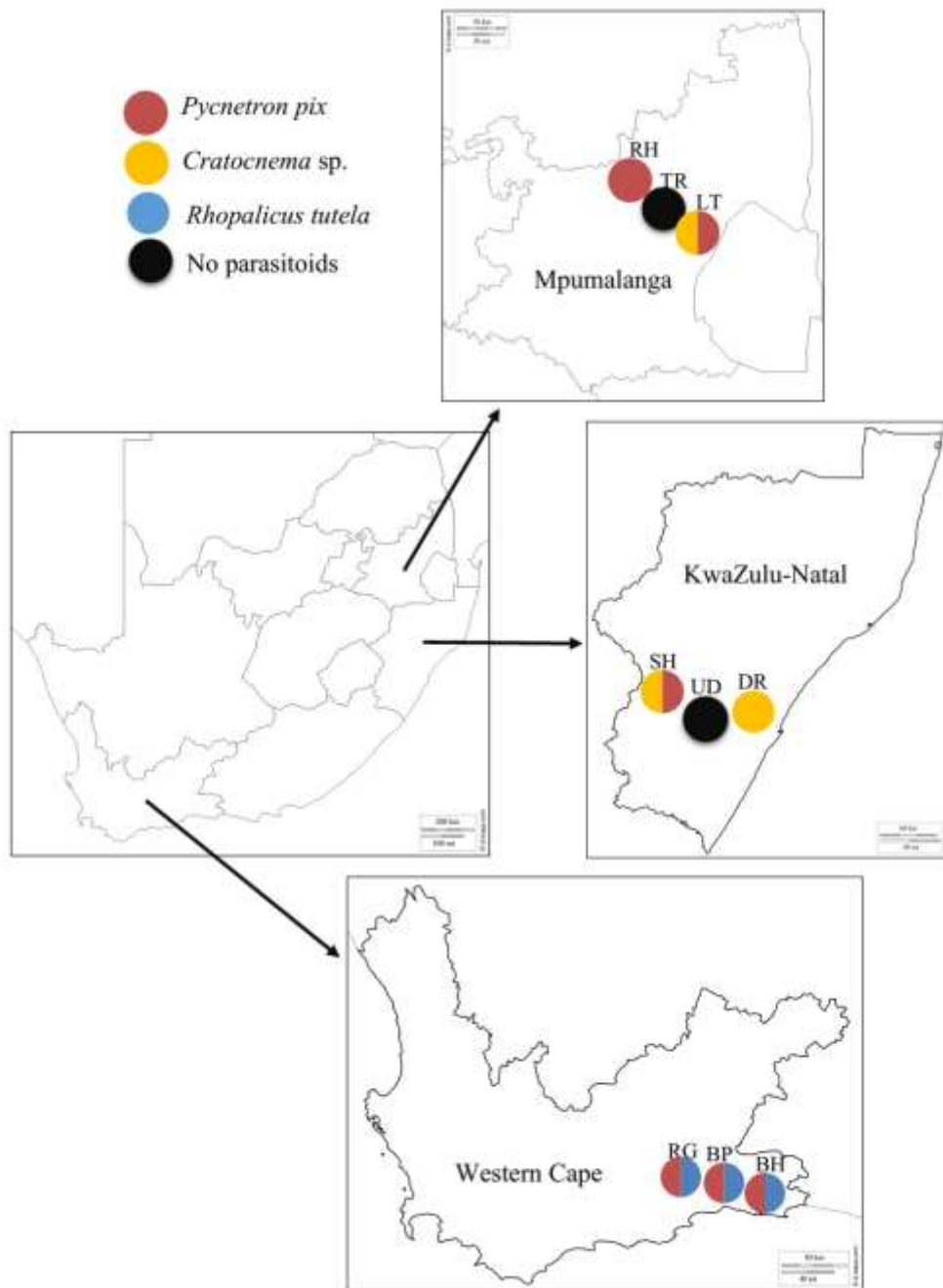
Figure 1. Distribution of parasitoids of *Pissodes* sp. in nine *Pinus* plantations across three major *Pinus*-growing provinces of South Africa. Plantations are represented by the following abbreviations: BP = Bergplaas, RG = Ruigtevlei, BH = Braken Hill, SH = Shafton, DR = De Rust, UD = Underberg, RH = Rooihoogte, LT = Lothair and TR = Torburnlea. *Pycnetron pix* was distributed in all the three provinces, while *Rhopalicus tutela* was restricted to the Western Cape. *Cratocnema* sp. was detected both in Mpumalanga and KwaZulu-Natal.

Distribution and frequency of parasitoids

The native parasitoid species, *P. pix* was distributed in all three provinces sampled in South Africa (Mpumalanga, Western Cape and KwaZulu-Natal) (Table 2; Fig. 1). Compared to the other two provinces, *P. pix* was more frequent in the Western Cape. In this province it was detected from all the three surveyed plantations and had higher among site and tree frequency (55.6 % and 50 % of sites and 21.2 % and 35.7 % of trees in the two seasons sampled) compared to Mpumalanga (11.1 % and 28.6 % of sites and 5.3 % and 9.1 % of trees in the two seasons sampled) and KwaZulu-Natal (22.2 % and 0 % of sites and 4.4 % and 0 % of trees in the two seasons sampled). The parasitism rate of *P. pix* was very low in all the provinces (1.2 % and 5.4 % in Western Cape, 0.6 % and 1.7 % in Mpumalanga and 0.1 % and 0 % in KwaZulu-Natal in the two seasons sampled, respectively). The relative higher parasitism rate (5.4 ± 4.5 %) in the Western Cape in summer 2015 could be the result of a high number of *P. pix* (139 individuals) in a single tree in Ruigtevlei plantation. Exclusion of this outlier tree from the calculation decreased the parasitism rate to 3.8 ± 3.0 % .

The distribution of *R. tutela* was restricted to plantations in the Western Cape, where it was found in all the three surveyed plantations (Table 2; Fig. 1). The frequency of *R. tutela* was 66.7 % and 30 % among sites and 39.4 % and 28.6 % of the sampled trees contained *R. tutela* individuals in winter and summer, respectively. Parasitism rates of 2.8 % and 1.3 % were recorded in winter and summer, respectively.

Cratocnema sp. was distributed in Mpumalanga and KwaZulu-Natal (Table 2; Fig. 1). Among site and among tree frequency of *Cratocnema* sp. in the two seasons sampled was 0 % and 14.2 % and 0 % and 3 % in Mpumalanga and 22.2 % and 0 % and 4.4 % and 0 % in KwaZulu-Natal, respectively. *Cratocnema* sp. had a parasitism rate of 0 % and 0.1 % in Mpumalanga and 1 % and 0 % in KwaZulu-Natal, in the two seasons sampled, respectively.

Table 2. Distribution and parasitism rate of parasitoids of the invasive *Pissodes* sp. in South Africa.

Province	Plantation	Search season	No. of sites with parasitoids			No. of trees with parasitoids			Tree-level parasitism rate (%)		
			P. pix	*R. tutela*	*Cratocnema* sp.	*P. pix*	*R. tutela*	*Cratocnema* sp.	*P. pix*	*R. tutela*	*Cratocnema* sp.
	Braken Hill		0/3	3/3	0/3	0/14	8/14	0/14	0.0 ± 0.0	4.1 ± 2.6	0.0 ± 0.0
	Ruigtovlei	July 2013	4/4	2/4	0/4	5/13	4/13	0/13	3.3 ± 1.0	3.0 ± 2.4	0.0 ± 0.0
	Bergplaas	(Winter)	1/2	1/2	0/2	2/6	1/6	0/6	0.2 ± 0.2	1.3 ± 1.3	0.0 ± 0.0
	Total		**5/9**	**6/9**	**0/9**	**7/33**	**13/33**	**0/33**	**1.2 ± 1.1**	**2.8 ± 0.8**	**0.0 ± 0.0**
WC	Braken Hill		1/2	1/2	0/2	1/2	2/2	0/2	0.0 ± 0.0	2.9 ± 0.1	0.0 ± 0.0
	Ruigtovlei	November 2015	3/3	1/3	0/3	4/6	1/6	0/6	14.4 ± 6.6	0.2 ± 0.2	0.0 ± 0.0
	Bergplaas	(Summer)	1/5	1/5	0/5	1/6	1/6	0/6	1.8 ± 1.8	0.7 ± 0.7	0.0 ± 0.0
	Total		**5/10**	**3/10**	**0/10**	**6/14**	**4/14**	**0/14**	**5.4 ± 4.5**	**1.3 ± 0.8**	**0.0 ± 0.0**
	Rooihoogte		1/3	0/3	0/3	2/13	0/13	0/13	1.9 ± 1.9	0.0 ± 0.0	0.0 ± 0.0
	Torburnlea	August 2013	0/3	0/3	0/3	0/14	0/14	0/14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Lothair	(Winter)	0/3	0/3	0/3	0/11	0/11	0/11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Total		**1/9**	**0/9**	**0/9**	**2/38**	**0/38**	**0/38**	**0.6 ± 0.6**	**0.0 ± 0.0**	**0.0 ± 0.0**
MPU	Rooihoogte		1/2	0/2	0/2	1/15	0/15	0/15	0.8 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
	Torburnlea	April 2015	0/3	0/3	0/3	0/15	0/15	0/15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Lothair	(Autumn)	1/2	0/2	1/2	2/3	0/3	1/3	4.3 ± 3.5	0.0 ± 0.0	0.3 ± 0.3
	Total		**2/7**	**0/7**	**1/7**	**3/33**	**0/33**	**1/33**	**1.7 ± 1.3**	**0.0 ± 0.0**	**0.1 ± 0.4**
	Shafton		2/3	0/3	1/3	2/15	0/15	1/15	0.2 ± 0.1	0.0 ± 0.0	0.8 ± 0.8
	De Rust	September 2013	0/3	0/3	1/3	0/15	0/15	1/15	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 2.2
	Underberg	(Spring)	0/3	0/3	0/3	0/15	0/15	0/15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Total		**2/9**	**0/9**	**2/9**	**2/45**	**0/45**	**2/45**	**0.1 ± 0.1**	**0.0 ± 0.0**	**1.0 ± 0.2**
KZN	Shafton		NP	NP	NP	NP	NP	NP	NP	NP	NP
	De Rust	April 2015	NP	NP	NP	NP	NP	NP	NP	NP	NP
	Underberg	(Autumn)	0/1	0/1	0/1	0/5	0/5	0/5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Total		**0/1**	**0/1**	**0/1**	**0/5**	**0/5**	**0/5**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**

[WC] Western Cape Province, [MPU] Mpumalanga Province, [KZN] KwaZulu-Natal Province, [NP] no *Pissodes* sp. infested trees

The male-to-female ratios were 0.47:1 for *P. pix*, 0.41:1 for *Cratocnema* sp. and 0.44:1 for *R. tutela* (Table 3).

Table 3. Male-to-female ratios of parasitoids of the *Pissodes* sp. in South Africa.

Parasitoid species	Total No.	No. male	No. female	Sex ratio
Pycnetron pix	162	52	110	0.47:1
Cratocnema sp.	24	7	17	0.41:1
Rhopalicus tutela	75	23	52	0.44:1

Discussion

We found that *P. pix*, a parasitoid native to South Africa, is widely distributed throughout the major *Pinus*-growing provinces of South Africa. In addition, we detected the presence of two other parasitoid species of *Pissodes* sp. We also confirmed feeding relationships for log-emerged insects by amplifying DNA from the gut contents of the parasitoids using *Pissodes* sp.-specific primers. *Cratocnema* sp. appears to be native to South Africa (http://www.waspweb.org/Ichneumonoidea/Braconidae/Braconinae/Cratocnema/index.htm, accessed on 08 March 2017), whereas *R. tutela* is recorded from the Holarctic region (Hougardy & Grégoire, 2004). Interestingly, analysis of DNA sequence data revealed that the *R. tutela* in South Africa is of European origin, and thus represents a case where the introduced invasive pest species and the introduced natural enemy are from different continents.

The recovery of *P. pix* from all regions sampled in South Africa stands in contrast with Prinsloo (2005) who noted that *P. pix* was not recorded from the Western Cape despite monitoring over many years. Whether the current result represents a more recent association in this region or whether prior monitoring was insufficient is not known. The wide distribution of *P. pix* detected in the current study is not unexpected as the wasp is native to

South Africa and known to parasitize other indigenous insects, such as the cycad weevil *Antliarhinus peglerae* and members of the genus *Porthetes* (Gebeyehu & Wingfield, 2003; Prinsloo, 2005). Only three species of *Pycnetron* have been described to date, including the South African *P. pix*. The two other species are *P. curculionidis* in Philippines and the Indo-Pacific region and *P. longicaudata* in Madagascar (Gahan, 1925; Risbec, 1952; Bouček, 1988; Prinsloo, 2005). *Pycnetron pix* is the only species of the genus *Pycnetron* that is reported parasitizing a member of the genus *Pissodes*, which is expected as the native ranges of these species do not overlap. According to Bouček (1988) and Prinsloo (2005), the distribution of the genus *Pycnetron* is limited to Old World tropics such as Sub-Saharan Africa, Madagascar, South Africa, southern and eastern Asia, the Philippines and Australia. Literature on the biology of *P. pix* is lacking.

This is the first report of a *Cratocnema* species parasitizing a member of the genus *Pissodes.* The genus *Cratocnema* is distributed in the Afrotropical region, more specifically in Cameroon, Democratic Republic of Congo, Equatorial Guinea, Madagascar, Mozambique, South Africa, Tanzania and Togo (http://www.waspweb.org/Ichneumonoidea/Braconidae/Braconinae/Cratocnema/index.htm, accessed on 08 March 2017), with fifteen recorded species. Only *C. postfurcale* has been previously recorded from South Africa. Literature on the biology and host species of the genus *Cratocnema* is lacking. In our study, *Cratocnema* sp. was reared from *Pissodes* sp. in *Pinus* plantations in KwaZulu-Natal and Mpumalanga, but not in the Western Cape. However, considering the very low parasitism rates observed in the other provinces, it is possible that this was merely an effect of sampling. Future monitoring will add to our understanding of the spatial dynamics of *Cratocnema* sp. and its association with *Pissodes* sp.

Low rates of parasitism were recorded from both of the native parasitoid species, *P. pix* and *Cratocnema* sp. Similar low parasitism rates have been recorded from many native parasitoids attacking introduced insects (Zappala et al., 2012; Grabenweger et al., 2010; Matošević & Melika, 2013; Francati et al., 2015). The lower parasitism rate and species richness of native natural enemies on exotic hosts can be explained by the longer time required to adapt to novel hosts. According to Cornell and Hawkins (1993), parasitism by native parasitoids only weakly increases with time and full acquisition of native parasitoids by some introduced hosts may require 100 to 10, 000 yr. Taxonomic distinctiveness of the new hosts from the indigenous community and behavioural, as well as biological and phenological adjustments required of the native natural enemies to successfully exploit new hosts require a longer period of time (Cornell & Hawkins, 1993; Grabenweger et al., 2010; Zappala et al., 2013).

This was the first report of *R. tutela* in South Africa and most likely also in the Afrotropical region. *Rhopalicus tutela* is a Holarctic generalist ecto-parasitoid of bark- and wood-feeding insects, with wide distribution in Europe, Asia and North America (Krüger & Mills, 1990; Kenis et al., 2004; Noyes; 2017). It has a wide host range within beetle family Curculionidae and has been most frequently reared from conifer bark beetles (Scolytinae) (Kenis & Mills, 1994; Kenis et al., 2004; Noyes, 2017). In the bark beetle, *Ips typographus*, *R. tutela* was often observed ovipositing on third instar and occasionally on pre-pupae and young pupae (Krüger & Mills, 1990). Co-introduction of *R. tutela* and *Pissodes* sp. in to South Africa seems unlikely as their geographic origins differ according to current knowledge (Wondafrash et al., 2016). While the pathway of *R. tutela* into South Africa is not known, co-introduction along with one or more of the exotic European bark beetles that occur in South African *Pinus* plantations, namely *Hylastes angustatus*, *Hylurgus ligniperda*, or

Orthotomicus erosus, is the most likely possibility, though the wasp is not been reported in these systems. In its native range in Europe and North America it is a known parasitoid of *Pissodes* weevils (*P. castaneus*, *P. pini*, *P. piniphilus* and *P. harcyniae* and *P. strobi*) on both *Pinus* and spruce (Kenis & Mills, 1994; Kenis et al., 2004; Noyes; 2017). Its congener, *R. guttatus*, of European origin, is recorded as a parasitoid of *P. castaneus*, *P. pini*, *P. validirostris* and *P. piceae* (Kenis & Mills, 1994; Kenis et al., 2004).

Rhopalicus tutela was detected only in the Western Cape province of South Africa, and at very low levels. The limited distribution is unlikely to be due to narrow environmental constraints given the wide range distribution of this species in the Holarctic region (Krüger & Mills, 1990) and its generalist behaviour (Kenis & Mills, 1994; Kenis et al., 2004). However, this could be a recent introduction, or alternatively spread could have been limited by climatic and other environmental factors of the specific genotype present.

The current study revealed novel natural enemies of the introduced *Pissodes* sp. in its invasive range in South Africa, where the weevil was parasitized by *P. pix* and *Cratocnema* sp., both native parasitoids to South Africa, and *R. tutela*, an accidentally introduced parasitoid. The *Pissodes* sp. and the accidentally introduced parasitoid (*R. tutela*) have originated from different continents, the weevil being from North America (Wondafrash et al., 2016) and the parasitoid from Europe. Interestingly all the other organisms in this association are also novel, including with the tree host and native parasitoids. Despite the accumulation of both native and exotic parasitoid species on the invasive *Pissodes* sp. through time, the collective parasitism rate remained extremely low compared to native populations of *Pissodes* (Kenis & Mills, 1994). For example, parasitism in *P. castaneus* is an important mortality factor, ranging from 25 % to 75 % in France and the United Kingdom.

The extremely low parasitism rate on *Pissodes* sp. is unlikely to have any significant effect on the population, thus leaving the weevil still in a relatively 'enemy-free' space. *Pissodes* sp. is not currently considered a major pest in South Africa, but if this status should change, for example if the weevil is confirmed to be a major vector of the pitch canker pathogen in South Africa (Coutinho et al., 2007), then management would be required to reduce populations. Once the true identity of this weevil is confirmed, importation of exotic parasitoids associated with *Pissodes* species from North America could be considered for its future management. This could include the North American parasitoids *Dolichomitus terebrans nubilipennis* (Ichneumonidae), *Bracon pini* (Braconidae) and *Eurytoma pissodis* (Eurytomidae) (Mills & Fischer, 1986).

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