The distribution and diversity of *Leptocybe invasa* (Hymenoptera: Eulophidae) and its gall associates in South Africa

Caitlin R Gevers[1], Bernard Slippers[2], Ilaria Germishuizen[3], Gudrun Dittrich-Schröder[1] and Brett P Hurley[1]*

[1] *Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa*
[2] *Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa*
[3] *Institute for Commercial Forestry Research, Scottsville, South Africa*
* *Corresponding author: brett.hurley@up.ac.za*

***Leptocybe invasa* is an invasive gall wasp and pest of *Eucalyptus* trees, which has become widely distributed in Africa, Asia, Europe and the Americas. Several other wasp species have been found to co-occur in *L. invasa*-induced galls. In South Africa, this includes an introduced biological control agent, *Selitrichodes neseri*; two known, accidentally introduced parasitoids, *Megastigmus zebrinus* and *Quadrastichus mendeli*; and *M. pretorianensis*, whose role in the gall is uncertain. In addition to the gall associates, a second lineage of *L. invasa* or cryptic species was discovered in South Africa in 2015. To determine the distribution and prevalence of these species across South Africa, a national monitoring scheme was initiated. Galled *Eucalyptus* material was collected at infested sites and emerging adults were collected and identified. Morphology, DNA barcoding and polymerase chain reaction (PCR) Restriction Fragment Length Polymorphism analyses were used to differentiate between the species and lineages. Results from the first two sampling periods indicated that *L. invasa* lineage A has spread throughout South Africa while lineage B had a more limited distribution in the country. Subsequent samples recorded the further spread of *L. invasa* Lineage B, which now occurs in all provinces sampled. The *Leptocybe* lineages were found to co-occur on individual trees, increasing the potential for admixture. From the *Eucalyptus* genotypes sampled, there was no indication of differences in host association between the *Leptocybe* lineages. *Selitrichodes neseri, M. zebrinus* and *M. pretorianensis* were present throughout South Africa and emerged from trees that contained both *Leptocybe* lineages, but their frequency varied with site. This study will inform future distribution of parasitoids as well as monitoring of potential changes in plant host resistance, admixture and parasitoid resistance in future.**

Keywords: gall wasp, parasitoid, *Leptocybe*, *Selitrichodes*, *Megastigmus*, *Quadrastichus*

Introduction

Eucalypts are native to Australia but have become one of the most widely planted trees in the world (Rejmanek and Richardson 2011). Eucalypts are grown in intensively managed plantations (Wingfield et al. 2008) and the value of these trees lies in the production of timber, pulp, fuelwood and other products. Initially, eucalypts planted outside their native range remained relatively free of insect pests (Wingfield et al. 2008). This has gradually changed, and numerous insect pests have become serious threats to the sustainability of non-native eucalypt plantations (Hurley et al. 2016). These insect pests include non-native pests that have been accidentally introduced and native pests that have adapted to this new host (Hurley et al. 2016).

Leptocybe invasa Fisher & La Salle (Hymenoptera: Eulophidae) (Figure 1 A–B) (Mendel et al. 2004), commonly known as the blue gum chalcid, is one of the most economically important introduced pests of eucalypts (Csóka et al. 2017). This wasp is native to Australia and was first detected outside its native range in 2000 (Mendel et al. 2004). It is present in over 40 countries in Asia, Europe, Africa and the Americas (Zheng et al. 2014; Nugnes et al. 2015). Oviposition by *L. invasa* initiates abnormal plant growth resulting in gall formation.The resulting gall is lined with nutritive tissue which provides a food source for the developing larva. The galls are multi-chambered and each chamber contains a single developing wasp. In high densities this malformation can lead to stunted growth and in severe cases to the death of the plant (Mendel et al. 2004).

Nugnes et al. (2015) used molecular markers to show that the global distribution of *L. invasa* was represented by two different lineages (Lineage A and Lineage B) that potentially represent cryptic species. Lineage A was originally found in Israel (2000) but has since been reported from Europe, the Americas, eastern and Southern Africa and parts of Asia (Dittrich-Schröder et al. 2018). The distribution of Lineage B is more limited, namely in Asia, Ghana and South Africa (Dittrich-Schröder et al. 2018). Given the high rate of spread, the global distribution of the two lineages is likely to change over time. Although *L. invasa* favours parthenogenetic reproduction (thelytoky: genetically identical female offspring), males have been observed in the invasive range: China (Chen et al. 2009); Turkey (Nugnes et al. 2015); India (Akhtar et al. 2012;

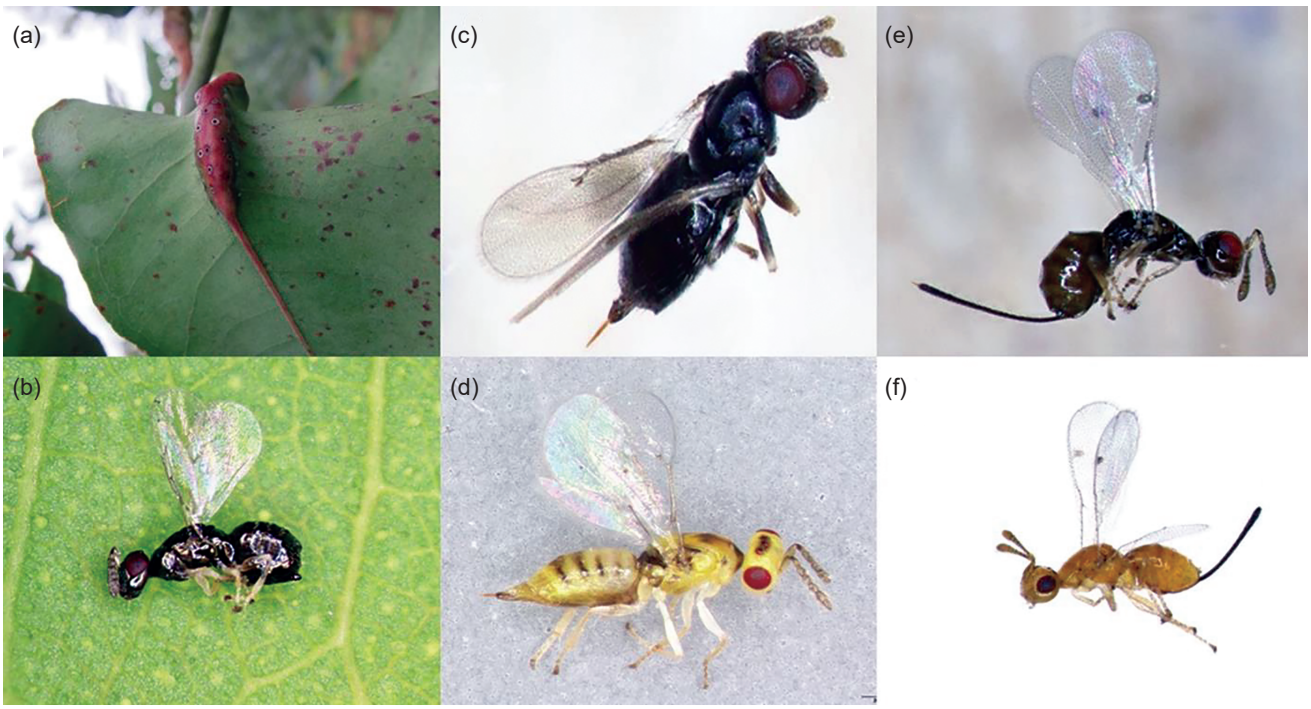

Figure 1: *Leptocybe invasa* and its gall community in South Africa. **A:** *L. invasa* gall on the midrib of a leaf with several adult emergence holes; **B:** *L.invasa* female; **C:** *S. neseri* female; **D:** *Q. mendeli* female; **E:** *M. pretorianensis* female; **F:** *M. zebrinus* female

Kumari et al. 2010); Taiwan (Tung & La Salle 2010); Thailand (Sangtongpraow et al. 2011); and South Africa (Dittrich-Schröder et al. 2018). The presence of both sexes may indicate the potential of sexual reproduction. Co-occurrence of these two lineages could potentially lead to genetic admixture, as reported by Dittrich-Schröder et al. (2018). Such admixture might increase the fitness and adaptability of this invader (Keller & Taylor 2010; Verhoeven et al. 2011; Dittrich-Schröder et al. 2018).

Biological control is one of the main strategies used to manage *L. invasa* in South Africa. The biological control agent, *Selitrichodes neseri* Kelly & La Salle (Kelly et al. 2012) (Hymenoptera: Eulophidae) (Figure 1C), a biparental larval ectoparasitoid, was released in 2012 (Dittrich-Schröder et al. 2014) and parasitises both *Leptocybe* lineages (Gevers et al. 2021). Another parasitoid of *L. invasa*, *Quadrastichus mendeli* (Hymenoptera: Eulophidae) Kim & La Salle (Kim et al. 2008), was reported in South Africa in 2016 despite no intentional release (Bush et al. 2017). Although *Q. mendeli* parasitises Lineage A (Gevers et al. 2021), its capacity to parasitise Lineage B remains to be confirmed. Two *Megastigmus* species (Hymenoptera: Megastigmidae), *M. zebrinus* Grissel (Grissel 2006) (confirmed parasitoid) (Klein et al. 2015) and *M. pretorianensis* Doğanlar (Doğanlar 2015) (unknown role), are associated with *L. invasa* galls in South Africa (Gevers et al. 2021). *Megastigmus zebrinus* parasitises both *Leptocybe* lineages as well as *S. neseri*. *Megastigmus pretorianensis* has frequently been found in a gall cavity containing both *Leptocybe* lineages and *S. neseri.* This indicates that *M. pretorianensis* may be an inquiline, but does not exclude the potential of it being a parasitoid.

The objectives of this study were to determine the distribution in South Africa of; (i) both lineage A and B of *L. invasa*; (ii) the *Leptocybe*-associated parasitoids; and (iii) to investigate the potential of the *Leptocybe* lineages in facilitating the distribution of the parasitoids. Distribution in a region, site (compartment/plantation) and tree level were investigated, and host plant species were also considered. We determined the presence and prevalence of the wasp species from field-collected galled material using morphological and molecular tools.

Materials and methods

Sampling

Sampling was conducted between November 2016 and November 2019, with two sampling periods conducted per year. There were a total of seven sampling periods. Sampling period 1 in November 2016 included four regions: Limpopo, Mpumalanga South and two areas in KwaZulu-Natal (KZN Midlands and Zululand). The Eastern Cape and Mpumalanga North regions were included from sampling period 2, May 2017. The plant material (galled branches) was collected from sites that contained trees infested with *L. invasa* galls. These compartments were represented either by trial plots or single species sites. Where possible, the same sites were selected for each of the subsequent sampling periods. However, alternative sites were selected when the trees became too tall to sample.

Trial plot sites were large compartments with blocks of different eucalypt hybrid clones as well as pure eucalypt species (*E. grandis* in this study). The blocks representing the different genotypes and *E. grandis* contained either 36 (6 × 6)

or 49 (7 × 7) trees. In each trial plot we sampled from three different species or hybrid clones. Where possible, the same clones were sampled across different trial plots to allow a comparison between sites and regions. Five random trees within the inner block of each genotype or pure species were selected. Six branches of approximately 30 cm, showing *L. invasa* galls, were collected from each tree.

In some areas, such as Limpopo, trial plots were not available and thus material was collected from commercial compartments planted with *E. grandis* or one of its hybrid clones. The same experimental procedures were followed as for the trial plots, except that five trees were randomly sampled along a zigzag transect. The transect consisted of 50 trees, with the direction changing by 45° every 5 trees. The transect started two to three trees into the compartment to prevent edge effects. Pure species and trial plots located in commercial compartments were at least 1 km apart.

Galled material (Figure 1A) collected from the trees was placed in ADDIS 9L containers with a piece of tissue paper lining the bottom, to absorb excess moisture. Three days a week (Monday, Wednesday and Friday) the emerged adult wasps were collected using a pooter and stored in 96% non-denatured ethanol in a fridge. In addition, the tissue paper was replaced and the quality of the galled leaf material was checked. Galled leaf material was kept for three weeks, unless it became mouldy, in which case it was discarded.

Identification of adults

The wasps that emerged from the galled material were identified morphologically and sexed using a Nikon SMZ1500 microscope (×20 magnification). *Leptocybe invasa* (Figure 1B) females were identified using the taxonomic description in Mendel et al. (2004) and the morphological table from Zheng et al. (2014). *Selitrichodes neseri* (Figure 1C) and *Q. mendeli* (Figure 1D) were identified using the taxonomic description found in Kelly et al. (2012) and Kim et al (2008) respectively. Grissell (2006) and Doganlar (2015) taxonomic descriptions were used to identify *M. zebrinus* (Figure 1F) and *M. pretorianensis* (Figure 1E) respectively.

Differentiation of* Leptocybe *lineages through molecular analysis

DNA from five *L. invasa* specimens per site was used to confirm the presence of the two lineages at that site. If only one lineage was confirmed from the five specimens used, bulk extractions using 10 wasps were used, with a maximum of 5 reactions. If there was still only one lineage detected the other lineage was considered not confirmed at that site. If both Lineage A and Lineage B were confirmed at a site, DNA from 10 to 15 further specimens were separately extracted to obtain an estimate of the proportion of the two lineages at that site. Where possible, the 10 to 15 specimens were selected from the same tree to test if the 2 lineages co-occurred on a single tree. Both positive and negative controls were used in every test. Positive controls included confirmed DNA for Lineage A and Lineage B.

DNA was extracted from the entire specimen using the *Prep*GEM™ insect DNA extraction kit (ZyGem kit). Each reaction was prepared with 20 µl polymerase chain reaction (PCR) grade water, 4 µl 10× Buffer (Black), 1 µl PrepGEM™. This reaction was then incubated at 75 °C for 15 minutes and 95 °C for 5 minutes using the Bio-Rad iCycler. The same kit was used for bulk/pooled DNA extraction with modifications to the amounts of reagent used as follows: to 40 µl PCR grade water, 6 µl of 10× Buffer and 1.5 µl PrepGEM.

PCR was performed using the *cytochrome* b primers CP1 (5′-GAT GAT GAT GAA ATT TTG GAT C -3') (Harry et al. 1998) and CB2 (5'-ATT ACA CCT CCT AAT TTA TTA GGA AT -3') (Jermiin and Crozier 1994) resulting in amplification of a 716 bp fragment of the *cytochrome* b (*cyt* b) region of mitochondrial DNA. Each PCR reaction mix was prepared using 10.8 µl 10× PCR grade water, 2.5 µl PCR Buffer, 2.5 µl dNTP's, 3 µl $MgCl_2$, 0.2 µl FastStart TAQ, 1 µl CP1 and CB2 primers (10uM of each), 4 µl of extracted DNA. The PCR amplifications were performed in a Bio-Rad iCycler with PCR conditions as follows: 95 °C for 7 minutes, 35 cycles of (95 °C for 1 minute, 48 °C for 1 minute, 72 °C for 1 minute), with a final extension step of 10 minutes at 72 °C. The QIAquick® PCR purification kit was used to purify the samples following the manufacturers protocol.

The restriction enzyme *AseI* (New England BioLabs) cleaves DNA between thymine nucleotides at sites containing the motif ATTA. *In silico* analysis indicated that this restriction enzyme could be used to differentiate between the gall-former and the various associated hymenopterans. The reaction was prepared with 18 µl PCR grade water, 0.5 µl *AseI*, 2.5 µl 10× NE Buffer and 4 µl purified PCR product generated using the primers CP1 and CB2 (as indicated above). The reaction was then placed in a Bio-Rad iCycler under the following conditions: 37 °C for 2.5 hours, 65 °C for 20 minutes, 4°C to hold. The product was run on a 2% agarose gel. Restriction sites using *AseI* were unique for each lineage and therefore the pattern of bands on the gel could be used to identify both lineages of *Leptocybe*.

Statistical analysis

The total number of wasps that emerged from every sampled tree was recorded for each species (*L. invasa*, *S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis*). These data were used to calculate the proportion of the total emerged wasps per tree, for each species. The parasitism levels for each tree were calculated using the emergence data of the parasitoid species (*S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis*).

$$\text{Parasitism level} = \frac{\text{Total number of parasitoid species emerged}}{\text{Total wasps emerged}}$$

For this study we assumed *M. pretorianensis* was a parasitoid. Although its role has not been confirmed and it may be an inquiline (Gevers et al. 2021), most *Megastigmus* species associated with *Leptocybe invasa* around the world have been described as parasitoids (Le et al. 2018).

The emergence rates from some of the trees were very low because of the condition (too few galls, material became mouldy) of the collected material. Galled material that produced fewer than ten wasps was not included in the data analysis.

The Kruskal-Wallis test was used to compare the mean parasitism levels per tree between the different regions, using the combined data from all seven sampling periods and a separate analysis for *S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis*. This test was selected because the Shapiro Wilk test of normality indicated that the data were not

normally distributed (p-value < 0.05) and Levene's test indicated that the assumption of homogeneity of variance was not met (p-value < 0.05). Pairwise comparisons using the Dunn-Bonferroni were conducted for dependent variables in which the Kruskal-Wallis test was significant. The Friedman test was used to compare the mean parasitism levels per tree between *S. neseri*, *M. zebrinus* and *M. pretorianensis* within the different sampling periods, using a separate analysis for each region. All statistical analyses were conducted using SPSS version 17 (IMB Corp 2017). The Wilcoxon signed-rank test was conducted as the post hoc test.

Results

Distribution of Leptocybe *lineages*

We collected a total of 54 222 *L. invasa* specimens between 2016 and 2019. *Leptocybe invasa* was detected in all six sampled regions of South Africa. Lineage A was found in all six of the sampled regions for the duration of the study. Lineage B was found in four regions between 2016 and 2017, namely Zululand, KwaZulu Natal Midlands, Mpumalanga South and Eastern Cape (Figure 2). It increased its distribution into all six regions by 2019 (Figure 2). The two lineages were found to co-occur on a single tree in a few of the sampled sites. In Zululand, both lineages were present at most sites, with only a single site where Lineage B was not found.

Distribution of L. invasa *parasitoids and inquilines*

Data from all seven sampling periods was used to determine the distribution of the *L. invasa* parasitoids and inquilines. A total of 88 875 wasps were collected across a total of 180 sites and from 947 trees. Of the wasps that emerged, 61.52% were *L. invasa*, 12.78% *S. neseri*, 2.32% *Q. mendeli*, 4.49% *M. zebrinus* and 13.96% *M. pretorianensis* (Table 1).

Selitrichodes neseri, *M. zebrinus* and *M. pretorianensis* were found in each of the sampled regions (Table 2). The region had a significant effect on parasitism levels of each parasitoid: *S. neseri* (Kruskal-Wallis (KW): $H(5) = 9.65$, $p = 0.086$), *Q. mendeli* (KW: $H(5) = 73.880$, $p = 0.015$), *M. zebrinus* (KW: $H(5) = 146.04$, $p = 0.001$) and *M. pretorianensis* (KW: $H(5) = 86.281$, $p = 0.001$). For *S. neseri*, total parasitism and mean parasitism per tree was highest in Limpopo, where mean parasitism was significantly higher than in the KZN Midlands, Eastern Cape and the Mpumalanga South (Table 1, Figure 3). *Quadrastichus mendeli* was found in most regions except the KZN Midlands and Eastern Cape from the fourth sampling period (2017) onwards (Figure 4). For *Q. mendeli*, total parasitism and mean parasitism per tree were highest in Mpumalanga North, where mean parasitism was significantly higher than in Zululand ($H(3) = 62.82$, $p = 0.003$), Limpopo ($H(3) = -49.89$, $p = 0.017$) and Mpumalanga South ($H(3) = -112.14$, $p = 0.001$) (Table 1, Figure 3). Total parasitism and mean parasitism per tree for *M. zebrinus* was also highest in Mpumalanga North, where mean parasitism was significantly higher than in the Eastern Cape ($H(3) = 52.37$, $p = 0.001$ and KZN Midlands ($H(3) = 38.70$, $p = 0.002$ (Table 1, Figure 3). For *M. pretorianensis*, total parasitism and mean parasitism was highest in Zululand, where it was significantly higher than in Eastern Cape ($H(3) = -2020$, $p = 0.001$), KZN Midlands ($H(3) = -116$, $p = 0.002$), Mpumalanga North ($H(3) = -60.92$, $p = 0.03$ and Mpumalanga South ($H(3) = -146.4$, $p = 0.001$) (Table 1, Figure 3).

The parasitoid species had a significant effect on parasitism levels between the sampling periods (1 to 7), in each region (p-value < 0.05). *Selitrichodes neseri* and *M. pretorianensis* were generally the dominant parasitoids (Table 1, Figure 4). *Selitrichodes neseri* emerged from over 50% of the trees sampled in each region. *Megastigmus pretorianensis* emerged from about 48% of the trees sampled in each region. Mean parasitism per tree for *S. neseri* was significantly higher than the other three parasitoids between sampling periods 3 (2017) and 7 (2019) in the Eastern Cape (Figure 4). *Megastigmus pretorianensis* mean parasitism per tree was significantly higher than the other three parasitoids in Mpumalanga South (sampling period 1; 2016) and the KZN Midlands (sampling period 1; 2016) (Figure 4). Often, parasitism levels between *S. neseri* and *M. pretorianensis* were not significant. Mean parasitism per tree for *M. zebrinus* was significantly lower than *S. neseri* and *M. pretorianensis* in the KZN Midlands and Eastern Cape throughout the duration of the study, and in Mpumalanga South after sampling period 5 (2018 to 2019). *Quadrastichus mendeli* was first detected in the survey in Zululand and Limpopo in sampling period 4 (2017), but its parasitism level was significantly higher than the other three parasitoids in Zululand by the next year (2018) and in Mpumalanga North by the following year (2019) (Figure 4).

All four wasps, namely *S. neseri*, *Q. mendeli, M. zebrinus* and *M. pretorianensis,* emerged from regions where both *Leptocybe* lineages were present and emerged from trees that contained both *Leptocybe* lineages.

Discussion

We investigated the distribution of two genetically distinct *Leptocybe* lineages (Lineage A and Lineage B) and their associated gall community in South Africa. Both these *Leptocybe* lineages as well as the four other gall associates we identified (three parasitoids and one wasp of unknown role) were present throughout the country. They all co-occurred with each other on the same trees and in the same regions at times but varied significantly in their frequency over time and site.

Leptocybe invasa lineage A and B have spread successfully into all six sampled regions in South Africa. As expected, Lineage A was present in more regions than Lineage B when the study commenced in 2016. Lineage A was first reported in South Africa in 2007 (Neser et al. 2007) while Lineage B was only reported in 2015 (Dittrich-Schröder et al. 2018), and thus had not had the same amount of time to become established and spread. However, since 2018, Lineage B was found in all sampled regions, indicating its rapid spread. It is possible that Lineage B was present in all the sampled regions before 2018 but was not detected in this study due to the limitation in the number of sites and wasps sampled. The relatively short period in which the *Leptocybe* lineages, especially Lineage B, have spread to the main forestry areas of the country is of concern.

The thelytokous reproductive mechanism of *L. invasa* is thought to have contributed to its global rapid spread. The parthenogenetic (genetically identical female offspring) reproductive ability means that Allee effects, such as mate finding, do not hinder successful establishment (Nugnes et al. 2015; Dittrich-Schröder et al. 2018). Recently, males have been found in the invasive range of *L. invasa* in (China, Turkey,

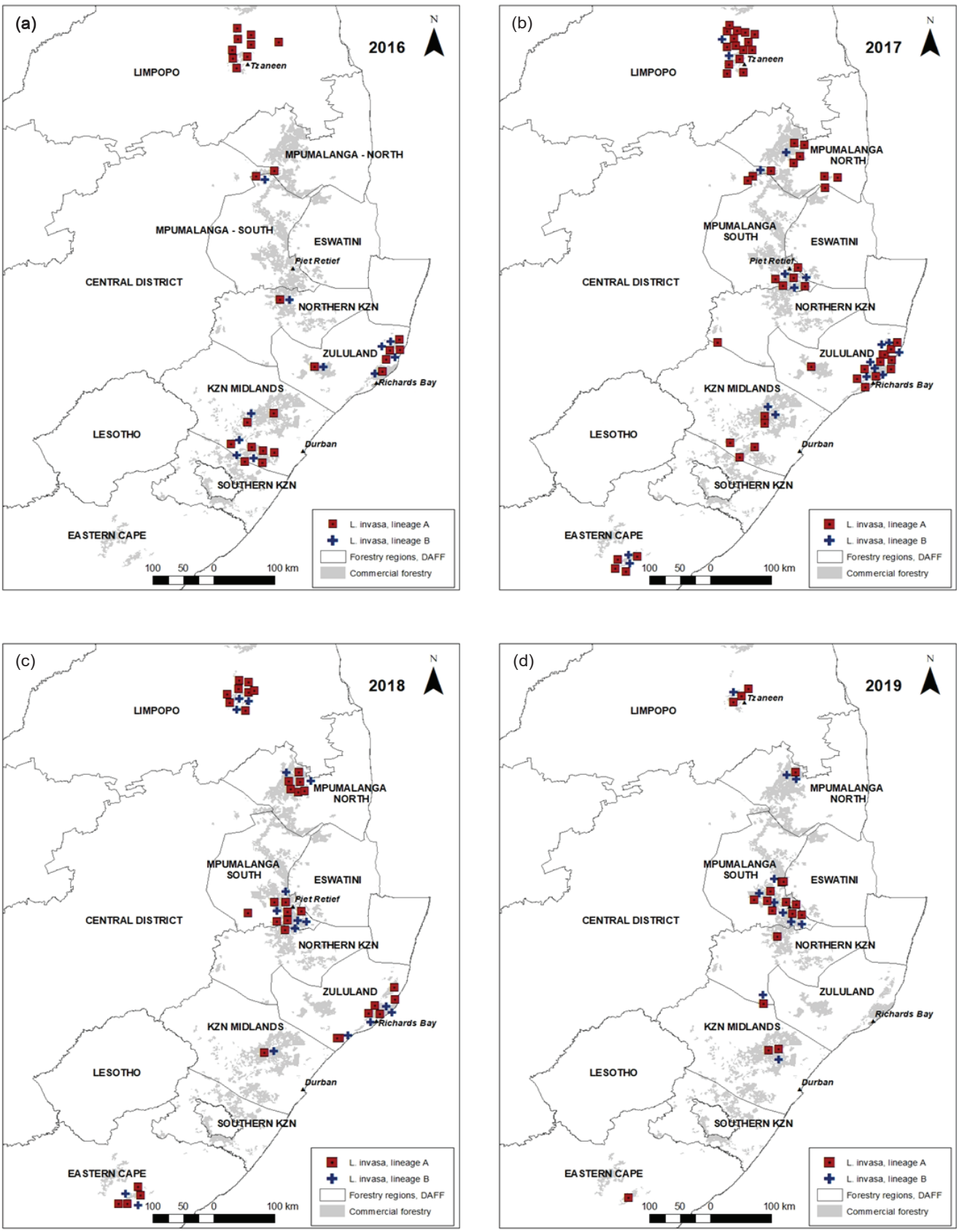

Figure 2: The occurrence of *L. invasa* Lineage A and B in the plantation forests located in the summer rainfall region of South Africa in (a) 2016, (b) 2017, (c) 2018, and (d) 2019

Table 1: Occurrence and prevalence of the different wasp species

			Percentage of each wasp species				
	Number of sites	Number of wasps	Li	Sn	Qu	Mz	Mp
Eastern Cape	11	9 110	84.48	10.17	0.00	0.54	4.81
Limpopo	43	12 510	42.36	20.01	4.55	6.26	26.82
KZN Midlands	28	19 482	83.86	7.05	0.01	0.14	8.94
Zululand	41	19 852	56.19	14.09	3.08	6.15	20.49
Mpumalanga South	41	18 895	68.10	14.27	1.97	2.51	13.15
Mpumalanga North	16	9 026	45.82	18.10	6.38	17.70	12.00
Total	180	88 875	63.47	13.95	2.67	5.55	14.37

Li = *L. invasa*, Sn = *S. neseri*, Qu = *Q. mendeli*, Mz= *M. zebrinus*, Mp= *M. pretorianensis*

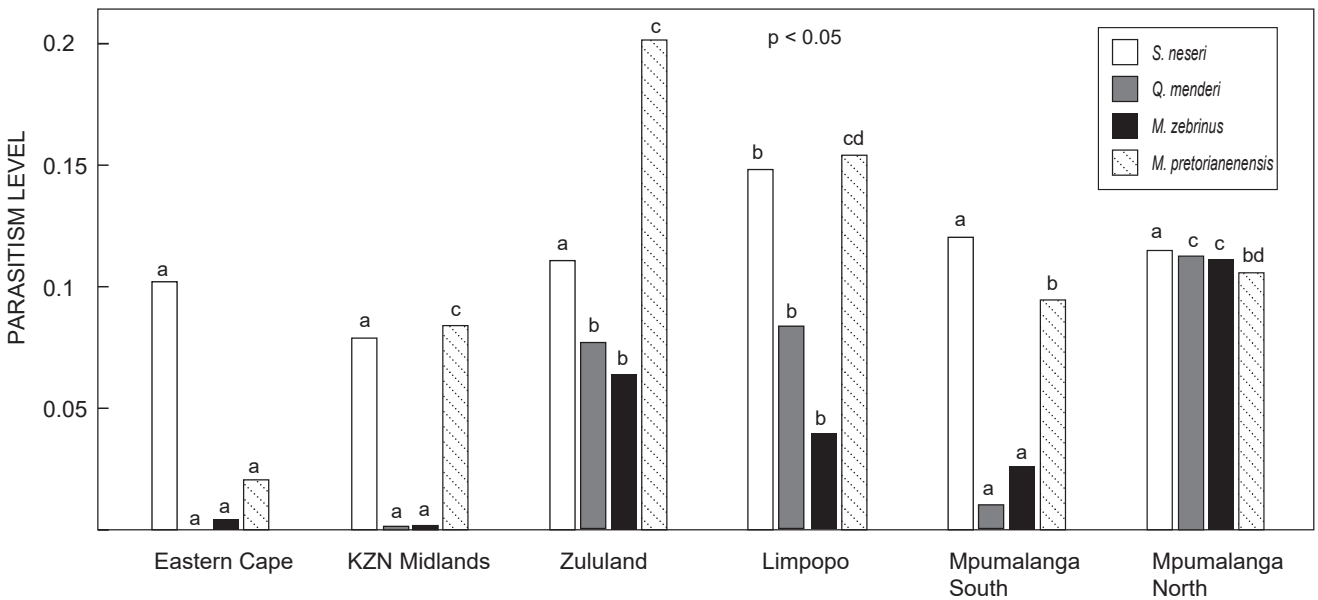

Figure 3: Mean parasitism rates of *S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis* for each forestry region (sampling periods combined). Different letters indicate significant differences ($p < 0.05$) in parasitism within a single species between regions

India, Taiwan, Thailand and South Africa) (Chen et al. 2009; Kumari et al. 2010; Tung & La Salle 2010; Sangtongpraow et al. 2011; Akhtar et al. 2012; Nugnes et al. 2015; Dittrich-Schröder et al. 2018). The presence of both male and female specimens would suggest the possibility of sexual reproduction, which could result in increased genetic variation. Increased genetic variation may increase the ability of the wasp to adapt and survive in a range of environmental conditions, and the potential to overcome tree resistance. It is currently unknown whether the reproductive mechanism between the *Leptocybe* lineages differs. The two *Leptocybe* lineages were found to co-occur within a single site and on a single tree. This co-occurrence is potentially problematic for control efforts as it increases the possibility of admixture. This is especially of concern as admixture has been observed in Laos, where the two lineages also co-occur (Dittrich-Schröder et al. 2018). Admixture in introduced ranges can be beneficial for the invader due to potential increased fitness and adaptability (Keller and Taylor 2010; Verhoeven et al. 2011). Admixture between the two *Leptocybe* lineages may result in potential changes in environmental adaptation, response to host resistance mechanisms and resistance to biological control agents (Dittrich-Schröder et al. 2018).

Selitrichodes neseri and *M. pretorianensis* were found in all six of the sampled regions and in most cases were the two dominant species (besides *Leptocybe invasa*). These data confirm that *S. neseri*, the biological control agent released in 2012 as an ectoparasitoid of *L. invasa* (Kelly et al. 2012), has successfully established and has rapidly spread throughout the forestry areas of South Africa. *Megastigmus pretorianensis* was described as a South African species (Doganlar et al. 2015), but its recent discovery in Australia suggests a possible origin in Australia (Le et al. 2020). Although *M. pretorianensis* may potentially be an inquiline, in this study it was assumed to be a parasitoid, as most *Megastigmus* species associated with *L. invasa* galls around the world have been confirmed to be parasitoids (Le et al. 2018).

This study was the first to confirm the presence of *Q. mendeli* in eucalypt plantations in South Africa, since its first detection in the country in 2016 (Bush et al. 2017). *Quadrastichus mendeli* was first detected in a 2017 survey, but by 2019 it was recorded as the dominant parasitoid species in some of the

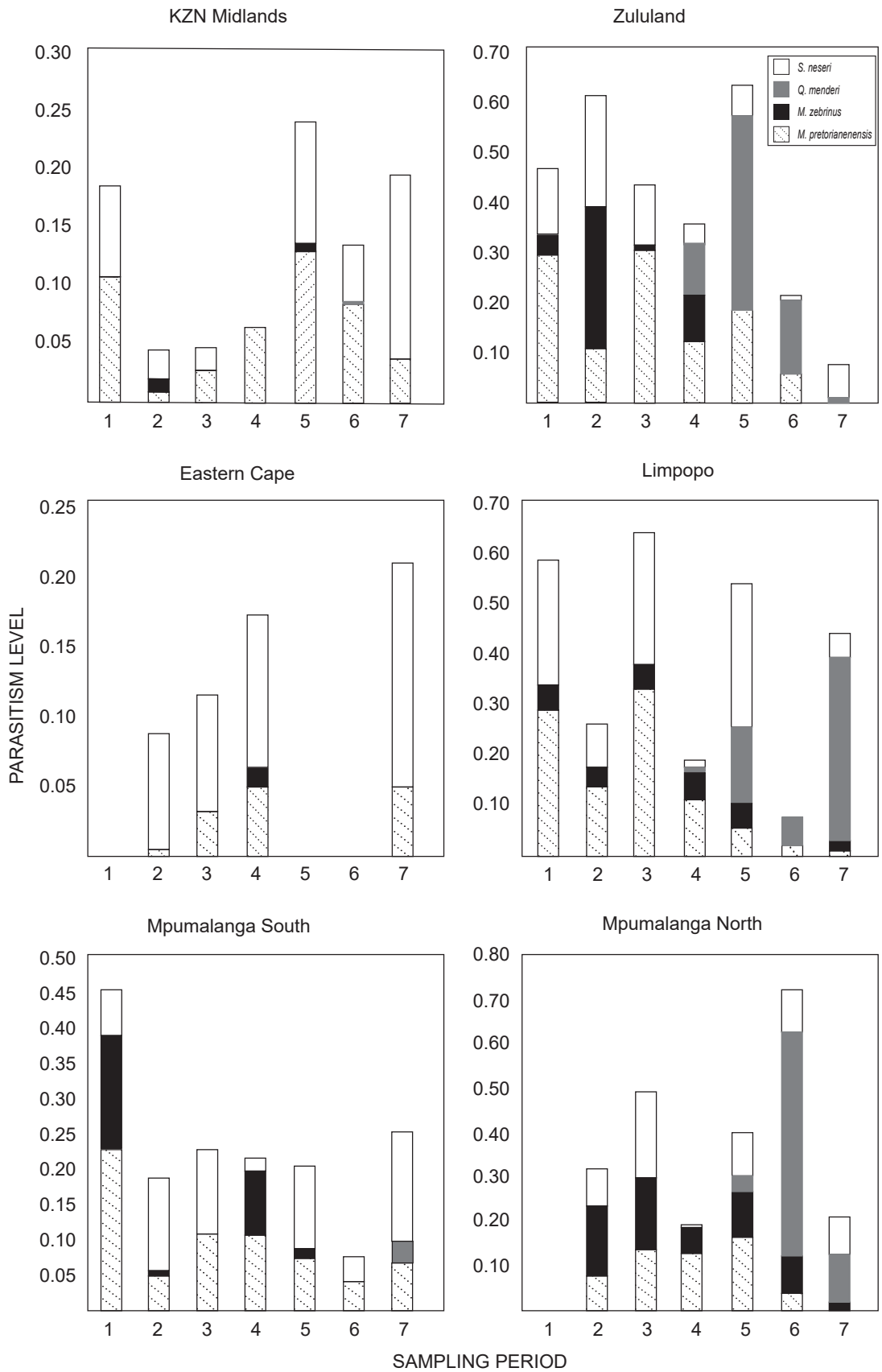

Figure 4: Mean parasitism levels of *S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis* between sampling periods (1 to 7), in each forestry region

forestry regions. *Quadrastichus mendeli* is a parasitoid that has been used in Israel as a biological control agent of *L. invasa* and has been unintentionally introduced into South Africa. It has also been recorded from Laos (ACIAR 2016; Bush et al. 2017; Le et al. 2018).

Megastigmus zebrinus was present in all six forestry regions, but parasitism levels varied considerably and were lower than parasitism levels observed for *S. neseri, Q. mendeli* and *M. pretorianensis*. This wasp was first recorded in the Western Cape in 1998 (Grissel 2006). *Megastigmus zebrinus* has a broader host range than *S. neseri* and *M. pretorianensis* and is part of other gall communities. For example, Klein (2009) has shown that it is a parasitoid in the hymenopteran community associated with a gall wasp of *E. camaldulensis* seeds, *Quadrastichodella nova.* The reasons for lower parasitism levels are not known. However, it may be attributed to its broader host range in comparison to the other parasitoids or potentially being outcompeted by the other parasitoids because they are more aggressive and therefore better competitors within the *Leptocybe invasa* gall complex.

All four wasps (*S. neseri, Q. mendeli*, *M. pretorianensis* and *M. zebrinus*) emerged from trees where both *Leptocybe* lineages were present. Gevers et al. (2021) confirmed that *S. neseri* and *M. zebrinus* were able to parasitise both *Leptocybe* lineages, and *M. pretorianensis* was found co-inhabiting a gall cavity with both *Leptocybe* lineages. *Quadrastichus mendeli* was found to only parasitise Lineage A by Gevers et al. (2021), however, at the time of that study it was not found in the areas where Lineage B was present. It now co-occurs with Lineage B, but needs to be confirmed as a parasitoid of this lineage.

This study highlights the value of long-term national monitoring schemes. Implementation of such national monitoring schemes improve pest management by providing data that can be used to understand future pest invasions, rate of spread, distribution patterns of new and existing biological control agents, and the presence of local natural enemies. Over the four years of the collections for this study, rapid and significant changes occurred in the distribution of the pest and its natural enemies. These data will inform human assisted spread of the various parasitoids as well as monitoring potential changes in plant host resistance, admixture and parasitoid resistance in future.

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ORCID iDS

Caitlin R Gevers — https://orcid.org/0000-0001-8467-8967
Bernard Slippers — https://orcid.org/0000-0003-1491-3858
Ilaria Germishuizen — https://orcid.org/0000-0003-0078-1137
Gudrun Dittrich-Schröder — https://orcid.org/0000-0001-5700-6276
Brett P Hurley — https://orcid.org/0000-0002-8702-5547

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