Diversity of entomopathogenic nematodes and their symbiotic bacteria in South African plantations and indigenous forests

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Summary - The occurrence and diversity of entomopathogenic nematodes (EPNs) and their symbiotic bacteria was evaluated in commercial forestry plantations (*Eucalyptus* spp., *Pinus* spp. and *Acacia mearnsii*) and indigenous forests in South Africa. EPNs were most prevalent in *A. mearnsii* plantations, accounting for 60.7 % of the isolates, while indigenous forests, plantations of *Pinus* spp. and *Eucalyptus* spp. accounted for 35.7 %, 3.6 % and 0 % of the isolates, respectively. DNA sequences of the internal transcribed spacer (ITS) and D2-D3 28S rDNA regions were used to identify the nematode species. Four *Steinernema* spp. were identified, including *S. citrae, S. sacchari*, two undescribed species, as well as *Heterorhabditis bacteriophora* and *H. baujardi*. *Heterorhabditis baujardi* is reported here from South Africa for the first time. Analysis of 16S rRNA of the bacteria confirmed the presence of at least three *Xenorhabdus* species from *Steinernema* isolates and two subspecies of *Photorhabdus luminescens* from *Heterorhabditis* species.

Keywords: *Heterorhabditis*, *Photorhabdus*, *Steinernema*, *Xenorhabdus*, biological control, forestry plantation, survey

Entomopathogenic nematodes (EPNs) residing in the family Steinernematidae and Heterorhabditidae are obligate insect parasites that occur in mutualistic associations with bacteria in the genera *Xenorhabdus* and *Photorhabdus*, respectively. The ability to mass rear these nematodes using solid media and liquid culture bioreactor technology (Ehlers, 2007), as well as their broad host range (Koppenhöfer, 2007) and ease of application (Grewal, 2002) have rendered them useful biological control agents of insect pests on various crop systems (Grewal *et al*., 2005; Koppenhöfer, 2007). Surveys have been conducted in many parts of the world to find locally-adapted EPN species or isolates, largely driven by the commercialization of EPNs as biocontrol agents (Hominick *et al*., 1996; Hominick, 2002). This has contributed to the number of described EPN species increasing from 13 (10 *Steinernema* and 3 *Heterorhabditis*) in the late 1980's (Kaya and Gaugler, 1993) to 66 (55 *Steinernema* and 11 *Heterorhabditis*) in 2007 (Nguyen and Hunt, 2007; Nguyen *et al*., 2007). In a comprehensive monograph, Hunt and Nguyen (2016) updated the species list of *Heterorhabditis* and *Steinernema* and several species names were found not to be valid. New synonymies were proposed resulting in a current 100 valid *Steinernema* and 16 *Heterorhabditis* species. The bacterial symbionts of only 27 *Steinernema* and 12 *Heterorhabditis* species have been described (Stock, 2015).

Despite a recent increase in surveys to discover native EPN species, knowledge on the diversity of these nematodes and their associated bacterial species remains rather limited, especially in less developed countries of the world. In Africa, only a few studies have focused on documenting the diversity of EPNs. These include surveys in Cameroon (Kanga *et al*., 2012a), Ethiopia (Mekete *et al*., 2005), Kenya (Mwaniki *et al*., 2008), Nigeria (Akyazi *et al*., 2012), South Africa (Hatting *et al*., 2009; Malan *et al*., 2011) and Tanzania (Mwaitulo *et al*., 2011). These surveys have resulted in reports of a number of previously described species, including

Heterorhabditis bacteriophora Poinar, *Heterorhabditis zealandica* Poinar, *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens, *Steinernema feltiae* Wouts, Mráček, Gerdin & Bedding and *Steinernema weiseri* Mrácek, Sturhan & Reid (Mekete *et al*., 2005; Mwaniki *et al*., 2008; Hatting *et al*., 2009; Malan *et al*., 2011; Akyazi *et al*., 2012; Kanga *et al*., 2012b). As of 2017, 19 EPN species have been described from the African continent (Table 1). In only nine cases have their bacterial symbionts also been identified. This relatively large number of reports, many of them in recent years, illustrates a growing interest in EPNs in Africa. The discovery of native EPN species in this region could promote the development and use of these species as biocontrol agents against local insect pests.

Recording the environmental factors associated with the presence / absence of EPNs during surveys will contribute to a better understanding of the factors that drive the distribution of these species. Soil is the natural habitat of EPNs, and it therefore is not surprising that parameters such as soil texture, moisture, pH, and organic matter, are important factors that determine the distribution and persistence of EPNs (Alekseev *et al*., 2006; Koppenhöfer and Fuzy, 2006; Kanga *et al*., 2012b). Although response to these environmental factors may vary with nematode species, prevalence of EPNs is generally higher in sandy and acidic soils (Stock *et al*., 1999; López-Núñez *et al*., 2007; Hatting *et al*., 2009).

Plantations of commercially propagated non-native trees and indigenous forest present potentially habitats in which to find and apply EPN species. Most surveys and applications of EPNs have thus far focused on agricultural fields and orchards. In South Africa, forest trees including both indigenous and non-native species account for about 2 % of the total land area (i.e. 2.3 million ha), of which about 1.3 mil. ha (55 %) is dedicated to plantation forestry utilising

non-native species including *Eucalyptus* spp., *Pinus* spp. and *Acacia mearnsii* (Edwards, 2012). Planted forests are of great socioeconomic importance in the country, with exported forest products in 2013 amounting to $1.7 billion and with the industry responsible for the employment of 170, 000 people (http://www.forestry.co.za, accessed 11 July 2017).

The sustainability of plantation forestry in South Africa, as with other regions of the world, is threatened by native and non-native insect pests and pathogens (Wingfield *et al*., 2008; 2015). Amongst these are insect pests of establishment (i.e. those that attack newly planted trees), which are normally the immature stages of native insects that feed on roots of seedlings. The most devastating of these establishment pests are white grubs (Coleoptera: Scarabaeidae) and cutworms (Lepidoptera: Noctuidae, *Agrotis* spp.), where infestations have been reported to result in up to 59 % seedling loss in wattle (*Acacia mearnsii*) (Govender, 2014; Harrison and Wingfield, 2016). Insecticides currently provide the most important means to control these pests, but their use in plantation forestry is under increasing pressure due to high costs, environmental contamination, and restriction by certification bodies such as the Forestry Stewardship Council (https://ic.fsc.org, accessed 10 July 2017; Garnas *et al*., 2012). However, EPNs have been successfully used to control white grubs (Grewal *et al*., 2005; Koppenhöfer *et al*., 2004, 2006) and cutworms (Ebssa and Koppenhöfer, 2011) in other crop systems, and they consequently offer a possible alternative for the management of these insect pests in forestry.

In this study, we conducted a survey in South African forest plantations and indigenous forests to determine whether EPN species were present in these habitats, and if so, to investigate how their distribution was influenced by various environmental factors such as soil pH, texture,

organic matter content, altitude and tree species. In addition, we isolated the bacteria and compared the nematode-bacteria association with those from previous reports.

Materials and methods

Sites sampled and sampling strategy

The survey was conducted in two provinces of South Africa, namely KwaZulu-Natal (KZN) and Mpumalanga, between February and May 2014. In each province, four vegetation/plantation types were considered, three in plantations (*Eucalyptus* spp., *Pinus* spp. and *A. mearnsii*) and one in indigenous forests (Appendix 1). In total 32 sites were sampled, with eight sites randomly selected per vegetation type. From each site, 20 soil samples were taken at least 10 m apart, giving a total of 640 samples (32 x 20). Each soil sample (approximately 1.5 kg) consisted of a composite of five random sub-samples taken in a 2 m^2 area at a depth of 0-20 cm. Samples were placed in polyethylene bags to prevent water loss and kept in cold boxes (at *ca.* 15 °C) during transport to the laboratory.

Nematode recovery and propagation

EPNs were recovered from soil samples that were stored in a cold room (at *ca*. 10 °C) prior to baiting using the last instars of *Galleria mellonella* L. (Lepidoptera; Phyralidae), following the technique described by Stock and Goodrich-Blair (2012). Samples were thoroughly mixed with tap water (amount depending on the dryness of the sample) to moisten the soil. Ten last-instar *G. mellonella* larvae were placed in 300 ml plastic containers with approximately 1 kg soil obtained from each sample at room temperature (23 ± 2 °C). *Galleria mellonella* larvae were monitored every three days (i.e. three times over nine days) and dead larvae were collected and placed in modified White traps (Kaya and Stock, 1997). Infective

juvenile (IJs) nematodes collected in the White traps were maintained by re-infection through *G. mellonella* larvae and stored in approximately 150 ml sterilized distilled water in 500 ml-vented tissue culture flasks at 14 °C for subsequent identification and establishment of stock cultures.

Soil physical and chemical properties

From each site sampled, a portion of the soil (*ca*. 100 g) was used to analyse physical and chemical properties of the soil. Soil pH was measured using standard methods in a water suspension (soil:water ratio of 1:2.5, w/v). Soil organic carbon was determined using the Walkley-Black method (Walkley, 1935) and texture (sand, clay, and silt percentage) was determined using the Bouyoucous hydrometer method (Bouyoucos, 1962). Altitude and vegetation type at each site was also recorded.

Characterisation of nematodes

Total genomic deoxyribonucleic acid (DNA) was isolated from pooled samples of IJs using a modified phenol chloroform protocol described by Goodwin *et al*. (1992), where IJs were ground with Retsch MM 301 Mixer Mill (Retsch GmbH, Rheinische, Germany) using metal beads. Polymerase chain reaction (PCR) amplification and sequencing of a region of the 28S (D2-D3) and internal transcribed spacer (ITS) regions of the ribosomal DNA gene was used to identify and determine the phylogenetic relationship between *Steinernema* and *Heterorhabditis* species, respectively, as described in Hatting *et al*. (2009) and Thanwisai *et al*. (2012). DNA was PCR amplified in 25 µl final volume with addition of 3 µl 10x PCR buffer (containing 3 mM $MgCl_2$), 1 µl 5 mM dNTP's, 0.25 µl Taq polymerase (Fast star), 16.75 µl of SABAX pure water (Adcock Ingram) and 1.0 µM of each primer set and 2 µl of DNA template. The primers used to amplify the ITS region were TW81 (F) and AB28 (R) as reported by Stock (2009). The primer

set used for the D2-D3 region for *Steinernema* spp. were D2F and 536R (Nguyen *et al*., 2006). The PCR cycling profile for the ITS and D2D3 regions was the same as those described by Stock (2009) and Nthenga *et al*. (2014), respectively. Sequence data for the forward and reverse DNA strands were edited manually using CLC Main Workbench v.6 (available online at http://www.clcbio.com) and compared with those present in GenBank by means of a Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI).

The ITS (for *Heterorhabditis* and *Steinernema)* and D2-D3 (for *Steinernema*) sequences and corresponding nucleotide sequences of other representatives of *Heterorhabditis* and *Steinernema* species and *Caenorhabditis elegans* Maupas (as the outgroup) available in GenBank were aligned using MAFFT (available online at http://mafft.cbrc.jp/alignment). Phylogenetic analyses (Maximum Likelihood) of ITS and D2-D3 sequence data were conducted using the software package PhyML 3.1 (Guindon *et al*., 2010). For phylogenetic analysis of *Steinernema* the TVM+I+G and TIM3+G models were selected by jModeltest 2.1.5 (Darriba *et al*., 2012) for the ITS and D2-D3, respectively. The model GTR+I+G was selected for the analysis of the ITS region of *Heterorhabditis* species.

Isolation and characterization of bacteria

Bacterial symbionts of *Steinernema* and *Heterorhabditis* were isolated following the procedure described by Koppenhöfer (2007). Bacteria were obtained from the haemolymph of infected *G. mellonella* larvae and isolated by plating onto nutrient bromothymol blue agar (NBTA) (nutrient agar; Oxoid LTD., Basingstock, Hampshire, England) supplemented with 0.004 % (w/v) triphenyltetrazolium chloride and 0.0025 % (w/v) bromothymol blue. Plates incubated at 25 °C for 48 h. Blue and blue-green colonies were randomly selected from the

plates and re-plated to obtain single colonies. Total genomic DNA was extracted from 22 bacterial isolates from *Steinernema* spp. and four isolates from *Heterorhabditis* spp. using PrepMan^{TM} Ultra reagent (Applied Biosystems, Woolston Warrington, UK).

The 16S ribosomal ribonucleic acid (rRNA) gene region of extracted DNA was PCR-amplified in 25 μl final volume with the addition of 2.5 μl of 10 × PCR buffer, 2 mM $MgCl_2$, 2.5 μl of 5 mM dNTP 0.15 μl of *Taq* polymerase, 16.35 μl of SABAX pure water and 0.25 μM of each primer set and 1 μl of DNA template. PCR cycling parameters were an initial step of 95 ºC for 5 min, followed by 30 cycles of 95 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min and a final extension of 72 ºC for 5 min using the 27F and 1492R primers (Frank *et al*., 2008). Two *Steinernema* isolates (DM4 and NTC19) were lost in culture and thus the bacteria could not be isolated from these nematodes. The 16S rRNA sequences for type strains of *Xenorhabdus* spp. and *Photorhabdus* sub-species were downloaded from the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (http://www.bacterio.net, accessed 18 August 2017) from the NCBI database for comparative purposes. *Photorhabdus luminescens* and *X. nematophila* were used as an outgroup for *Photorhabdus* spp. and *Xenorhabdus* spp., respectively. Sequence editing, alignment and phylogenetic analyses were conducted in the same manner as described above for identification of the nematodes. However, for both bacterial genera the model GTR was selected for the analysis of 16s rRNA.

Results

Sampling results

Of the 32 sites sampled, EPNs were found in four sites from Mpumalanga and five from KZN. These nematodes were isolated from 28 of the 640 soil samples (4 %).

Characterization of nematodes

Maximum likelihood (ML) analysis of ITS rDNA sequence placed 24 isolates of *Steinernema* in four groups. Seven isolates (DM4, DM14, DIN1, DIN5, DIN10, NTC7 and NTC19) exhibited high similarity (99 %) to NCBI deposited sequences of *S. citrae*. Fourteen isolates (ML1, ML2, ML4, ML5, ML9, ML15, ML16, DW1, DW3, DW7, DW8, DW9, DW12 and DW15) did not match available sequence data for previously described *Steinernema* species. Isolate ML15 was used to describe this clade as representing, *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley (Abate *et al*., 2016). Two isolates (Duk and MT5) were highly similar (99 %) to *S. sacchari* (Nthenga *et al*., 2014). The remaining isolate (Tito13) did not match other *Steinernema* species, having less than 92 % similarity with its closest known relative, *Steinernema nyetense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens (Kanga *et al*., 2012b), and is thus considered to be an undescribed species. All the *Steinernema* species collected in this study belonged to a new monophyletic group all described from the African continent, namely the 'Cameroonense-clade that includes *S. cameroonense*, *S. nyetense*, *S. sacchari* and *S. fabii*, except for *S. citrae* which is in the feltiae-group (Stokwe *et al*., 2011) (Figure 1 A). The ML analysis of the more conserved D2-D3 rDNA gene region (Stock, 2009) showed similar grouping of isolates as the ITS gene region, except that strong differentiation among species in the sub-group in the 'Cameroonian' group was not observed (Figure 1 B).

Maximum likelihood (ML) analysis of ITS rDNA sequence placed four isolates of *Heterorhabditis* in two groups. Three isolates (MT10, MT17 and MT19) showed high similarity (99 %) to *H. baujardi*. The single remaining isolate (MK) was most similar to *H. bacteriophora* (99 %) (Figure 2).

Prevalence of recovered nematode species

Steinernema isolates accounted for 24 (86 %) of all nematode isolates and *Heterorhabditis* isolates represented the remaining 4 (14 %). Of the four *Steinernema* species, *S. fabii* represented the majority of the isolates (n = 14), followed by *S. citrae* (n = 7), *S. sacchari* (n = 2) and a *Steinernema* sp. (n = 1). Of the two *Heterorhabditis* species, *H. baujardi* represented the majority of isolates (n = 3) followed by one *H. bacteriophora* isolate.

The recovered nematode species were most prevalent in wattle plantations, accounting for 60.7 % of the samples. In contrast, indigenous forests, plantations of *Pinus* spp. and *Eucalyptus* spp., accounted for 35.7 %, 3.6 % and 0 % of the isolates, respectively. Most *Steinernema* species were found in wattle plantations and indigenous forests, with one species, *S. sacchari* found in a pine plantation. The *Heterorhabditis* species were found only in indigenous forests (Appendix 1).

In general, EPNs were recovered in soils having a high sand content (50 - 92 %); no EPNs were recovered in clay or silt soils. Organic matter content was higher in the habitats where most of the EPNs were isolated, namely indigenous forest (6.9 ± 3.9 %) and wattle (4.9 ± 2.9 %), as compared to pine (2.5 ± 2 %) and eucalypts (1.4 ± 1.4 %). However, there was a high level of variation in organic matter content for the nematode-positive soil samples, ranging from 2.49 - 11.8 % in indigenous forest to 2.25 - 5.9 % in wattle plantations. No pattern was observed

for pH among the different plantations or between EPN positive and negative sites. A total of 18 % of the samples were recovered in lower altitude (<300 m asl), 50 % in higher altitude (>1200 m asl), and the remaining 32 % were recovered from an altitude of between 900 and 1200 m asl (Table 2).

Characterization of symbiotic bacteria

The symbiotic bacteria of all 22 *Steinernema* isolates were confirmed to represent species of *Xenorhabdus* based on the analysis of their 16S rRNA sequences. Maximum likelihood analysis of 16S rRNA placed the *Xenorhabdus* isolates into three groups (Figure 3A). The first group, which includes five isolates from *S. citrae*, was closely related to *X. bovienii*. The second group, which included one isolate (BTito13) from the undescribed *Steinernema* species, did not match any available sequence and most likely represents a new *Xenorhabdus* species. The third group, which included the majority of isolates (n = 16), was most closely related to *X. khoisanae*, with 99 % similarity. Two of the latter isolates were from *S. sacchari* (BDUK and BMT5) and clustered together with an 80 % bootstrap, and sister group to the remaining 14 isolates, which were from *S. fabii*. The associations of the species of symbiotic bacteria with the *Steinernema* species found in this study were previously unknown, although these bacteria have been recorded to be associated with other EPN species (Table 3).

The symbiotic bacteria of all four *Heterorhabditis* isolates were confirmed to be *Photorhabdus* species based on the analysis of their 16S rRNA sequences. Maximum likelihood analysis of 16S rRNA placed the *Photorhabdus* isolates into two groups (Figure 3 B). Three of the isolates from *H. baujardi* clustered with *P. luminescens* subsp. *luminescens*. The remaining isolate from *H. bacteriophora* clustered with *P. luminescens* subsp. *laumondii*. *Photorhabdus*

luminescens had been previously reported as an associate of both the *Heterorhabditis* species found in this study, as well as an associate with other *Heterorhabditis* species (Table 3).

Discussion

This study represents the first survey in South Africa to determine the prevalence of native EPN species and their symbiotic bacteria in plantations of commercially propagated non-native trees. A total of 28 EPN isolates were found, representing four *Steinernema* and two *Heterorhabditis* species, and their associated bacteria. Of these, two *Steinernema* species and one *Xenorhabdus* species appeared to represent undescribed species. Most of the nematode-bacteria associations that were identified represent newly identified associations. EPNs were more prevalent in wattle and indigenous forest sites, and sites that had soil with a high sand content, varying from acidic to neutral pH and with both low and relatively high organic carbon content.

Results of this study revealed a greater number of *Steinernema* spp. than *Heterorhabditi*s spp. The higher proportion of *Steinernema* species found in the surveys is similar to that found in a survey from diverse vegetation types in South Africa, where *Steinernema* accounted for 80 % of the recovered samples (Hatting *et al*., 2009). The result is, however, in contrast to a survey in a citrus orchard of South Africa where *Heterorhabditis* species were dominant, accounting for 89 % of samples (Malan *et al*., 2011). Given that there is a higher diversity of *Steinernema* species reported in Africa and worldwide (Nguyen *et al*., 2007; Hunt and Nguyen, 2016), the results of this study are not unexpected.

Of the *Heterorhabditis* species, *H. bacteriophora* has been recovered previously in South Africa from various sites (Hatting *et al*., 2009; Malan *et al*., 2011). This is also one of the most widely distributed EPNs in the world (Nguyen and Hunt, 2007). The present study, however,

represents the first record of *H. baujardi* in South Africa. The nematode was first described in Vietnam, and has subsequently been reported in Cameroon (Kanga *et al*., 2012b), Brazil (Dolinski *et al*., 2008) and Thailand (Thanwisai *et al*., 2012). These broad distributions, across continents, are likely due to human mediated dispersal (Abate *et al*., 2017).

The *Steinernema* species collected in this study were found in diverse habitats, including indigenous South African forests and non-native wattle and pine. This is in contrast to the *Heterorhabditis* species that were only found in the indigenous forest samples. *Steinernema citrae* was the only species that was isolated from both provinces sampled and in two habitats (wattle and indigenous forest). This species has also been reported from Western Cape province, demonstrating its wide distribution in South Africa (Malan *et al*., 2011). *Steinernema sacchari* was also recovered from two habitats (indigenous and unmanaged pine plantation), but only in the KZN province. The isolation of these different species from different habitats and geographic areas could relate to their specific adaptation to sites and a preference for certain environmental conditions or the availability of host insects.

A greater number of nematode species were recovered from indigenous forest sites and wattle plantations than those of eucalypts and pine. The prevalence of EPNs from indigenous forest and wattle sites could be influenced by factors such as soil type, availability of suitable hosts, and physiological and behavioural adaptations (Adams *et al*., 2006). The absence of EPNs from eucalypt sites could be due to unavailability of suitable insect hosts. This would be consistent with a study in South Africa that has shown that species richness and diversity of invertebrates in eucalypt and pine plantations is generally lower as compared to indigenous forest (Samways *et al*., 1996). In addition, it has been shown that soil dwelling invertebrates are

less diverse in eucalypt plantations as compared to other native forest in Australia (Bonham *et al*., 2002). The higher numbers of nematode species in indigenous forest and wattle plantations could also be related to relatively higher soil organic matter content at these sites as compared to that in pine and eucalypt plantations. Further studies to investigate potential biotic factors, as well as abiotic factors such as soil moisture, are required to better understand the patterns of distribution and diversity of nematodes in natural forests and plantations.

Analysis of 16S rRNA bacterial sequences confirmed the presence of at least three *Xenorhabdus* species (*X. bovienii*, *X. khoisanae* and *Xenorhabdus* sp.) from *Steinernema* isolates and *Photorhabdus luminescens* from *Heterorhabditis* species. The latter bacterial species could represent two sub-species, including *P. luminescens* subsp. *luminescens* and *P. luminescens* subsp. *laumondii*. Many previous surveys have focused only on isolating EPNs and not their symbiotic bacteria. However, isolating and confirming the identity of the symbiotic bacteria is becoming increasingly important due to their potential valuable antimicrobial, insecticidal and nematicidal traits (Webster *et al*., 2002). For example, toxin complexes from *Xenorhabdus* and *Photorhabdus* spp. include several insecticidal proteins (Brown *et al*., 2004; ffrench-Constant *et al*., 2007, 2010; Hinchliffe *et al*., 2010; Sheets *et al*., 2011). For this reason, they are being considered as potential alternatives to *Bacillus thuringiensis* to provide genes for incorporation into plants for insect resistance (ffrench-Constant *et al*., 2007). Knowledge of the bacterial associate of EPNs that will be applied over wide areas as biological control agents should thus be fully understood.

The majority of the bacterial isolates identified in this study were clustered with *X. khoisanae.* These bacteria were isolated from *S. fabii* and *S. sacchari*, but *X. khoisanae* is also a

known symbiont of *Steinernema khoisanae* Nguyen, Malan & Gozel from South Africa (Nguyen *et al*., 2006; Ferreira *et al*., 2013a). Thus, *X. khoisanae* is carried by at least three different *Steinernema* species. This is not uncommon as several species of bacteria are known to be associated with more than one nematode species, such as *X. bovienii* which has been isolated from nine species of *Steinernema* (Lee and Stock, 2010; Tailliez *et al*., 2010). The two nematode species collected in this survey and *S. khoisanae* were collected in the same geographic region, which might explain the sharing of similar bacterial symbionts. Molecular analysis showed that the EPNs that share these bacteria are distantly related, where *S. fabii* and *S. sacchari* cluster in the Cameroonense-clade and *S. khoisanae* belongs to the glaseri-group (Nguyen *et al*., 2006).

Results of this study confirmed that the bacterial symbiont of *H. baujardi* is closely related to *P. luminescens* subsp. *luminescens*. This is in agreement with studies in Brazil and Thailand where *H. baujardi* was found to carry *P. luminescens* of unknown sub-species (Thanwisai *et al*., 2012; Ferreira *et al*., 2011). However, *H. bacteriophora* has been found in association with at least eight sub-species of both *P. luminescens* and *P. temperate*, including *P. luminescens* subsp. *akhurstii P. luminescens* subsp. *kayaii, P. luminescens* subsp. *laumondii, P. luminescens* subsp. *caribbeanensis, P. temperata* subsp. *khanii, P. temperata* subsp. *thracensis, P. temperata* subsp. *tasmaniensis* and *P. temperata* subsp. s*tackebrandtii* (Lewis and Clarke, 2012; Maneesakorn *et al*., 2011; Stock, 2015). This is unlike the situation with *Steinernema* species where the symbiosis is highly specific and in which single nematode taxa carry a single *Xenorhabdus* sp. In a previous study, *H. bacteriophora* and *Heterorhabditis safricana* Malan, Nguyen, de Waal & Tiedt from South Africa were also shown to be associated with *P. luminescens* subsp. *laumondii* (Geldenhuys *et al*., 2016).

Although phylogenetic analysis based on 16S rRNA gene sequences confirmed the presence of two *P. luminescens* sub-species, clades resolved in the phylogeny were not consistent with species level taxonomic designations for *Photorhabdus*. In particular, three isolates in the present study together with *P. luminescens* subsp. *luminescens* formed a monophyletic group with *Photorhabdus asymbiotica* subsp. *asymbiotica*, but *P. asymbiotica* subsp. *asymbiotica* and *P. asymbiotica* subsp. *australis* did not form a monophyletic group. This is in agreement with previous studies (Akhurst *et al*., 2004; Shapiro-Ilan *et al*., 2009; Tailliez *et al*., 2010) in Australia, France and the USA. While 16S rRNA sequence data can be used to identify the sub-species of *P. luminescens*, they do not resolve the phylogenetic relationships between the subspecies of *P. luminescens*. In order to resolve these taxonomic uncertainties, multilocus sequence analysis will be required (Tailliez *et al*., 2010; Ferreira *et al*. 2013a, b, 2014, 2016).

This study revealed a substantial diversity of EPN species and their associated bacteria in plantation and indigenous forests of South Africa. Potential environmental factors as drivers of diversity of these EPNs were identified, but these require further investigations including targeted field surveys and laboratory studies. This study included a relatively low number of sites and it is likely that further surveys including additional geographic areas and climatic conditions both in plantations and indigenous forests of South Africa will reveal additional EPN species. The isolation and results nevertheless provides the opportunity to now consider the potential of these nematodes to be used as biological control agents for establishment pests in forestry. In this regard, effectively utilising native EPNs against these pests would reduce the reliance on insecticides. It is also the preferred option compared to introducing exotic EPNs that may have

possible unintended side effects on non-target organisms and the environment (Abate *et al*., 2017).

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List of tables

Table 1. Entomopathogenic nematodes and associated *Photorhabdus* and *Xenorhabdus* bacteria in Africa.

Nematode species	Symbiotic bacteria	Country of isolation	References
H. noenieputensis	*P. luminescens* subsp. *noenieputensis*	South Africa	Malan *et al*., 2012; Ferreira *et al*., 2013a
H. safricana	*P. luminescens* subsp. *laumondii*	South Africa	Malan *et al*., 2008; Geldenhuys *et al*., 2016
H. taysearae	Unknown	Egypt	Shamseldean *et al*., 1996
S. cameroonense	Unknown	Cameroon	Kanga *et al*., 2012a
S. citrae	*Xenorhabdus bovienii**	South Africa	Stokwe *et al*., 2011
S. beitlechemi	*X. khoisanae*	South Africa	Çimen *et al*. 2016a
S. biddulphi	Unknown	South Africa	Çimen *et al*., 2016b
S. ethiopiense	Unknown	Ethiopia	Tamiru *et al*., 2012
S. fabii	*X. khoisanae**	South Africa	Abate *et al*., 2016
S. jeffreyense	Unknown	South Africa	Malan *et al*., 2016a
S. innovationi	Unknown	South Africa	Çimen *et al*., 2015
S. karii	*X. hominickii*	Kenya	Waturu *et al*., 1997; Lewis & Clarke, 2012
S. khoisanae	*X. khoisanae*	South Africa	Nguyen *et al*., 2006; Ferreira *et al*., 2013b
S. nguyeni	Unknown	South Africa	Malan *et al*., 2016b
S. nyetense	Unknown	Cameroon	Kanga *et al*., 2012a
S. pwaniensis	Unknown	Tanzania	Půža *et al*., 2017
S. sacchari	*X. khoisanae**	South Africa	Nthenga *et al*., 2014
S. tophus	Unknown	South Africa	Çimen *et al*, 2014
S. yirgalemense	*X. indica*	Ethiopia	Nguyen *et al*.,2004; Tamiru *et al*., 2012; Ferreira *et al*., 2016

*Bacteria species confirmed from the current study in South Africa

Table 2. Distribution of entomopathogenic nematodes considering different environmental factors.

Categories (total site/total samples)	Recovery[a] frequency (%)	Positive samples	
		No.	Percent [b]
pH			
< 3 (0)	-	0	-
3 – 3.9 (5/100)	9	9	32.1
4 – 4.9 (15/300)	2.7	8	28.6
5 – 5.9 (4/80)	1.25	1	3.6
6 – 6.9 (8/160)	6.25	10	35.7
Organic carbon (%)			
0 – 3 (19/380)	4.47	17	60.7
3.1 – 6 (6/120)	1.7	2	7.15
6.1 – 9 (4/80)	-	0	-
9.1 – 12 (3/60)	15	9	32.15
Texture			
Sandy clay loam (4/80)	12.5	10	35.7
Sandy loam (9/180)	6	11	39.3
Sand (7/140)	0.7	1	3.6
Loam(5/100)	2	2	7.15
Loamy sand (2/40)	10	4	14.3
Silt loam (2/40)	-	0	-
Clay loam (2/20)	-	0	-
Clay (1/20)	-	0	-
Habitat type			
Indigenous forest (8/160)	6.25	10	35.7
Wattle (8/160)	10.6	17	60.7
Pine (8/160)	0.6	1	3.6
Eucalyptus (8/160)	-	0	-
Altitude (m asl)			
≤300 (10/ 200)	2.5	5	18
>300 – 600 (0)	-	0	-
>600 -900 (2/ 40)	-	0	-
>900-1200 (5/ 120)	7.5	9	32
>1200 (13/ 260)	5.4	14	50

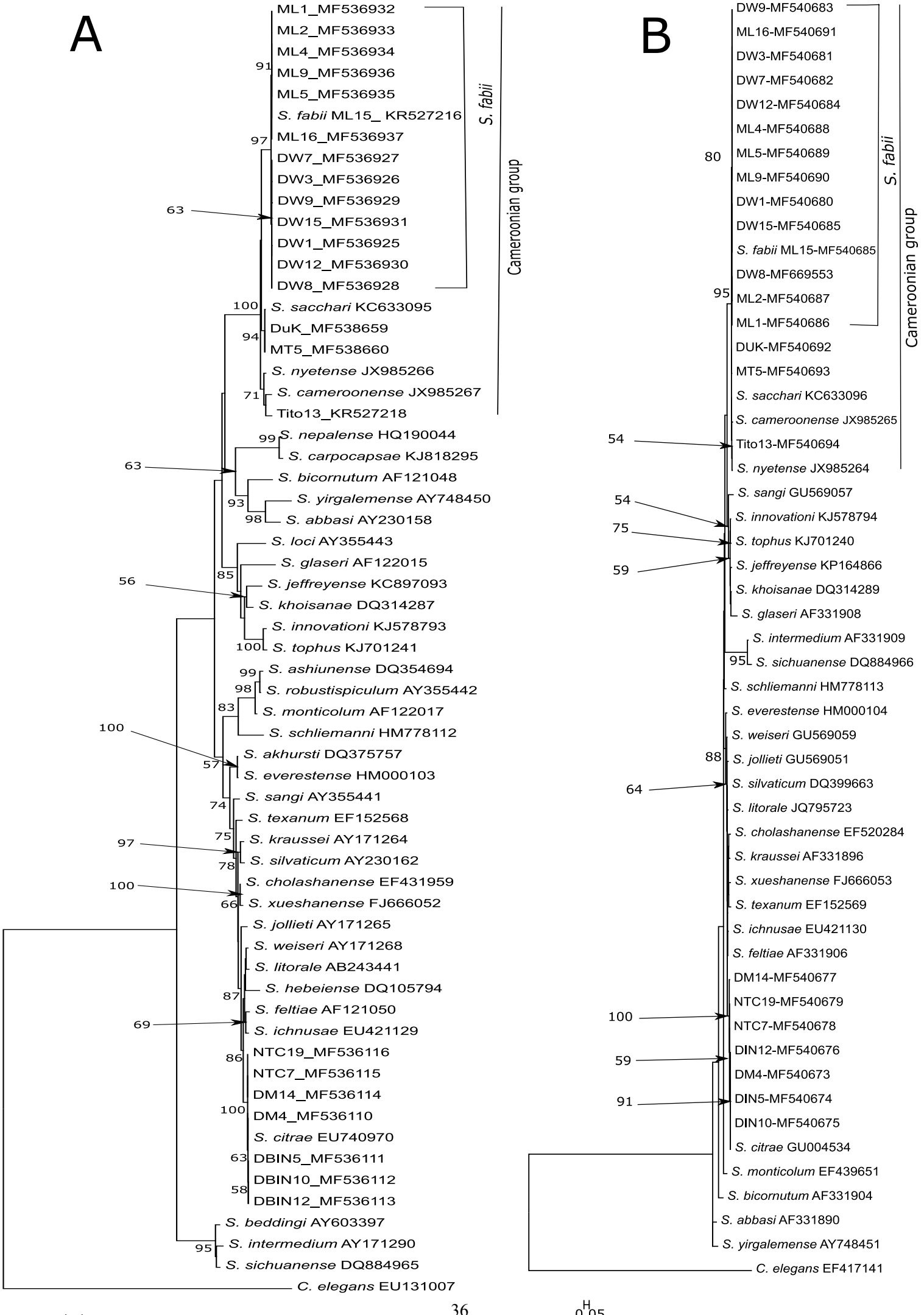
[a] Recovery frequency (number of positive samples/ total number of samples)

[b] Percent positive samples (number positive samples per category variable/ number total positive samples)

Table 3. Entomopathogenic nematodes and associated *Xenorhabdus* or *Photorhabdus* spp. from South African indigenous and plantation forestry.

EPN species recovered in our study	Associated bacteria	Previous bacteria associates (References)	Previous / other EPN associates of the bacteria (References)
Steinernema citrae	*Xenorhabdus bovienii*	Unknown	*S. affine*, *S. feltiae*, *S. intermedium*, S. *jollieti*, *S. kraussei*, *S. oregonense*, *S. puntauvense*, *S. sichuanense*, *S. weiseri* (Tailliez *et al*., 2010, Lee and Stock, 2010)
S. fabii	*X. khoisanae*	Unknown	*S. khoisanae* (Ferreira *et al*., 2013a)
S. sacchari	*X. khoisanae*	Unknown	*S. khoisanae* (Ferreira *et al*., 2013a)
Steinernema sp.	*Xenorhabdus* sp.	Unknown	Unknown
Heterorhabditis baujardi	*Photorhabdus luminescens* subsp. *luminescens*	*P. luminescens* (Thanwisai et al., 2012, Ferreira et al., 2011)	*H. bacteriophora, H. georgiana, H. indica* (Lewis and Clarke, 2012)
H. bacteriophora	*P. luminescens* subsp. *laumondii*	*P. luminescens, P. temperate* (Lewis and Clarke, 2012, Maneesakorn et al., 2011)	*H. georgiana, H. indica, H. baujardi* (Lewis and Clarke, 2012, Thanwisai *et al*., 2012), *H. bacteriophora, H. safricana* (Geldenhuys *et al*., 2016)

List of figures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DW3_MF536926
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DW15_MF536931
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DW12_MF536930
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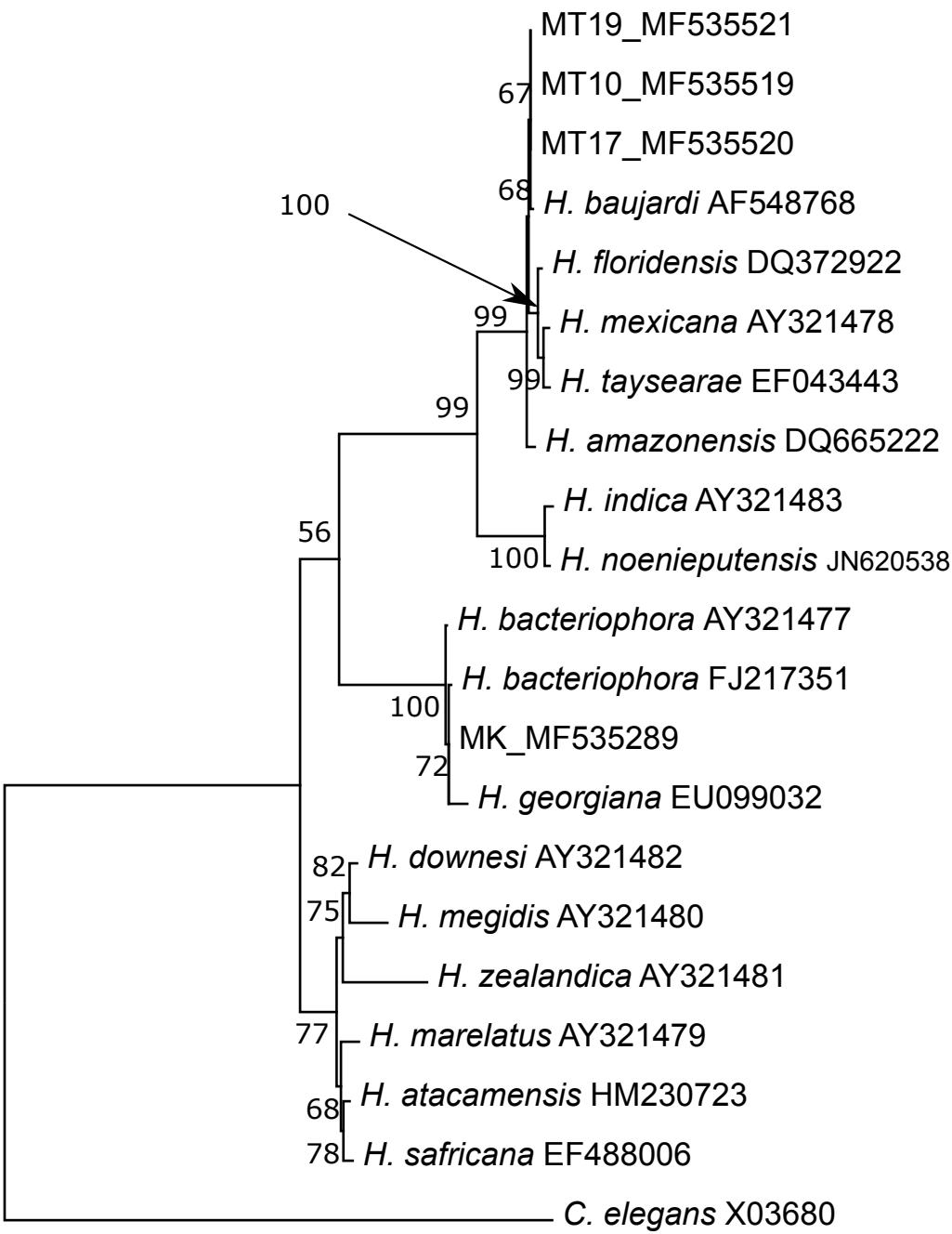
Figure 1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H. safricana EF488006
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0.05

Figure 2

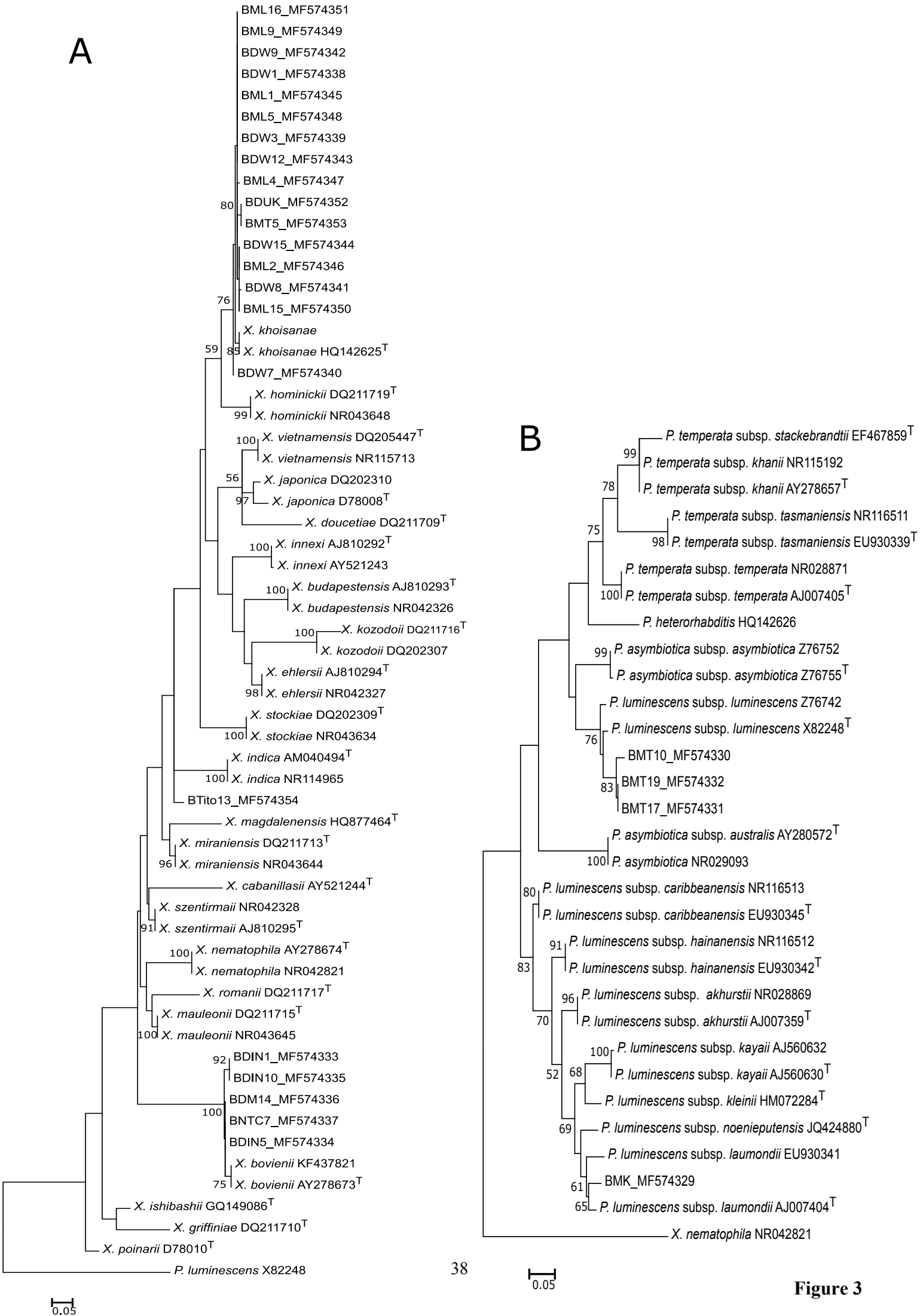
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Figure 3

Appendix A. Sampling sites: number of samples/site, geographic location, soil property and vegetation/plantation types.

Provinces	Sampling sites	EPN positive samples/ total no. of samples (Species)	GPS Coordinate	Soil property			Altitude	Vegetation/ plantation type
				Sand/Clay/ Silt % (texture)	%C	PH		
Kwazulu-Natal	Demagteneberg	2/20 (*S. citrae*)	S29^{0}17'26" E30^{0}10'50"	60/22/18 (Sandy clay loam)	11.8	6.66	1436	Indigenous forest
	Mkomazi river	1/20 (*H. bacteriophora*)	S30^{0}00'60" E30^{0}14'10"	66/18/16 (Sandy loam)	2.49	6.37	1020	Indigenous forest
	Pietermaritzburg	0/20	S29^{0}33'25" E30^{0}22'73"	13/28/59 (Silt loam)	8.05	3.52	837m	Wattle
	Underberg (PW)	0/20	S29^{0}38'16" E30^{0}03'88"	45/16/39 (Loam)	2.50	4.70	1405m	Pine
	Underberg (PW)	0/20	S29^{0}38'16" E30^{0}03'88"	55/16/29 (Sandy loam)	4.79	4.07	1405m	Eucalypt
	Underberg (PW)	0/20	S29^{0}38'16" E30^{0}03'88"	47/16/37 (Loam)	8.11	6.66	1405m	Indigenous
	Underberg (LW)	0/20	S 29^{0}34' 17" E 30^{0}05' 29"	30/36/34 (Clay loam)	2.7	4.27	1492	Pine
	Underberg (PV)	0/20	S29^{0}34'33" E30^{0}09'38"	22/28/50 (Silt loam)	3.50	4.35	1153m	Indigenous
	Ingwe-NTC	0/20	S29^{0}24'23" E30^{0}06'20"	36/28/36 (Loam)	7.31	3.52	1211m	Pine
	Ingwe-NTC	2/20 (*S. citrae*)	S29^{0}23'31" E30^{0}07'38"'	50/20/30 (Loam)	5.90	3.77	1211m	Wattle
	Mtunzini- T016	0/20	S29^{0}01'07" E30^{0}37'69"	91/8/1 (Sand)	0.13	4.49	50 m	Eucalypt
	Mtunzini- Q004	0/20	S29^{0}01'10" E31^{0}40'76"	82/14/4 (Loamy sand)	0.85	4.36	98 m	Eucalypt
	Mtunzini-1	0/20	S28^{0}59'82" E31^{0}42'40"	73/12/15 (Sandy loam)	3.74	4.70	41	Indigenous
	Mtunzini-2	4/20 (*H. baujardi*) & (*S. sacchari*)	S28^{0}58'01" E31^{0}45'34"	87/8/15 (Loamy sand)	9.95	6.77	22 m	Indigenous
	Kwambonambi	0/20	S28^{0}38'55" E32^{0}09'84"	92/8/0 (Sand)	2.49	4.07	12m	Pine
	Kwambonambi	0/20	S28^{0}38'55" E32^{0}09'84"	91/8/1 (Sand)	1.61	4.12	12m	Eucalypt Sappi

Provinces	Sampling sites	EPN positive samples/ total no. of samples (Species)	GPS Coordinate	Soil property			Altitude	Vegetation/ plantation type
				Sand/Clay/ Silt % (texture)	%C	PH		
	Kwambonambi	0/20	S28^{o}35'94" E32^{o}06'33"	90/8/2 (Sand)	0.66	6.40	55m	Eucalyptus Mondi
	Mtubatuba (B001)	0/20	S28^{o}20'81" E32^{o}14'71"	93/6/1 (Sand)	0.88	5.05	60m	Eucalypt1
	Mtubatuba (A100)	0/20	S28^{o}21'03" E32^{o}15'41"	92/8/0 (Sand)	0.30	6.79	69 m	Eucalypt2
	Mtubatuba	1/20 (*S. sacchari*)	S28^{o}21'25" E32^{o}14'46"	92/8/0 (Sand)	0.59	5.31	58m	Pine
Mpumalanga	Moolman-1	7/20 (*S. fabii*)	S27^{o}12'30" E31^{o}01'04"	77/14/9 (Sandy loam)	2.76	3.88	1220	Wattle
	Moolman-2	0/20	S27^{o}8'30" E30^{o}51'52"	37/44/19 (Clay)	2.42	3.77	1222	Wattle
	Dumbe	3/20 (*S. citrae*)	S27^{o}27'03" E30^{o}27'85"	58/16/26 (Sandy loam)	11.8	6.04	1614m	Indigenous
	Dumbe (5049)	7/20 (*S. fabii)*	S27^{o}19'00" E30^{o}42'32"	67/24/9 (Sandy clay loam)	2.27	4.20	1112m	Wattle
	Dumbe (Comp.5050)	0/20	S27^{o}19'00" E30^{o}42'32"	70/20/10 (Sandy clay loam)	4.54	4.15	1112m	Eucalypt1
	Dumbe	0/20	S27^{o}33'471" E30^{o}28'86"	72/18/10 (Sandy loam)	0.96	4.20	1500m	Pine
	Dumbe (Com. A041)	0/20	S27^{o}33'47" E30^{o}28'86"	81/14/5 (Sandy loam)	2.44	5.48	1500m	Eucalypt2
	Piet Retief	0/20	S26^{o}56'15" E30^{o}45'49"	56/14/30 (Sandy loam)	6.33	5.25	1329m	Wattle
	Piet Retief	0/20	S26^{o}57'04" E30^{o}46'39"	36/40/24 (Clay loam)	2.27	4.79	1240m	Pine
	Tito	1/20 (*Steinernema* sp. 2)	S27^{o}33'49" E30^{o}28'85"	51/32/17 (Sandy clay loam)	2.25	4.53	1184m	Wattle
	Tito	0/20	S27^{o}33'49" E30^{o}28'85"	76/18/6 (Sandy loam)	1.77	4.70	1184m	Pine
	DELFT	0/20	S27^{o}18'74" E31^{o}07'89"	35/26/39 (Loam)	3.74	6.74	745m	Indigenous