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Molecular characterization and species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the *penetrans* group (Nematoda: Pratylenchidae)

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ABSTRACT

Root-lesion nematodes of the genus *Pratylenchus* are an important pest parasitizing a wide range of vascular plants including several economically important crops. However, morphological diagnosis of the more than 100 species is problematic due to the low number of diagnostic features, high morphological plasticity and incomplete taxonomic descriptions. In order to employ barcoding based diagnostics, a link between morphology and species specific sequences has to be established. In this study, we reconstructed a multi-gene phylogeny of the *Penetrans* group using nuclear ribosomal and mitochondrial gene sequences. A combination of this phylogenetic framework with molecular species delineation analysis, population genetics, morphometric information and sequences from type location material allowed us to establish the species boundaries within the *Penetrans* group and as such clarify long-standing controversies about the taxonomic status of *P. penetrans*, *P. fallax* and *P. convallariae*. Our study also reveals a remarkable amount of cryptic biodiversity within the genus *Pratylenchus* confirming that identification on morphology alone can be inconclusive in this taxonomically confusing genus.

1. Introduction

Root-lesion nematodes of the genus *Pratylenchus* Filipjev 1936 are migratory endoparasites belonging to the family Pratylenchidae (Nematoda, Tylenchida). Root-lesion nematodes are ranked as the third most important group of plant-parasitic nematodes in terms of economic loss in agriculture and horticulture (Castillo and Vovlas, 2007). In some cases, yield loss can extend up to 85% of the expected production (Nicol et al., 2011). Several species of *Pratylenchus* such as *P. penetrans*, *P. brachyurus*, *P. coffeae* and *P. vulnus* have a wide geographical distribution and can parasitize a wide range of host plants (for a detailed list see Castillo and Vovlas, 2007). Within the genus *Pratylenchus*, 98 species were recognized by Geraert (2013) after which three additional species have been described: *Pratylenchus oleae* (Palomares-Rius et al., 2014), *Pratylenchus quasitereoides* (Hodda et al., 2014), and *Pratylenchus parazeae* (Wang et al., 2015), bringing the total species number to 101. However, morphological diagnosis of root-lesion nematodes is problematic due to the low number of diagnostic features and high intraspecific variability (Castillo and Vovlas, 2007; Roman and Hirschmann, 1969; Tarte and Mai, 1976). These problems are clearly demonstrated by a recent study revealing the presence of different morphotypes within a single species, suggesting that several of the already scarce morphological diagnostic features might be dependent on the reproductive strategy of a population (Troccoli et al., 2016). In order to properly diagnose *Pratylenchus* spp., a wide variety of biochemical and molecular methods have been proposed, including isozyme electrophoresis (Andres et al., 2000; Ibrahim et al., 1995), restriction fragment analysis of the internal transcribed spacer (ITS) of ribosomal RNA gene (Waeyenberge et al., 2000), and sequencing of different fragments of the ribosomal gene cluster, including ITS (De Luca et al., 2011; Wang et al., 2012), 18S (Subbotin et al., 2008; van Megen et al., 2009) and 28S (Al-Banna et al., 2004; Handoo et al., 2001; Subbotin et al., 2008). Species-specific primers have been developed for a variety of different species and genes (Al-Banna et al., 2004; Handoo et al., 2001; Palomares-Rius et al., 2014; Subbotin et al., 2008; Troccoli et al., 2016) as well as in combination with duplex PCR (De Luca et al.,

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Table 1
Species and populations of *Pratylenchus* used in the study. * Subbotin et al. (2008), [1] No morphological voucher available, [2] Topotype material.

Species	Locality	Specimen or sample code	*COI* haplotype	Host	GenBank accession number		
					D2-D3 of 28 S rRNA	ITS of rRNA	*COI*
P. brachyurus	China, F0983	T162	Br1	*Ficus* sp.	–	KY828251-KY828252	KY817010
P. crenatus	The Netherlands, Baale-Nassau F0683-2	T133	Cr1	Grasses	KY828371	–	KY817014
P. crenatus	The Netherlands, Arkel F1145	T183	Cr1	*Pyrus* sp.	–	–	KY817006
P. crenatus	The Netherlands, Smilde F0712	T275	Cr1	Grasses	–	–	KY816997
P. crenatus	The Netherlands, Hei en Boeicop F1247	T297	Cr1	*Pyrus* sp.	–	–	KY816989
P. crenatus	The Netherlands, Heersch F2468	T702	Cr1	*Vitis* sp.	–	–	KY816969
P. crenatus	UK, England, Rothemstadt, broadbalk	T761	Cr1	Grasses	KY828370	–	KY816943
P. convallariae	Belgium, Wetteren	T267	Co1	Grasses	–	KY828256	KY817000
P. convallariae	Belgium, Wetteren	T268	Co1	Grasses	KY828373	–	KY816999
P. convallariae	Belgium, Wetteren	T269	–	Grasses	–	–	–
P. convallariae	USA, Ohio, Tipp City	CD1813[1]	Co2	Unknown plant	KY828372	–	KY817025
P. dunensis	The Netherlands, Groote Keeten	T67	Du1	*Ammophila arenaria*	KY828369	KY828244-KY828245	KY817019
P. dunensis	The Netherlands, Groote Keeten	T68	–	*Ammophila arenaria*	KY828368	–	–
P. dunensis	The Netherlands, Groote Keeten	T69	–	*Ammophila arenaria*	–	–	–
P. dunensis	The Netherlands, Groote Keeten	T70	–	*Ammophila arenaria*	–	–	–
P. fallax	The Netherlands, Middelharnis	T85	Fa1	Grasses	KY828367	–	KY817017
P. fallax	The Netherlands, Middelharnis	T87	–	Grasses	KY828366	–	–
P. fallax	The Netherlands, Smilde F0712	T272	Fa1	Grasses	KY828365	–	KY816998
P. fallax	The Netherlands, Baale-Nassau F0683-3	T283	Fa1	Grasses	KY828364	–	KY816996
P. fallax	The Netherlands, Baale-Nassau F0683-3	T284	Fa1	Grasses	–	–	KY816995
P. fallax	The Netherlands, Uddel F0689	T286	Fa1	Grasses	–	–	KY816994
P. fallax	The Netherlands, Uddel F0689	T290	Fa1	Grasses	–	–	KY816993
P. fallax	The Netherlands, Doornenburg[2]	T353	Fa1	*Malus pumila*	KY828363	KY828258	KY816988
P. fallax	The Netherlands, Doornenburg[2]	T354	Fa1	*Malus pumila*	–	–	KY816987
P. fallax	The Netherlands, Heersch F2468	T700	Fa1	*Vitis vinifera*	–	–	KY816971
P. fallax	The Netherlands, Andelst F2420	T706	Fa1	*Prunus domestica*	–	–	KY816967
P. fallax	The Netherlands, Andelst F2420	T707	Fa1	*Prunus domestica*	–	–	KY816966
P. fallax	The Netherlands, Andelst F2420	T708	Fa1	*Prunus domestica*	–	–	KY816965
P. fallax	The Netherlands, Winssen F2399	T709	Fa1	*Prunus domestica*	–	–	KY816964
P. fallax	The Netherlands, Ochten F2421	T710	Fa1	*Prunus avium*	–	–	KY816963
P. fallax	The Netherlands, Ochten F2421	T712	Fa1	*Prunus avium*	–	–	KY816962
P. fallax	The Netherlands, Terwolde F2432	T717	Fa1	*Vitis vinifera*	–	–	KY816957
P. fallax	The Netherlands, Terwolde F2432	T718	Fa1	*Vitis vinifera*	–	–	KY816956
P. fallax	The Netherlands, Terwolde F2432	T719	Fa1	*Vitis vinifera*	–	–	KY816955
P. fallax	The Netherlands, Papendrecht F2473	T727	Fa1	*Prunus domestica*	–	–	KY816947
P. fallax	The Netherlands, Papendrecht F2473	T728	Fa1	*Prunus domestica*	–	–	KY816946
P. fallax	The Netherlands, Papendrecht F2473	T729	Fa1	*Prunus domestica*	–	–	KY816945
P. fallax	The Netherlands, Ysbrechtum F2455	V4 C	Fa1	*Vitis vinifera*	KY828362	KY828272-KY828273	KY816938
P. fallax	The Netherlands, Ysbrechtum F2455	V5 C	Fa1	*Vitis vinifera*	KY828361	–	KY816937
P. fallax	The Netherlands, Ysbrechtum F2455	V8 C	Fa1	*Vitis vinifera*	KY828360	–	KY816935
P. fallax	The Netherlands, Doornenburg	T376	Fa2	*Malus pumila*	–	–	KY816986
P. fallax	The Netherlands, Doornenburg	T682	Fa2	*Pyrus* sp.	–	–	KY816978
P. fallax	The Netherlands, Heersch F2468	T701	Fa2	*Vitis vinifera*	–	–	KY816970
P. fallax	The Netherlands, Winssen F2399	T705	Fa2	*Prunus domestica*	–	–	KY816968
P. fallax	The Netherlands, St.Oedenrode F2470	T685	Fa3	*Vitis vinifera*	–	–	KY816977
P. penetrans	The Netherlands, Kloetinge F2466	T720	Pe1	*Prunus avium*	–	–	KY816954
P. penetrans	The Netherlands, Vredepeel F770	T44	Pe2	*Daucus carota*	KY828359	–	KY817020
P. penetrans	Ethiopia, Shashamane	T225[1]	Pe2	*Zea mays*	KY828354	–	KY817001
P. penetrans	The Netherlands, Hei en Boeicop F1247	T296	Pe2	*Pyrus* sp.	–	–	KY816990
P. penetrans	The Netherlands, Schimmert F2433	T697	Pe2	*Prunus domestica*	–	–	KY816974
P. penetrans	The Netherlands, St.Oedenrode F2470	T730	Pe2	*Vitis vinifera*	–	–	KY816944
P. penetrans	France, Britany	CA192[1]	Pe2	*Malus pumila*	EU130859*	KY828242-	KY817022

(continued on next page)

Table 1 *(continued)*

Species	Locality	Specimen or sample code	*COI* haplotype	Host	GenBank accession number		
					D2-D3 of 28 S rRNA	ITS of rRNA	*COI*
						KY828243	
P. penetrans	The Netherlands, Baarlo F2584	V3 A	Pe3	*Malus pumila*	KY828347	KY828268-KY828269	KY816941
P. penetrans	The Netherlands, Stramproy F2425	T714	Pe4	*Prunus avium*	–	–	KY816960
P. penetrans	USA, California, Stanislaus County	CA91[1]	Pe5	*Vigna unguiculata*	EU130862*	–	KY817023
P. penetrans	The Netherlands, Zoetermeer F0716-1	T132	Pe6	Grasses	KY828358	–	KY817015
P. penetrans	The Netherlands, Kloetinge F2466	T722	Pe6	*Prunus avium*	–	–	KY816952
P. penetrans	Rwanda, Nyakiriba	T143	Pe7	*Allium cepa*	KY828357	KY828249-KY828250	KY817013
P. penetrans	Rwanda, Nyakiriba	T144	Pe7	*Allium cepa*	–	–	KY817012
P. penetrans	Rwanda, Nyakiriba	T145	Pe7	*Allium cepa*	–	–	KY817011
P. penetrans	The Netherlands, Arkel F1145	T184	Pe7	*Pyrus* sp.	–	–	KY817005
P. penetrans	Rwanda, Bushoki	T200[1]	Pe7	*Solanum tuberosum*	KY828355	–	KY817004
P. penetrans	The Netherlands, Hei en Boeicop F1247	T295	Pe7	*Pyrus* sp.	KY828352	–	KY816991
P. penetrans	The Netherlands, Schimmert F2433	T698	Pe7	*Prunus domestica*	–	–	KY816973
P. penetrans	The Netherlands, Kloetinge F2466	T721	Pe7	*Prunus avium*	–	–	KY816953
P. penetrans	The Netherlands, Wemeldinge F2472	T724	Pe7	*Prunus avium*	–	–	KY816950
P. penetrans	The Netherlands, Wemeldinge F2472	T726	Pe8	*Prunus avium*	–	–	KY816948
P. penetrans	The Netherlands, Stramproy F2425	T713	Pe9	*Prunus avium*	–	–	KY816961
P. penetrans	The Netherlands, Kloetinge F2466	T723	Pe10	*Prunus avium*	–	–	KY816951
P. penetrans	The Netherlands, Zoetermeer F0716-1	T181	Pe11	Grasses	–	–	KY817007
P. penetrans	Colombia, San Vicente	T666	Pe11	*Physalis peruviana*	KY828351	–	KY816982
P. penetrans	Colombia, San Vicente	T677	Pe11	*Physalis peruviana*	KY828350	–	KY816981
P. penetrans	Colombia, San Vicente	T678	Pe11	*Physalis peruviana*	KY828349	–	KY816980
P. penetrans	Colombia, San Vicente	T679	Pe11	*Physalis peruviana*	–	–	KY816979
P. penetrans	The Netherlands, Wemeldinge F2472	T725	Pe11	*Prunus avium*	–	–	KY816949
P. penetrans	Japan, Aichi	CA85[1]	Pe11	*Brassica oleraceae*	EU130860*	–	KY817021
P. penetrans	The Netherlands, Baarlo F2584	V8 A	Pe11	*Malus pumila*	KY828342	KY828274-KY828275	KY816936
P. penetrans	The Netherlands, Meijel F2537	V4B	Pe11	*Malus pumila*	KY828345	–	KY816939
P. penetrans	The Netherlands, Nagele F2612	V3 F	Pe11	*Malus pumila*	KY828346	KY828270-KY828271	KY816940
P. penetrans	Japan, Chiba	CA193[1]	Pe12	*Daucus carota*	EU130857*	–	KY817024
P. penetrans	The Netherlands, Arkel F1145	T172	Pe13	*Pyrus* sp.	KY828356	–	KY817009
P. penetrans	The Netherlands, Apeldoorn F1179	T293	Pe13	*Pyrus* sp.	KY828353	KY828257	KY816992
P. penetrans	The Netherlands, Sambeek F2583	T686	Pe13	*Malus pumila*	–	–	KY816976
P. penetrans	The Netherlands, Sambeek F2583	T687	Pe13	*Malus pumila*	–	–	KY816975
P. penetrans	The Netherlands, Schimmert F2433	T699	Pe13	*Prunus domestica*	–	–	KY816972
P. penetrans	The Netherlands, Meijel F2537	V1B	Pe14	*Malus pumila*	KY828348	KY828266-KY828267	KY816942
P. penetrans	The Netherlands, Stramproy F2425	T715	Pe15	*Prunus avium*	–	–	KY816959
P. penetrans	The Netherlands, Stramproy F2425	T716	Pe16	*Prunus avium*	–	–	KY816958
P. penetrans	The Netherlands, Meijel F2537	V6B	–	*Malus pumila*	KY828343	–	–
P. penetrans	The Netherlands, Baarlo F2584	V5A	–	*Malus pumila*	KY828344	–	–
P. penetrans	The Netherlands, Nagele F2612	V12F	–	*Malus pumila*	KY828341	–	–
P. penetrans	The Netherlands, Nagele F2612	V13F	–	*Malus pumila*	KY828340	–	–
P. penetrans	The Netherlands, Nagele F2612	V15F	–	*Malus pumila*	KY828339	–	–
P. pinguicaudatus	UK, England, Rothemstadt, broadbalk[2]	T572[1]	Pi1	*Triticum* sp.	–	KY828261-KY828263	KY816984
P. pinguicaudatus	UK, England, Rothemstadt, broadbalk[2]	CA191, CD2361[1]	–	*Triticum* sp.	KY828338	KY828239-KY828241	–
Pratylenchus sp. 1	Italy, Sicily, Villalba	T617	Sp1	*Lens culinaris*	KY828337	KY828264-KY828265	KY816983
Pratylenchus sp. 1	Italy, Sicily, Villalba	V13L	Sp1	*Lens culinaris*	KY828336	–	KY816934
Pratylenchus sp. 1	Italy, Sicily, Villalba	V15L	Sp1	*Lens culinaris*	KY828335	–	KY816933
Pratylenchus sp. 1	Italy, Sicily, Villalba	V29L	–	*Lens culinaris*	KY828334	–	–
Pratylenchus sp. 2	Nigeria, Ibadan, IITA trail field	T175	Sp2	Unknown	KY828333	–	KY817008
Pratylenchus sp. 2	Nigeria, Ibadan, IITA trail field	T194	–	Unknown	KY828332	–	–
Pratylenchus sp. 2	Nigeria, Ibadan, IITA trail field	T569	Sp2	Unknown	–	KY828259-KY828260	KY816985
Pratylenchus sp. 3	Rwanda, Nyamata	T201	Sp3	*Zea mays*	KY828331	KY828253-KY828255	KY817003
Pratylenchus sp. 3	Rwanda, Nyamata	T202	Sp3	*Zea mays*	KY828330	–	KY817002
Pratylenchus sp. 3	Rwanda, Nyamata	T278	–	*Zea mays*	–	–	–
Pratylenchus sp. 4	Tunisia	T71a	–	*Phoenix dactylifera*	KY828329	KY828246-KY828248	–

(continued on next page)

Table 1 *(continued)*

Species	Locality	Specimen or sample code	*COI* haplotype	Host	GenBank accession number		
					D2-D3 of 28 S rRNA	ITS of rRNA	*COI*
Pratylenchus sp. 4	Tunisia	T72a	Sp4	*Phoenix dactylifera*	–	–	KY817018
Pratylenchus sp. 4	Tunisia	T97[1]	–	*Phoenix dactylifera*	KY828328	–	–
Pratylenchus sp. 4	Tunisia	T103a	–	*Phoenix dactylifera*	KY828327	–	–
Pratylenchus sp. 4	Tunisia	T104a	Sp4	*Phoenix dactylifera*	KY828326	–	KY817016
Pratylenchus sp. 4	Tunisia	T106a[1]	–	*Phoenix dactylifera*	KY828325	–	–

2012; Waeyenberge et al., 2009) and quantitative PCR methods (Mokrini et al., 2013). Despite the existence of a wide spectrum of identification methods, no specific approach has been widely accepted yet and in fact most species identification methods have been tested for only a limited number of *Pratylenchus* taxa (Al-Banna et al., 2004; Mokrini et al., 2013; Palomares-Rius et al., 2014; Troccoli et al., 2016).

Most importantly, in order to employ molecular identification, traditional morphospecies would ideally be linked to molecular barcodes. In recent years, several species of *Pratylenchus* have been matched to molecular sequences, revealing the existence of cryptic species complexes (De Luca et al., 2011; De Luca et al., 2012). Collecting topotype material has often proved to be the only way to confidently connect DNA sequences to formerly described morphospecies (De Luca et al., 2010; Inserra et al., 2007; Troccoli et al., 2016). However, despite these efforts the vast majority of morphospecies remains unlinked to DNA sequences (Geraert, 2013).

Another problem is that the taxonomic status of several morphospecies is currently contested. This is especially true for the *Penetrans* group (clade IV) (Subbotin et al., 2008), in which species appear to share many morphological characteristics (Subbotin et al., 2008). Along *Pratylenchus penetrans* this clade IV contains *P. fallax*, *P. convallariae*, *P. pinguicaudatus*, *P. arlingtoni*, *P. dunensis*, *P. oleae* and *P. brachyurus* (Palomares-Rius et al., 2014); however, the validity of several of these taxa has been questioned (Subbotin et al., 2008). The taxonomic debate to clarify the validity of *P. penetrans* and *P. fallax* is an excellent example to illustrate the difficulties faced. *P. fallax* was originally described and differentiated from *P. penetrans* by Seinhorst (1968). Tarte and Mai (1976) subsequently considered both species to be conspecific, as morphological traits showed considerable intraspecific variability as a result of variable environmental conditions. Later *P. fallax* was re-erected as a separate species as breeding experiments yielded infertile offspring in interspecific breeding experiments (Perry et al., 1980). *P. penetrans* and *P. fallax* were also differentiated using isozyme electrophoresis (Ibrahim et al., 1995) and PCR-ITS-RFLP analysis (Waeyenberge et al., 2000). Subsequently, sequence analysis of the D3 region of the 28S rRNA gene revealed that *P. fallax* and *P. convallariae* are 99% identical, both species were shown to be closely related to *P. penetrans* (96–97% similarity) (Carta et al., 2001; Handoo et al., 2001). Additional molecular characterization by Subbotin et al. (2008) further brought into question the validity of several species within the *Penetrans* group. Surprisingly, ITS rRNA gene phylogenies strongly suggested that *P. fallax* was also very closely related to *P. lentis* (Palomares-Rius et al., 2010; Palomares-Rius et al., 2014). At the same time, Holterman et al. (2006) and Jones et al. (2013) argued *P. convallariae*, *P. penetrans* and *P. fallax* to be conspecific. Despite this taxonomic confusion *P. fallax*, is currently recognized as a quarantine species in the USA. This regulation is affecting the trade of plants and plant products, adding even more importance to clarification of the taxonomic status of this species (Handoo et al., 2001).

Therefore the goals of this study were to: (i) explore the biodiversity of the *Penetrans* group species based on a combination of morphological and molecular data; (ii) elucidate the molecular phylogeny of the *Penetrans* group; (iii) use this phylogenetic framework to delimitate species and clarify the taxonomic status of *P. arlingtoni*, *P. convallariae*, *P. dunensis*, *P. fallax, P. penetrans, P. pinguicaudatus* and four newly discovered undescribed species based on a link between sequence data and morphology; (iv) evaluate the correctness of species identification for sequences available in public databases; (v) assess the potential of using morphometric characters to distinguish species within the *Penetrans* group.

2. Material and methods

2.1. Nematode populations

Nematodes were extracted from soil and root material using a modified Baermann funnel and a mistifier (Hooper, 1986; van Bezooijen, 2006). Populations were sampled globally, however, the majority of samples were taken in the Netherlands focusing on fruit trees (*Malus* sp., *Prunus* spp., *Pyrus* sp.), grapes (*Vitis* spp.) and grasslands. The species: *P. dunensis*, *P. fallax* and *P. pinguicaudatus* were collected from the type localities, additional information on studied nematode species is given in Table 1.

2.2. Morphological study

Nematodes were studied in temporary slides sealed with nail-polish. Specimens were studied using an Olympus BX5 DIC microscope (Olympus Optical) and morphological vouchers were made using a combination of movies and photomicrographs made by an Olympus C5060Wz camera. To secure a link of morphometrics and sequences, measurements of these vouchers were made at the microscope or using ImageJ (Schneider et al., 2012). Vouchered nematodes were subsequently picked from temporary mounts and processed for DNA extraction. The resulting digital specimen vouchers are available online at http://nematodes.myspecies.info/taxonomy/term/10645/specimens.

Remaining unvouchered nematodes were fixed in TAF at 70 °C and processed to anhydrous glycerin, following the method of Seinhorst (1962) modified by Sohlenius and Sandor (1987) and measured using an Olympus BX5 DIC microscope (Table 2). Only measurements (L, V, a, b, c, c', stylet length, body width, post-uterine sac length, tail length, and position of metacorpus, cardia, pharyngeal lobe, excretion pore, vulva in respect to anterior end) from vouchers with a direct link with the molecular data were analyzed using a Cluster, a Principal Component Analysis (PCA) and a Canonical Discriminant Analysis (CDA). The Cluster analysis was done based on the default settings using Primer 6 (Clarke, 1993), after standardization and calculating an Euclidean distance matrix. The CDA and PCA analyses were only performed for those species with sufficient sequence-linked representatives (> 3 individuals, i.e. *P. penetrans*, *P. fallax* and *Pratylenchus* sp. 4) using the CANDISC procedure in SAS® 9.4 to find the sets of variables that discriminate most between the populations, based on the pooled within variance–covariance matrix.

For Scanning Electron Microscopy (SEM) nematodes were fixed in 600 µl fresh 4% Paraformaldehyde fixative buffered with Phosphate Buffer Saline (PBS) and 1% glycerol and heated for 3 s in a 750 W microwave. Subsequently, specimen were dehydrated in a seven-step graded series of ethanol solutions and critical-point dried using liquid

Table 2
Morphometric measurements of females of *P. penetrans*, *P. fallax*, *P. convallariae* and *Pratylenchus* sp. 3 females. All measurements were made on glycerin fixed specimen, measurements are in µm and given as mean ± standard deviation (range).

	Baarlo (F2584)	Nagele (F2612)	Meijel (F2537)	Ysbrechtum (F2455)	Uddel (F0689)	Doornenburg Type locality	Wetteren	Rwanda
Species Character	*P. penetrans*	*P. penetrans*	*P. penetrans*	*P. fallax*	*P. fallax*	*P. fallax*	*P. convallariae*	*Pratylenchus* sp. 3
n	6	12	6	8	10	7	10	14
L	659 ± 54(580–721)	684 ± 71(555–792)	593 ± 82(464–703)	527 ± 32(487–594)	447 ± 46(386–554)	471 ± 40(412–524)	411 ± 20(378–438)	531 ± 44(469–600)
Stylet length	16 ± 0.7(15–17)	16 ± 0.6(15–17)	15 ± 1.2(14–17)	16 ± 1(14–19)	15 ± 0.5(14–16)	16 ± 0.6(15–17)	15 ± 1(14–17)	13.6 ± 0.5(13–14.6)
Anterior end to center of metacorpus	61 ± 4(55–66)	60 ± 4(53–66)	55 ± 8(46–65)	57 ± 5(46–63)	45 ± 4(41–53)	46 ± 3(40–50)	44 ± 2(41–47)	54.7 ± 2.7(50.9–59.6)
Anterior end to cardia	76 ± 5(70–83)	76 ± 6(67–87)	70 ± 8(59–79)	70 ± 5(61–78)	53 ± 4(49–62)	56 ± 5(47–62)	51 ± 2(48–55)	91.5 ± 6.6(82.5–107.4)
Anterior end to pharyngeal gland lobe	146 ± 15(120–161)	147 ± 15(115–173)	93 ± 18(70–110)	127 ± 9(108–137)	78 ± 12(64–103)	79 ± 9(72–98)	106 ± 13(84–119)	111 ± 15.3(80–132)
Anterior end to EP	97 ± 11(85–112)	99 ± 7(88–110)	120 ± 23(85–137)	87 ± 8.3(78–96)	91 ± 11(71–104)	108 ± 14(78–117)	63 ± 6(51–71)	76 ± 12.2(49.8–90.5)
Anterior end to vulva	513 ± 66(421–584)	540 ± 52(431–631)	484 ± 76(365–594)	413 ± 23(384–460)	360 ± 34(309–427)	369 ± 25(336–409)	323 ± 153(308–353)	409 ± 40(338–464)
Maximum body width	28 ± 3(24–33)	27 ± 4(20–36)	21 ± 1.2(20–23)	21 ± 2(20–25)	16 ± 1(15–18)	16 ± 0.7(15–17)	16 ± 1(14–19)	21.6 ± 1.8(19–24.8)
Anal body width	13 ± 0.8(12–14)	15 ± 2(13–19)	12 ± 1(11–13)	11 ± 0.7(10–12)	9.6 ± 1(8 – 11)	10 ± 0.5(9 – 11)	10 ± 1(8 – 12)	13.6 ± 1.7(10.9–18)
Vulva-anus distance	106 ± 11(84–115)	97 ± 9(87–117)	80 ± 9(67–90)	87 ± 12(74–109)	63 ± 12(52–91)	78 ± 16(55–101)	63 ± 6(49–70)	79.7 ± 11.4(64–101.4)
Tail length	31 ± 3(25–35)	32 ± 4(27–41)	29 ± 2(25–31)	26 ± 2(23–31)	21 ± 2(17–24)	24 ± 2(22–27)	24 ± 3(18–27)	31.7 ± 2.9(26.2–36.6)
V	77 ± 6(66 – 82)	79 ± 5(67–84)	81 ± 2(78–84)	78 ± 1(77–80)	80 ± 2(77–83)	78 ± 2(74–81)	78 ± 2(75–83)	77.6 ± 1.4(75.3–79.7)
a	24 ± 2(20–26)	25 ± 5(18–35)	27 ± 3(23–32)	25 ± 3(19–29)	28 ± 2(25–32)	29 ± 3(24–33)	25 ± 2(19–28)	24.5 ± 3.1(19.8–29.6)
b	4.5 ± 0.1(4.3 – 4.8)	4.6 ± 0.6(3.7–5.5)	6.4 ± 0.7(5.6–7.7)	4.1 ± 0.4(3.8–4.8)	4.9 ± 0.7(3.9–6)	6 ± 0.4(5.3–6.6)	4 ± 0.5(3.3–5)	5.7 ± 0.4(5.2–6.8)
c	25 ± 9(17–41)	22 ± 3(15–27)	20 ± 1(18–23)	20 ± 2(16–23)	21 ± 3(17–25)	20 ± 2(17–22)	17 ± 2(14–22)	16.3 ± 1.9(12.8–20.1)
c′	2.1 ± 0.4(1.3–2.5)	2.1 ± 0.3(1.7–2.6)	2.5 ± 0.2(2.2–2.72)	2.3 ± 0.2(1.9–2.5)	1.3 ± 0.2(1–1.6)	2.5 ± 0.2(2.1–2.7)	2.5 ± 0.5(1.5–3)	2.4 ± 0.3(1.8–2.7)

CO_2, mounted on stubs with carbon discs, coated with gold (25 nm). Specimen were studied and photographed with a JSM − 840 EM (JEOL) electron microscope at 12 kV.

2.3. DNA extraction, PCR amplification and sequencing

Genomic DNA of individual nematodes was extracted using the quick alkaline lysis protocol described by Janssen et al. (2016). Briefly, individual nematodes were transferred to a mixture of 10 µl 0.05 N NaOH and 1 µl of 4.5% tween. The mixture was heated to 95 °C for 15 min, and after cooling to room temperature 40 µl of double-distilled water was added. PCR amplification was performed using TopTaq DNA polymerase (QIAGEN, Germany), in a volume of 25 µl using a Bio-Rad T100™ thermocycler. PCR mixtures were prepared according to the manufacturer's protocol with 0.4 µM of each primer and 2 µl of single nematode DNA extraction. The 28S rDNA fragment D2 A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers were used according to the protocol of De Ley et al. (1999). The internal transcribed rRNA gene spacer (ITS) was amplified using VRAIN2 F (5′-CTT TGT ACA CAC CGC CCG TCG CT-3′) and VRAIN2 R (5′-TTT CAC TCG CCG TTA CTA AGG GAA TC-3′) (Vrain et al., 1992), subsequently cloned using pGEM ® -T easy vector systems (Promega) and sequenced using universal M13 F and M13 R primers. The cytochrome c oxidase subunit 1 (*COI*) gene fragment was amplified using JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) according to the protocol of Derycke et al. (2010). Sanger sequencing of purified PCR fragments was carried out in forward and reverse direction by Macrogen (Europe). Contigs were assembled using GENEIOUS R6.1.8 (Biomatters; http://www.geneious.com). All contigs were subjected to BLAST searches to check for possible contaminations on http://www.ncbi.nlm.nih.gov. The newly obtained sequences were submitted to the GenBank database, accession numbers are displayed in Table 1 and in the phylogenetic trees.

2.4. Sequence and phylogenetic analyses

The newly obtained and published nematode sequences for each gene (De Luca et al., 2004; De Luca et al., 2011; Handoo et al., 2001; Kushida and Kondo, 2015; Majd Taheri et al., 2013; Mekete et al., 2011; Mokrini et al., 2016; Palomares-Rius et al., 2014; Subbotin et al., 2008; Troccoli et al., 2008; Troccoli et al., 2016; Wang et al., 2012) were aligned using ClustalX using default parameters. The best fit models of DNA evolution were obtained for each dataset using the program jModeltest 0.1.1 (Posada, 2008) under the Akaike information criterion. Bayesian phylogenetic analysis (BI) was carried out using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). BI analysis for each gene was initiated with a random starting tree and was run with four chains for 3.0×10^6 generations for *COI* and ITS datasets and 6.0×10^6 generations for the D2-D3 of 28S dataset. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples (20%), a 50% majority rule consensus tree was generated. Posterior Probabilities (PP) are given on appropriate clades. Pairwise divergences between the taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data using PAUP∗ 4.0b 10 (Swofford, 2002).

2.5. Species delimitation

Molecular species delimitation analyses were performed using the Generalized Mixed Yule Coalescent (GMYC) method (Pons et al., 2006) and the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al., 2012). For the GMYC species delimitation method an ultrametric tree was constructed under an uncorrelated lognormal relaxed clock using BEAST v1.7.5 (Drummond et al., 2012). Different codon positions

were treated as different partitions. Duplicated haplotypes were removed from the dataset using COLLAPSE 1.2 (Posada, 2004) and outgroup group sequences were removed from the alignment. A constant size coalescent prior was used because the GMYC method uses a coalescent null model to explain tree branching patterns (Pons et al., 2006). Default prior distributions were used except for the standard ucld.mean parameter which was changed into a uniform prior with initial value of 1, a lower bound of 0 and an upper bound of 100 for all three codon partitions. The analysis ran for 5×10^7 generations sampling every 5000th generation, 50% of the results were discarded as 'burnin'. From the resulting 20000 trees the maximum clade credibility tree was calculated with TreeAnnotator v1.7.5. On the resulting ultrametric tree both a single and a multiple-treshold GMYC model was optimized (Fujisawa and Barraclough, 2013) using the SPLITS package (Ezard et al., 2009) available for R. For the ABGD species delimitation method the alignment without outgroups and unique haplotypes was used. ABGD analyses were performed using the online version of the program (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html), using the default program settings. Distances were calculated using the Jukes-Cantor (JC69) substitution model and the Kimura (K80) substitution model.

3. Results and taxonomic notes

3.1. Dataset and taxonomic notes

Using a combination of morphological taxonomic characters and molecular criteria, seven valid species of *Pratylenchus* were distinguished within the studied samples: *P. brachyurus, P. crenatus, P. convallariae, P. dunensis, P. fallax, P. penetrans, P. pinguicaudatus* and four unidentified and putative new species. The taxonomic status of several of these species is highly contested (Castillo and Vovlas, 2007; Handoo et al., 2001; Subbotin et al., 2008) and as a result some long lasting misconceptions exist. In the following section an attempt is made to clarify the taxonomic status of each species. In order to establish a clear link between DNA sequences and morphology, each sequenced specimen is linked to a morphological voucher available on http://www.nematodes.myspecies.info/ (Table 1). The link between morphology and sequences is crucial for the identification of formerly described morphospecies and is essential for the reproducibility of the generated hypotheses (Pleijel et al., 2008). However, as species within the *Penetrans* group are morphologically very closely related, topotype material is often the only way to confidently link traditional morphospecies to DNA sequences (De Luca et al., 2012; De Luca et al., 2010; Inserra et al., 2007; Troccoli et al., 2016; Zamora-Araya et al., 2016).

Brief morphological descriptions with illustrations (Figs. 1–4), morphometric values (Table 2) and phylogenetic positions in the trees (Figs. 5–7) are given for several species of *Pratylenchus* below.

3.1.1. Pratylenchus arlingtoni Handoo, Carta & Skantar, 2001

Pratylenchus arlingtoni was described to be parasitizing grasses at the Arlington National Cemetery (VA, USA) (Handoo et al., 2001). While *P. arlingtoni* is morphologically closely related to *P. crenatus*, there is only a single DNA sequence of the D3 region of the 28S rRNA gene available for *P. arlingtoni* which indicates that this species belongs to the *Penetrans* group (Fig. 5). This phylogenetic position is highly surprising given the conserved morphology of the other species within this species complex. However, our phylogenetic analysis reveals that the sequence of *P. arlingtoni* (AF307328) differs only 4 base pairs from our *P. convallariae* population (Fig. 5), indicating that Handoo et al. (2001) accidentally sequenced a *P. convallariae* specimen while describing the morphology of *P. crenatus*. This is not unlikely given that *P. crenatus* and *P. convallariae* share the same habitat and can be expected to co-exist in many grass habitats. Furthermore, *P. arlingtoni* was described to have a filled spermatheca on rare occasions (Handoo et al., 2001), although, no males were reported for *P. arlingtoni*, and in the light of the current hypothesis this could point to the presence of *P. convallariae* in the sample. Additionally, re-examination of *P. arlingtoni* type material revealed a very close relationship with *P. crenatus*. Specifically, the same secretory-excretory duct swelling was observed in the type material as was described to be species-specific for *P. crenatus* (Karssen and Bolk, 2000). A morphometric study of *P. crenatus* (Kumari, 2015) revealed that several morphometric values are more variable than originally assumed by Loof (1960), suggesting that pharynx length and b value are not useful in separating *P. arlingtoni* from *P. crenatus* as proposed by Handoo et al. (2001). Also it is questionable if pharyngeal gland length, c′ value and post-uterine sac length relative to vulval-anal distance are useful to separate both species as they appear to be greatly variable and overlapping in range. Additionally, a SEM study of a *P. crenatus* population (Baarle-Nassau, the Netherlands, supplementary Fig. 1) matches exactly with the SEM characteristics described for *P. arlingtoni*. Finally, in this current study *P. crenatus* was found in five different locations in the Netherlands and one in Harpenden, Rothamsted, UK and all populations matched with the morphology of *P. arlingtoni.* Therefore we consider *P. arlingtoni* to be a *species inquirendae* until its morphology and molecular identity have been reassessed.

3.1.2. Pratylenchus brachyurus (Godfrey 1929) Filipjev and Schuurmans Stekhoven, 1941

Pratylenchus brachyurus is a widely distributed species in tropical and sub-tropical regions. A *P. brachyurus* population was recovered from a *Ficus* sp. sample from China, its *COI* sequence represents the first mitochondrial gene sequence of the species (Fig. 6). The population displayed all characteristic morphological features of *P. brachyurus*: a labial region with two annuli, stylet with broadly rounded knobs, typical tail point, empty spermatheca and posteriorly-positioned vulva. Originally *P. brachyurus* was considered to be part of clade III (Subbotin et al., 2008), later it was recovered as the earliest branching taxon of clade IV, albeit with poor support (Palomares-Rius et al., 2014). This phylogenetic position was confirmed by our D2-D3 of 28S rRNA gene phylogenetic tree (posterior probability = 99%, Fig. 5).

3.1.3. Pratylenchus convallariae Seinhorst, 1959 (Fig. 1)

Pratylenchus convallariae was originally described from Wassenaar, the Netherlands parasitizing *Convallaria majalis* L. Despite extensive sampling efforts in Wassenaar, *P. convallariae* was not recovered from the area. However, *P. convallariae* was recovered from grass in a sandy habitat (Wetteren, Belgium, GPS coordinates: 51.006643, 3.904379). The ITS sequences of this population are 92–95% identical with sequences deposited by Waeyenberge et al. (FJ712907- FJ712911, Fig. 7), originating from a *C. majalis* grower in Lisse, The Netherlands, which is situated approximately 20 km from the type locality in Wassenaar. Moreover, these sequences are also closely related to another *P. convallariae* ITS sequence from China (HM469448, 91.1–94.6% similar, Fig. 7). The morphology of our population matches the morphology of the original description very well including the characteristic head and tail point morphology (Fig. 1). Morphometrics also agree with the original description including the vulva position, although specimens of our population were smaller, 378–438 µm vs. 580–610µm (Table 2).

3.1.4. Pratylenchus dunensis de la Pena, Moens, van Aelst & Karssen, 2006 (Fig. 2)

Pratylenchus dunensis material in this study was sampled from the type host *Ammophila arenaria* and from the type locality Groote Keeten, the Netherlands (GPS coordinates: 52.865208, 4.703626). The 28S sequences from our population match (99.1–99.5% similarity) with previously deposited sequences of the species description (de la Peña et al., 2006).

3.1.5. Pratylenchus fallax Seinhorst 1968 (Fig. 2)

Pratylenchus fallax was originally described from an apple orchard

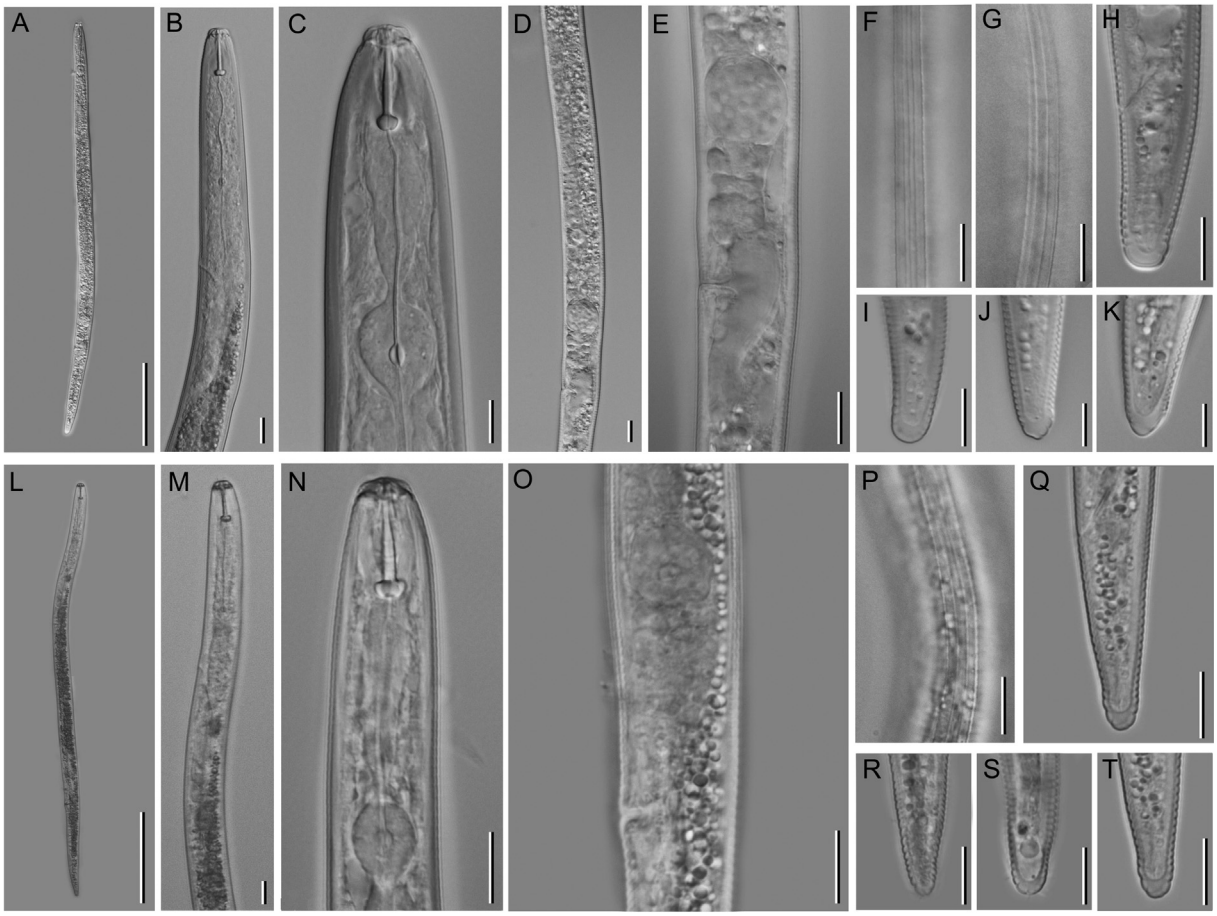

Fig. 1. Photomicrographs of specimens of *Pratylenchus penetrans* (A-K) and *P. convallariae* (L-T). A: Entire female body; B, C: Female anterior region; D, E: Vulval region; F, G: Lateral field; H-K: Tail region. L: Entire female body; M, N: Female anterior region; O: Vulval region; P: Lateral field; Q-T: Tail region. Scale bars A, L: 100 μm; B-K, M-T: 10 μm.

near Doornenburg, the Netherlands (Seinhorst, 1968). Thanks to the WNE type material collection (Nematode Collection Europe, http://www.nce.nu/), we were able to determine the exact type locality, this orchard being situated near the Castle of Doornenburg (GPS coordinates: 51.894251, 6.000240). Within the orchard *P. fallax* was recovered from the roots of two apple trees. Our *P. fallax* population matched very closely in morphology (Fig. 2) and morphometrics (Table 2) with the population described by Seinhorst (1968). On the type locality, several other species of *Pratylenchus* were found: *P. crenatus* and *P. thornei* associated with grass and *P. pratensis* associated with *Prunus avium* (L.). Beside the type location, *P. fallax* was found associated with *Malus pumila*, *Prunus avium, Prunus domestica, Vitis vinifera, Pyrus* sp. and grasses in the Netherlands, representing in total 11 separate populations (Table 1). Based on the results of our study, we can conclude that the 28S and ITS sequences of *P. fallax* sequences that are currently available on GenBank have been misidentified: the D3 28S sequence of *P. fallax* (AF264181) obtained from *Convallaria majalis* L. and originated from France (Handoo et al., 2001) does not match with our *P. fallax* topotype sequences. Instead this sequence is 99.5% similar to the *P. convallariae* population recovered in this study. Furthermore, five *P. fallax* ITS sequences (FJ712917- FJ712921) originating from Belgium do not match with our *P. fallax* topotype sequences but belongs to *P. lentis* (Palomares-Rius et al., 2010; Troccoli et al., 2008), *P. lentis* was recently considered a junior synonym of *P. pratensis* (Janssen et al., 2017a).

3.1.6. *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, 1941 (Fig. 1)

Pratylenchus penetrans was originally described as *Tylenchus penetrans* by Cobb (1917), and later transferred to the genus *Pratylenchus* (Filipjev and Schuurmans Stekhoven, 1941). Since then *P. penetrans* has been recorded from over 350 host plants and appears to have a worldwide distribution in temperate regions (Castillo and Vovlas, 2007). In this study *P. penetrans* was recovered from *Malus pumila, Prunus avium, Prunus domestica, Pyrus* sp., *Zea mays, Vitis vinifera, Vigna unguiculata, Allium cepa, Solanum tuberosum, Physalis peruviana, Brassica oleraceae, Daucus carota* and various grasses representing 20 separate populations of geographical widespread origin (Table 1). Morphometrics of the studied populations agree well with the original and other descriptions of this species (Castillo and Vovlas, 2007) (Table 2). One ITS sequence (FJ799117) has been uploaded as a *P. penetrans* sequence on GenBank but actually represents a *P. vulnus* sequence, suggesting that *P. vulnus* instead of *P. penetrans* (Chen et al., 2009) is present on strawberry in Taiwan.

3.1.7. *Pratylenchus pinguicaudatus* Corbett, 1969

Pratylenchus pinguicaudatus was descried from the Broadbalk trail field, Harpenden, Rothamsted, UK associated with wheat (Corbett, 1969). Subbotin et al. (2008) was the first who published 18S rRNA gene sequence of the topotype materials for *P. pinguicaudatus*. In this study we obtained the ITS, D2-D3 and *COI* gene sequences from this topotype material. The D2-D3 sequence did not match with those (AJ545014, KP289345-KP289347) of the root-lesion nematodes identified as *P. pinguicaudatus* from Tunisia and Morocco. Because these sequences clustered with those of *Pratylenchus* sp. 1, we consider these nematodes from North Africa as belonging to one or even two unidentified species.

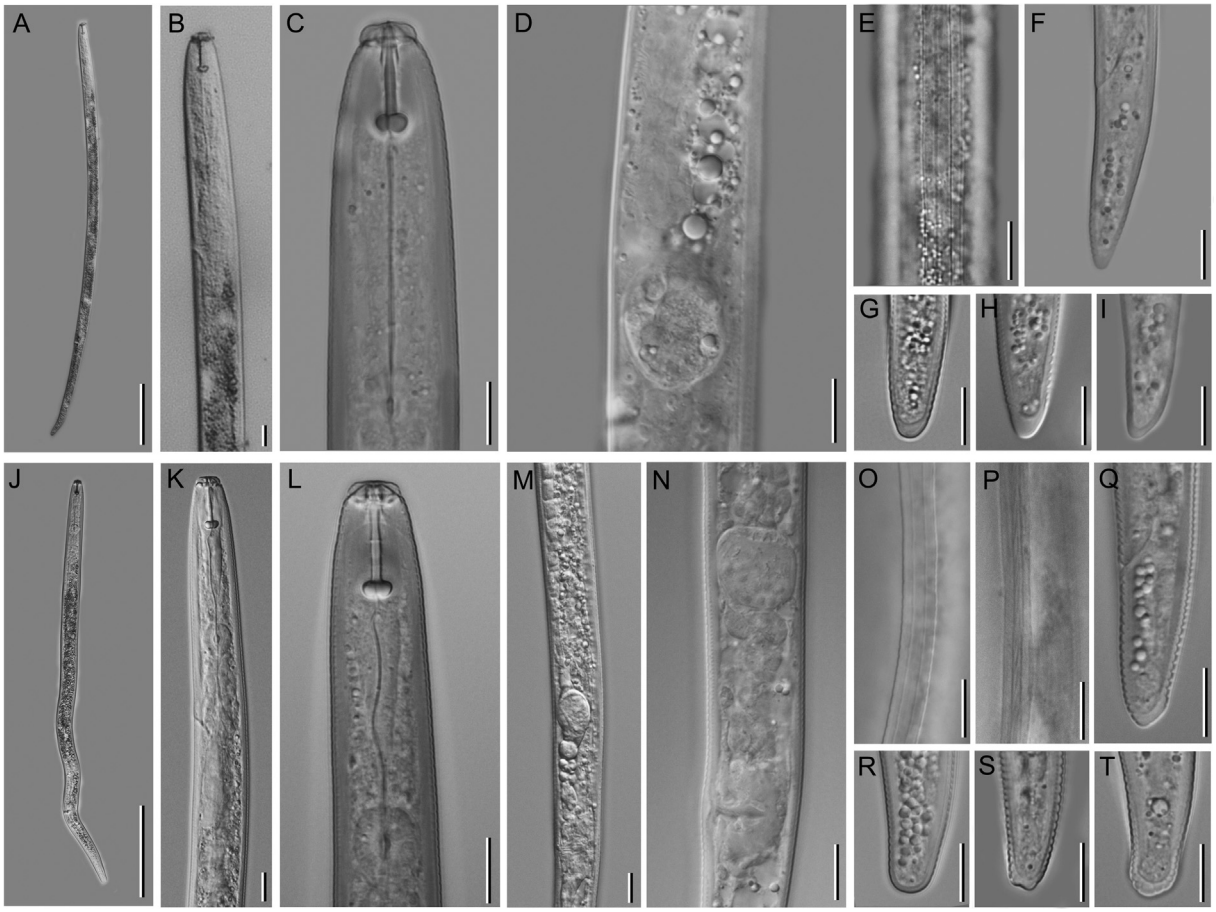

Fig. 2. Photomicrographs of specimens of *Pratylenchus dunensis* (A-I) and *P. fallax* (J-T). A: Entire female body; B, C: Female anterior region; D: Vulval region; E: Lateral field; F-I: Tail region. J: Entire female body; K, L: Female anterior region; M, N: Vulval region; O, P: Lateral field; Q-T: Tail region. Scale bars A, J: 100 μm; B-I, K-T: 10 μm.

3.1.8. *Pratylenchus sp. 1 (Fig. 3)*

In the summer of 2014 the type locality of *Pratylenchus lentis* was sampled in Villalba, Sicily, Italy (Troccoli et al., 2008). This sampling revealed *P. lentis* to be a junior synonym of *P. pratensis* (Janssen et al., 2017a). Besides *P. pratenis*, an unknown species of *Pratylenchus* was recovered from this location. Molecular species delimitation analyses indicate that this population represents a putative new species of the *Penetrans* group. *Pratylenchus* sp.1 is a bisexual species and morphologically similar to *P. penetrans,* sharing three lip annuli, rounded filled spermatheca, four lateral lines, a rounded tail tip and the same stylet morphology. Matrix code for the tabular key proposed by Castillo and Vovlas (2007): A2, B2, C3, D2, E3, F4, G2, H1, I4, J1, K1.

3.1.9. *Pratylenchus sp. 2 (Fig. 3)*

Pratylenchus sp. 2 was collected from an unknown host in a trial field of IITA, Ibadan, Nigeria. Although only a limited number of specimens were recovered, molecular analysis clearly separate this species from any other molecularly characterized *Pratylenchus* (Figs. 5–7). Morphologically this species is similar with *Pratylenchus* sp. 3, *P. oleae*, *P. pinguicaudatus* and *P. elamini* in sharing three lip annuli, four lateral lines and a similar stylet morphology. Moreover, no males were found for *Pratylenchus* sp. 2 and the spermatheca was always empty despite egg development in the gonads, indicating an asexual mode of reproduction which is also shared with the three aforementioned species. Matrix code for the tabular key proposed by Castillo and Vovlas (2007): A2, B1, C2, D1, E2, F1, G2, H1, I3, J1, K1.

3.1.10. *Pratylenchus sp. 3 (Fig. 4)*

Pratylenchus sp. 3 was recovered from Rwanda, Nyamata sector, Bugesera District, associated with *Zea mays*. Species delimitation analyses indicate this population to be a separate taxonomic entity. Morphologically this species is similar with *Pratylenchus* sp. 3, *P. oleae*, *P. pinguicaudatus* and *P. elamini*. With these species it shares the asexual mode of reproduction, empty reduced spermatheca, three lip annuli, and four lateral lines at times with oblique lines running in-between, and stylet morphology. Despite these similarities it differs by having a more variable and sometimes heavily crenate tail tip. Matrix code for the tabular key proposed by Castillo and Vovlas (2007): A2, B1, C2, D1, E2, F3(4), G3(2), H1, I1(2), J1, K2.

3.1.11. *Pratylenchus sp. 4 (Fig. 4)*

Pratylenchus sp. 4 was found from *Phoenix dactylifera* in Tunisia. Morphologically this species is very similar with *Pratylenchus fallax* and *P. penetrans*, with which it shares 3 lip annuli, a filled rounded spermatheca, four lateral lines sometimes with additional oblique striation, stylet morphology and a sexual mode of reproduction. It is also similar in having a variable tail tip, which is in general rounded but can also be crenate (Fig. 4). As *Pratylenchus* sp. 4 had a widespread geographic occurrence in Tunisia on *Phoenix dactylifera* (unpublished data), it is highly likely that this species was previously identified as *P. penetrans* by Troccoli et al. (1992) from Algeria and by Edongali (1996) from Libya, especially given that its morphology and morphometrics are very similar to *P. penetrans* and our population from Tunisia. Matrix code for the tabular key proposed by Castillo and Vovlas (2007): A2, B1, C3, D2, E2, F4, G2, H1(2), I3, J1, K1.

3.1.12. *Other unidentified species of Pratylenchus from the penetrans group*

Our sequence and phylogenetic analysis allowed to correct previous identification of two species of the group. The Iranian *Pratylenchus penetrans* D2-D3 of 28S rRNA gene sequence (JX261961) which was

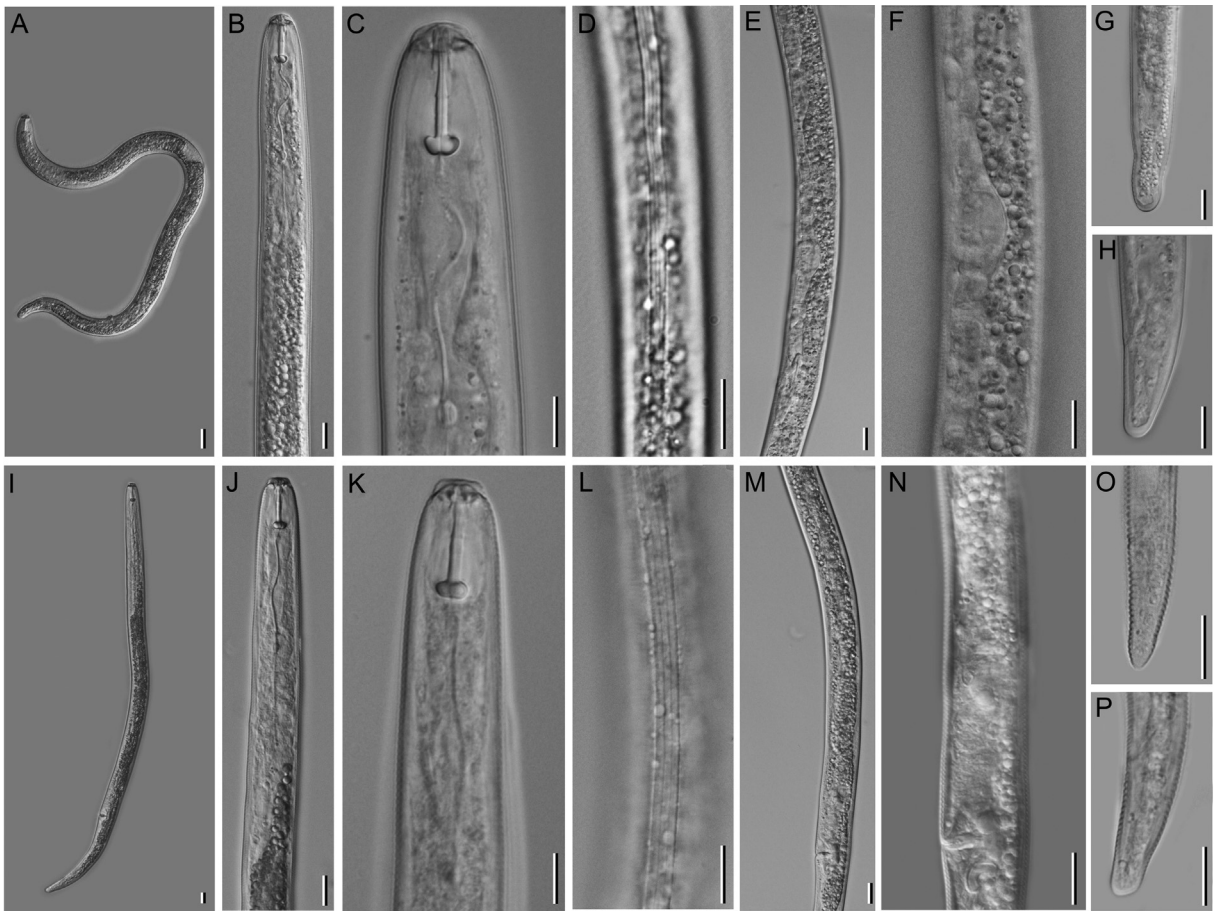

Fig. 3. Photomicrographs of specimens of *Pratylenchus* sp. 1 parasitizing *Lens culinaris* in Sicily (Italy (A-H) and *Pratylenchus* sp. 2 parasitizing an unknown host in Nigeria (I-P). A: Entire female body; B, C: Female anterior region; D: Lateral field; E, F: Vulval region; G, H: Tail region. I: Entire female body; J, K: Female anterior region; L: Lateral field; M, N: Vulval region; O, P: Tail region. Scale bars: 10 μm.

considered within the intraspecific range of *P. penetrans* by Majd Taheri et al. (2013) appeared in a fact to fall outside *P. penetrans* according to our data (Fig. 5). This species seems to be more closely related to *Pratylenchus* sp. 4 and *P. fallax* and is considered as an unidentified *Pratylenchus* in our tree. The D2-D3 sequences of Tunisian (AJ545014) and Moroccan samples (KP289344-KP289347) identified as *P. pinguicaudatus* by De Luca et al. (2004) and Mokrini et al. (2016), respectively, do not belong to this species, and, perhaps, belongs to *Pratylenchus* sp. 1 or an unidentified species. Additional morphological and molecular analyses must be carried out in order to diagnose these populations.

3.2. Phylogenetic relationships and evolution of parthenogenesis

In this study we analyzed the phylogenetic relationships within the *Penetrans* group using three gene fragments: the D2-D3 of 28S rRNA gene with the alignment of 163 *Pratylenchus* sequences, including 49 new ones (Fig. 5), the ITS rRNA gene with the alignment of 125 *Pratylenchus* sequences, including 37 new sequences (Fig. 6) and the *COI* gene with the alignment of 126 *Pratylenchus* sequences, including 91 new sequences (Fig. 7). The tree topologies obtained from these genes were generally congruent, except for the positions of several weakly supported clades. According to our analysis the *Penetrans* group contains following six valid species: *P. convallariae, P. dunensis, P. fallax, P. penetrans, P. oleae, P. pinguicaudatus* and several unidentified species. In all trees the *Penetrans* group was highly supported and according to the 28S rRNA gene, *P. brachyurus* forms the sister clade of this taxon. *Pratylenchus penetrans*, *P. fallax, P. convallariae, P. pinguicaudatus* and *Pratylenchus* sp. 4 form a monophyletic group in all trees and *P. fallax* is the sister taxon of *Pratylenchus* sp. 4 or an unidentified *Pratylenchus* sp. from Iran. The phylogenetic relationships between *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 have not yet been resolved in our analysis, however, both species have been shown to be the earliest branching species within the *Penetrans* group. Interestingly, asexual reproducing species *P. pinguicaudatus, P. oleae*, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 do not form a monophyletic grouping in our phylogenetic analyses. As a consequence, parthenogenetic lineages appear to have evolved several times independently from sexually reproducing taxa, indicating independent origins of parthenogenesis through loss of meiosis.

3.3. Molecular species delimitation

The *COI* alignment contained 95 sequences of the *Penetrans* group and was 405 base pairs long, revealing 30 different haplotypes. Molecular species delimitation using ABGD revealed 10–14 Operational Taxonomic Units (OTU's) (Table 3), differential operational taxonomic units were estimated within *P. penetrans* and *P. convallariae* according to different models and variable prior intraspecific divergence (Fig. 8, Table 3 and 4). The single threshold GMYC model predicted 11 operational taxonomic units, while the multiple threshold GMYC predicted two additional operational taxonomic units within *Pratylenchus penetrans*, yielding 13 operational taxonomic units (Fig. 8, Table 4). This variable number of estimated OTU's is not surprising given that molecular species delimitation analyses are known to generate a variety of different species hypotheses (Kekkonen and Hebert, 2014; Prevot et al., 2013). In this study we opted to follow the most stringent hypothesis, where only the OTU's that are supported by all ABGD and GYMC species delineation methods were retained. The minimal number

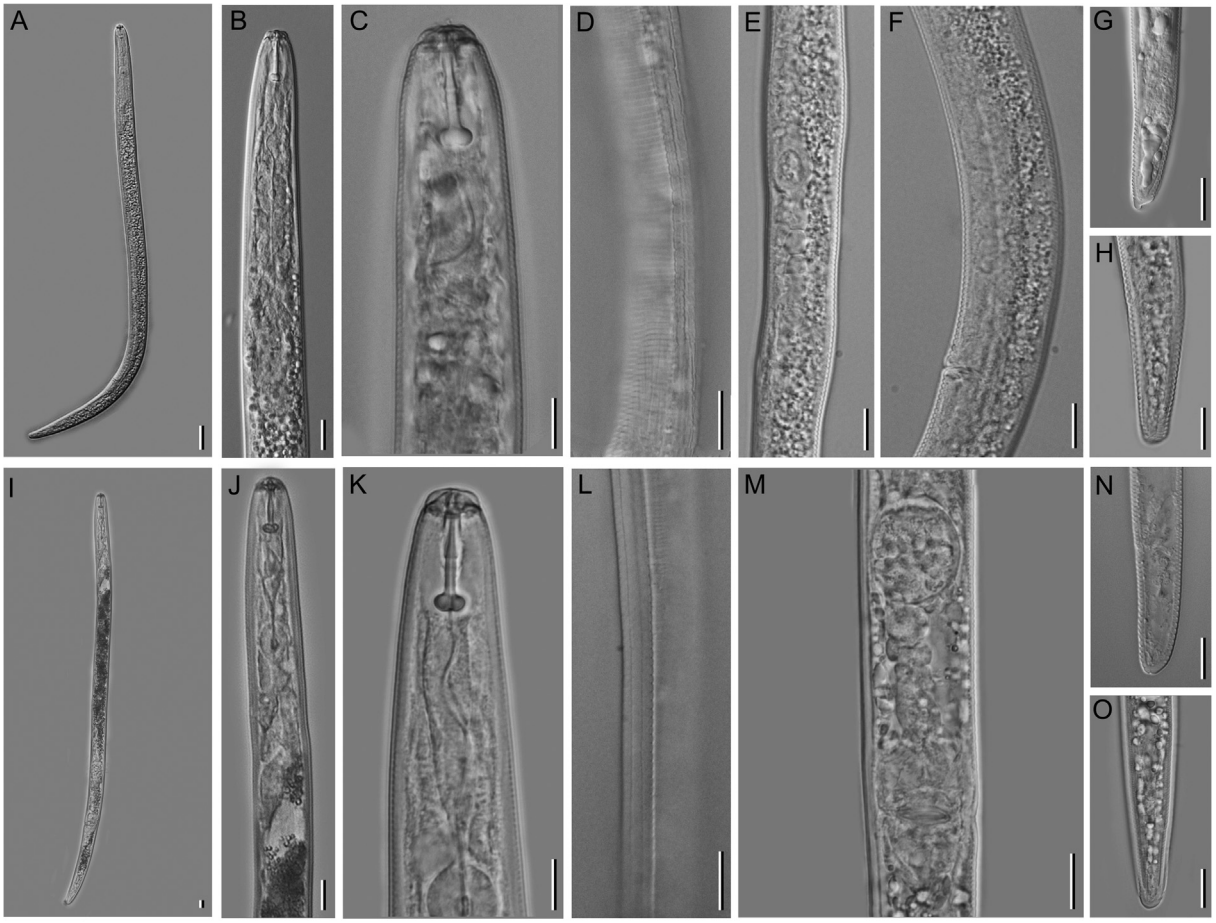

Fig. 4. Photomicrographs of specimens of *Pratylenchus* sp. 3 parasitizing *Zea mays* in Rwanda (A-H) and *Pratylenchus* sp. 4 parasitizing *Phoenix dactylifera* in Tunisia (I-O). A: Entire female body; B, C: Female anterior region; D: Lateral field; E, F: Vulval region; G, H: Tail region. I: Entire female body; J, K: Female anterior region; L: Lateral field; M: Vulval region; N, O: Tail region. Scale bars: 10 μm.

of OTU's as estimated by the ABGD analysis yielding 10 OTU's assuming a prior intraspecific divergence of 0.035938. Even following the most conservative estimate, *P. penetrans, P. fallax, P. convallariae, P. pinguicaudatus, P. oleae, P. dunensis*, and four new species are all delimitated as separate taxonomic entities, confirming signatures of independent evolution as predicted by population genetic theory (Fujisawa and Barraclough, 2013). Consequently, our species delimitation analyses confirmed the species hypotheses made by previous taxonomic studies (Cobb, 1917; Corbett, 1969; de la Peña et al., 2006; Palomares-Rius et al., 2014; Seinhorst, 1959, 1968). Importantly, all these OTU's are confirmed as being monophyletic groupings in the *COI*, ITS and 28S phylogenies (Figs. 5–7, Table 5). Additionally, all OTU's represented by more than a single *COI* sequence had a significant Rodrigo's P(RD) value, again suggesting that the observed degree of distinctiveness of these OTU's is not the product of random coalescent processes (Table 5) (Rodrigo et al., 2008). Also for the 28S rDNA region, all Rodrigo's P (RD) values were significant except those for *P. fallax* and *Pratylenchus* sp. 2 (Table 5). Furthermore, the Rosenberg's P(AB) probabilities were significant, rejecting the null hypothesis of random coalescence (Rosenberg, 2007), except for the *COI P. convallariae* and *Pratylenchus* sp.3 OTU's and the 28S rDNA *Pratylenchus* sp. 4 and *P. fallax* OTU's. All estimated *COI* OTU's are also associated with differential amino acid sequences. Amino acid variation between OTU's ranged from a minimum of 6 different amino acids (5% difference) between *Pratylenchus* sp. 4 and *P. fallax* to a maximum of 27 amino acid differences (19.7% difference) between *Pratylenchus* sp. 3 and *P. dunensis* (Supplementary Table 1).

Interestingly, the amount of intraspecific variability was found to be highly variable, for example, *P. fallax* populations form the Netherlands showed only low intraspecific variability (Table 5), while *P. penetrans* from the Netherlands were found to contain very diverse haplotypes (Fig. 9). For *P. penetrans* the *COI* haplotypes were separated into four distinct groups (A, B, C, D in Fig. 9), representing up to 8.5% nucleotide divergence. These haplotype groups were even recognized as different OTU's in some of the GMYC and ABGD species delimitation analyses (Fig. 8), providing some evidence for different divergence events within *P. penetrans*. However, these different haplotype groups appear to occur sympatrically in several locations, in Stamproy (the Netherlands) Pe4, Pe9, Pe15 and Pe16 were found in the same locality, and also in Arkel, Zoetermeer, Schimmert, Wemmeldinge, representatives from two different haplotype groups were found in a single population (Table 1). Interestingly, the variation in *P. penetrans* nucleotide haplotypes represents virtually no amino acid sequence variability (only Pe16, Pe3 and haplotype group C had a single amino acid replacement (Supplementary Table 1), indicating that nucleotide variation is mainly situated in the third codon position (codon 1 and codon 2 were 99.1% and 100% identical while codon3 was only 91.4% identical) and that the haplotype groups A, B, C and D belong to respectively the same species. Our analyses indicate *COI* to be a good barcode gene as all OTU's do not only form monophyletic groupings in the *COI* phylogeny (Fig. 7), but are also associated with high P(ID) values (Table 5). Remarkably, some *P. penetrans* haplotypes were found to occupy a wide geographical distribution; the Pe7 haplotype is widely distributed in the Netherlands but also occurs in Ethiopia and Rwanda, and the Pe11 haplotype occurs in the Netherlands, Japan and Colombia (Fig. 9).

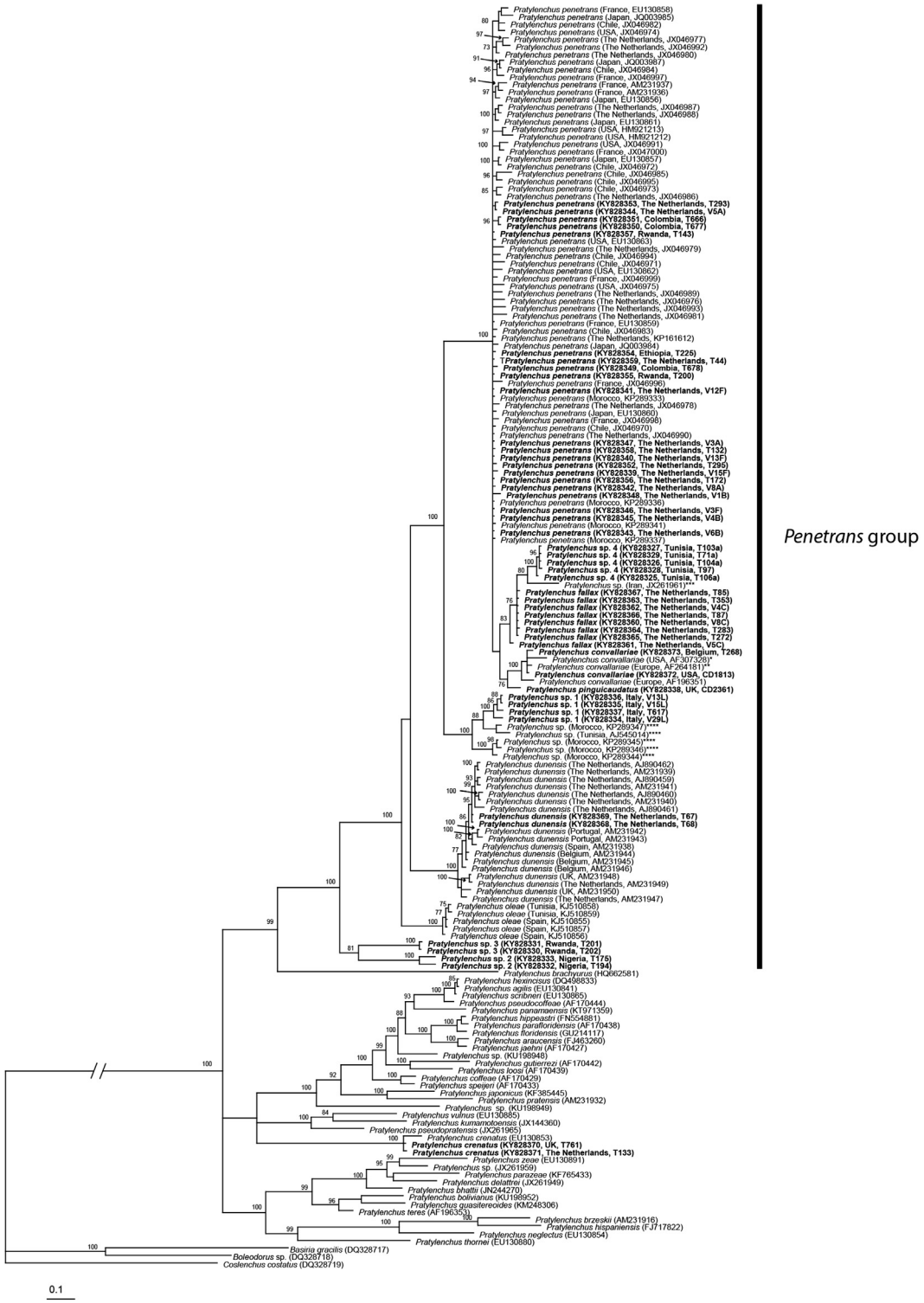

Fig. 5. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the D2-D3 of the 28 S rRNA gene sequences using the GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold. * – identified as *P. arlingtoni* by Handoo et al. (2001); ** – identified as *P. fallax* et al. (2001); *** – identified as *P. penetrans* by Majd Taheri et al. (2013); **** – identified as *P. pinguicaudatus* by De Luca et al. (2004) and Mokrini et al. (2016).

3.4. Morphometrics and morphology

A morphometric comparison of TAF fixed specimens of pure populations of *P. penetrans* (3), *P. fallax* (3), *P. convallariae* and *Pratylenchus* sp. 3 revealed both largely overlapping ranges between species and high intraspecific variability of morphometric characters (Table 2). The PCA analysis (Fig. 10) of pure populations *P. penetrans* (4), *P. fallax* (4), *P. convallariae*, *Pratylenchus* sp. 3 and *Pratylenchus* sp. 4 confirmed that morphometrics fail to separate different species within the *Penetrans* group species complex. The CDA plot did not show a clear pattern related to species and all analyzed species are not significantly different (Wilks' λ, P > 0.01). Based on the pooled within canonical structure, the morphometric characters that cause the relatively largest separation between the species, although insufficient, are b′ V (Can1: -0.33) and

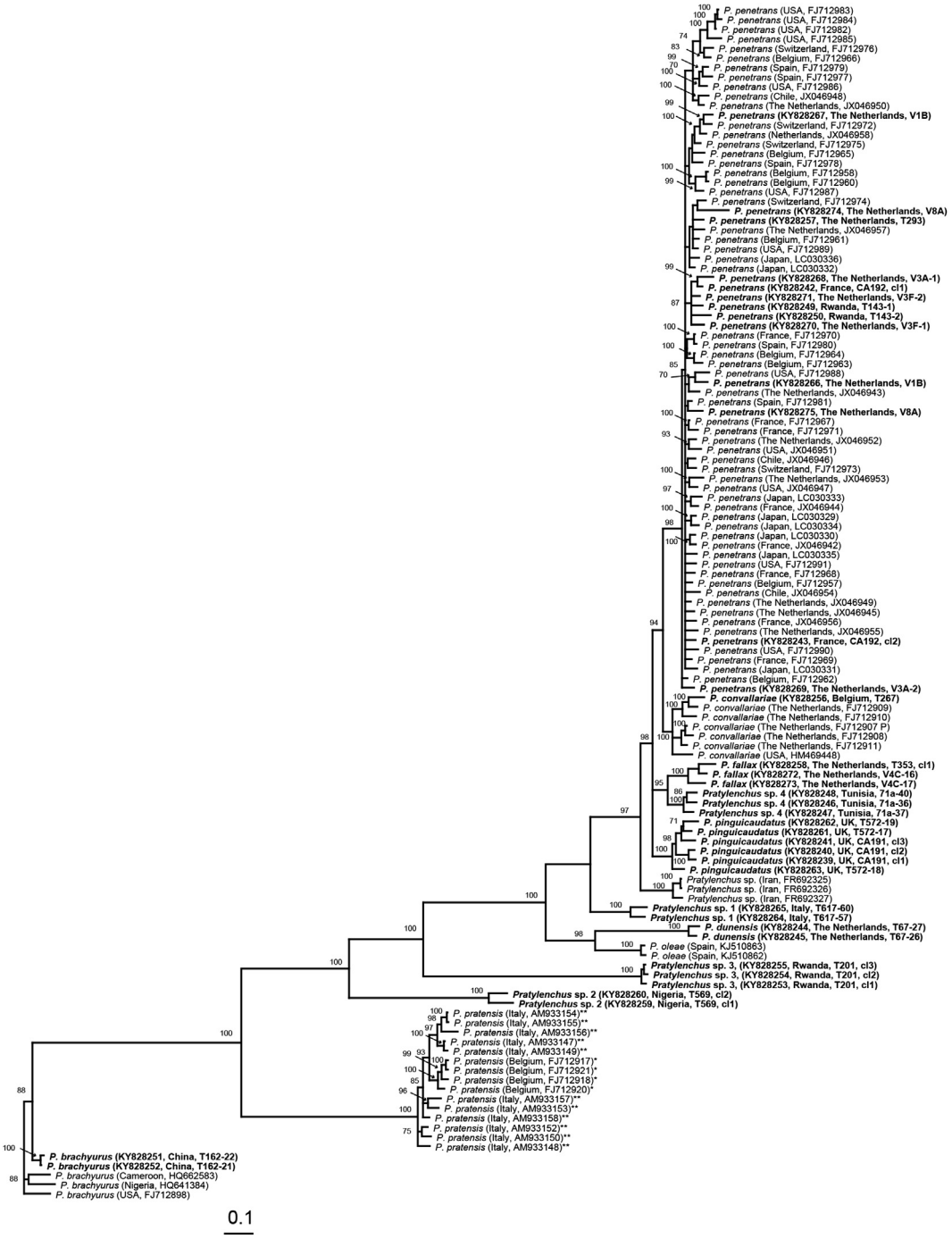

Fig. 6. Phylogenetic relationships within *Penetrans* group of the genus *Pratylenchus* as inferred from Bayesian analysis of the ITS of RNA gene sequences using the GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are displayed in bold. * - identified as *P. fallax* in the GenBank.

the position of the excretory pore (Can1: 0.25). Also the cluster analysis failed to group specimen of different species in a monophyletic cluster, again indicating that intraspecific morphometric variability is larger than interspecific morphometric variability (results not shown).

4. Discussion

Despite numerous claims in the past (Handoo et al., 2001; Holterman et al., 2009; Tarte and Mai, 1976) and a recent proposal by Helder in Jones et al. (2013) to synonymize *Pratylenchus fallax* and *P. convallariae* with *P. penetrans*, our study has clearly established these species as separate taxonomic entities with clear genetic boundaries according to ABGD and GMYC species delimitation. These different species were confirmed as being the result of speciation events rather than being the product of random coalescent processes through the use of Rosenberg's and Rodrigo's coefficients (Rodrigo et al., 2008; Rosenberg, 2007) and phylogenetic analyses of three different genes. The hypothesis that these OTU's are separate logical entities holds at least true for *P. penetrans* and *P. fallax* which are reproductively isolated (Perry et al., 1980). Moreover, Holterman et al. (2009) and Helder in Jones et al. (2013) concluded *P. penetrans*, *P. fallax* and *P. convallariae* to be conspecific based on a limited sequencing of 18S rDNA, a marker which is known to greatly underestimate biodiversity (Tang et al., 2012).

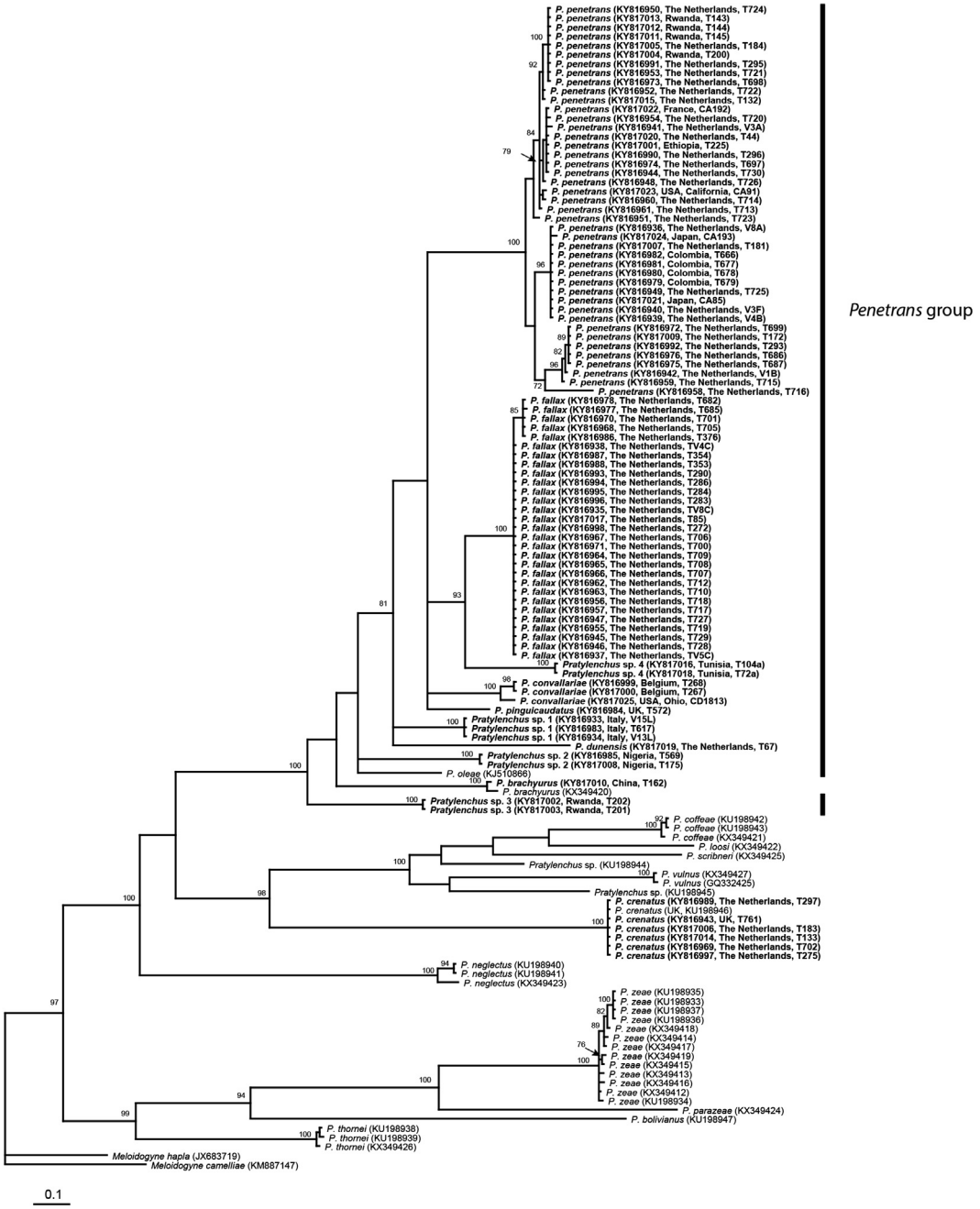

Fig. 7. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the *COI* gene sequences using the GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are displayed in bold.

Besides *P. penetrans*, *P. fallax* and *P. convallariae* our species delimitation methods also indicate *P. pinguicaudatus, P. dunesis, P. oleae* and four new species to be separate taxonomic entities. As *P. arlingtoni* has been shown to be a *species inquirenda,* this brings the total of valid species within the *Penetrans* group to 10, while the taxonomic status of some unidentified species from Iran, Morocco and Tunisia (Fig. 5) remain to be investigated. *P. brachyurus* was identified as the early-branching sister taxon of the *Penetrans* group, confirming the

Table 3
Summary of ABGD species delimitation analysis results, according to different models and differential prior intraspecific divergence (P).

			Prior intraspecific divergence (P)							
Model	X	Partition	0.001	0.00167	0.00279	0.00464	0.00774	0.01292	0.02154	0.03594
JC69	1.5	Initial	14	14	14	14	14	10	10	10
	1.5	Recursive	14	14	14	14	14	11	11	10
K80	1.5	Initial	14	14	14	14	14	10	10	10
	1.5	Recursive	14	14	14	14	14	11	11	10

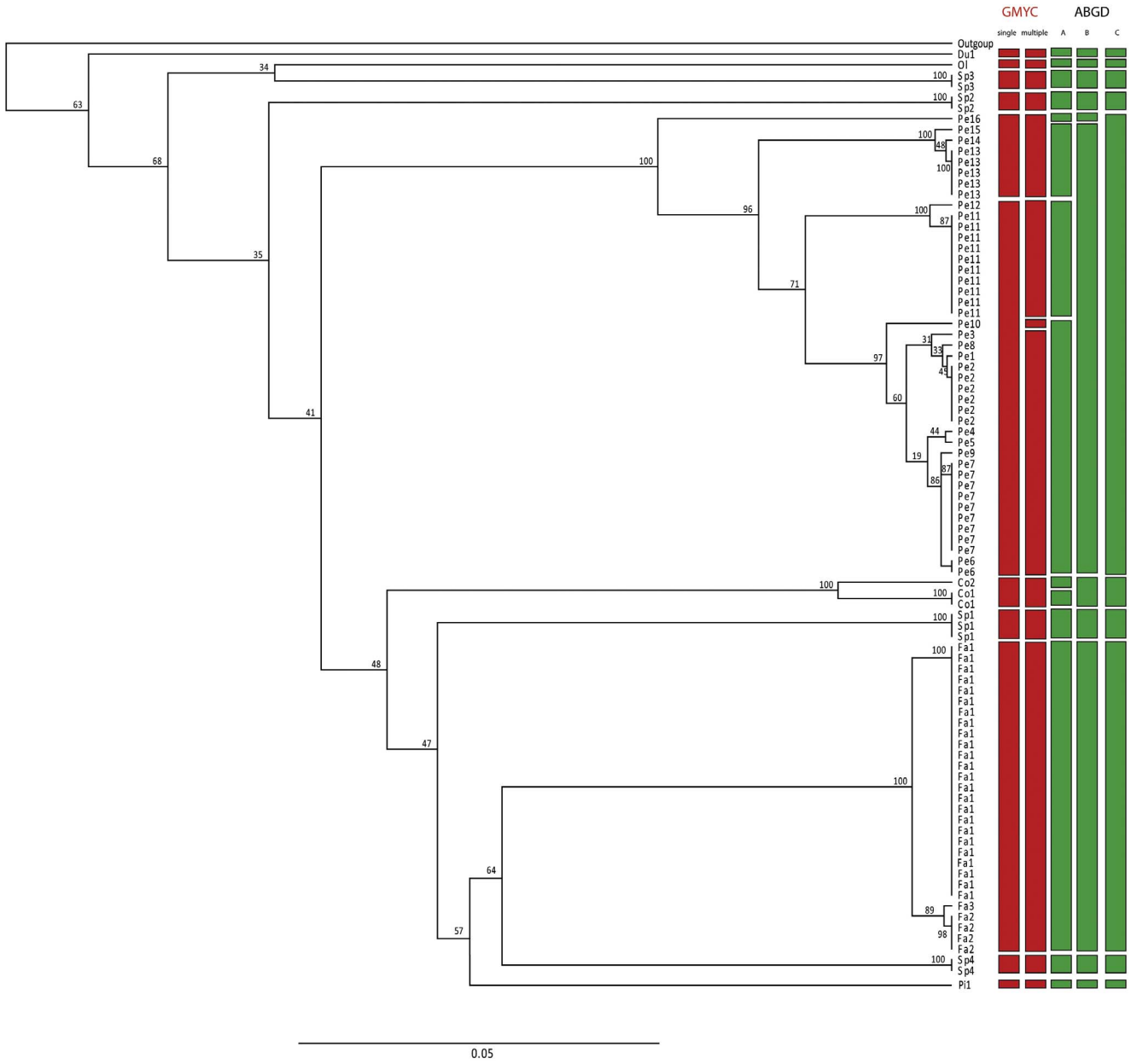

Fig. 8. Molecular species delimitation analysis of the Penetrans group. The resulting operational taxonomic units (OTU) as found by Generalized Mixed Yule Coalescent model (GMYC) and Automated Barcode Gap Discovery (ABGD) analyses are visualized as red and green bars on an ultrametric Bayesian tree of the *COI* gene. For the GMYC model the single and multiple threshold analyses are shown. For the ABGD analysis the A, B and C correspond to the 14, 11 and 10 OTU's, respectively as recovered for different prior intraspecific divergence assumptions (see also Table 3). Different haplotypes are displayed according to their *COI* haplotype name as summarized in Table 1. Bayesian posterior probabilities are indicated on the branches of the phylogenetic tree.

phylogenetic position found by Palomares-Rius et al. (2014). However, this phylogenetic position needs to be confirmed using multi-locus sequencing data in order to acquire satisfactory phylogenetic support values.

Alongside *P. brachyurus*, *P. oleae*, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 were recovered as early-branching asexual lineages of the *Penetrans* group (clade IV). Surprisingly, these parthenogenetic lineages together with *P. pinguicaudatus* do not form a monophyletic clade. Indicating that these parthenogenetic lineages evolved several times independently from sexually reproducing taxa. Mitotic parthenogenetic *Pratylenchus* lineages were previously shown to be associated with higher chromosome numbers in comparison to sexually reproducing species (Roman and Triantaphyllou, 1969): i.e. *P. scribneri* (2n = 25–26), *P. zeae* (2n = 21–26), *P. neglectus* (2n = 20) and *P.*

Table 4
Summary of GMYC species delimitation analysis results according to single and multiple threshold models.

GMYC	Clusters	Entities	Likelihood null model	Likelihood GMYC model	Likelihood ratio	Likelihood ratio test	Treshold
Single	4	11	107.7124	114.6923	13.95977	0.0009304112^{***}	−0.034947
Multiple	5	13	107.7124	114.9672	14.50958	0.000706782^{***}	−0.034947 to 0.012446

Table 5
Results of GENEIOUS species delimitation plugin (Masters et al., 2011). Posterior probability of each clade, intraspecific variability showing the genetic variability among members of a putative species, interspecific variability showing the variability between a putative species and its closest relative. Ratio between intraspecific and interspecific variability provides a measure of genetic differentiation between the focal species and it's nearest neighboring species. PID strict refers to the mean probability with the 95% confidence interval of correctly identifying an unknown specimen of the focal species using placement on a tree with the criterion that it must fall within, but not sister to, the species clade. Rosenbergs P(AB) is the probability of reciprocal monophyly under a random coalescent model (Rosenberg, 2007). Rodrigo's P (RD) is the value estimating the probability that a clade has the observed degree of distinctiveness due to random coalescent processes (Rodrigo et al., 2008).

COI

OTU	Closest species	Clade support	Intra dist	Inter dist	Intra/inter	PID strict (95% confidence interval)	Rodrigo's P(RD)	Rosenbergs P(AB)
P. penetrans	Co	100	0.167	1.407	0.12	0.95 (0.90,1.0)	< 0.05	2.4E−7
P. fallax	Sp4	100	0.030	0.905	0.03	0.98 (0.93,1.0)	< 0.05	1.4E−4
P. convallariae	Pe	100	0.107	1.407	0.08	0.74 (0.57, 0.92)	< 0.05	0.17
P. oleae	Sp1	–	0	1.301	–	–	–	–
P. dunensis	Co	–	0	1.614	–	–	–	–
P. pinguicaudatus	Co	–	0	0.172	–	–	–	–
P. sp. 1	Fa	100	0.014	1.253	0.01	0.78 (0.61,0.96)	< 0.05	2.9E−7
P. sp. 2	Ol	100	0.013	1.438	0.01	0.59(0.44,0.74)	< 0.05	6.4E−6
P. sp. 3	Ol	100	0.015	1.362	0.01	0.59(0.44,0.73)	< 0.05	0.33
P. sp. 4	Fa	100	0.015	0.905	0.02	0.58(043,073)	< 0.05	1.4E−4
28S rRNA								
OTU	Closest species	Clade support	Intra dist	Inter dist	Intra/inter	PID strict (95% confidence interval)	Rodrigo's P(RD)	Rosenbergs P(AB)
P. penetrans	Fa	98	0.246	0.622	0.40	0.87 (0.82,0.93)	< 0.05	1.3E−9
P. fallax	Sp4	/	0.052	0.367	0.14	0.88 (0.78,0.99)	–	–
P. convallariae	Fa	100	0.197	0.621	0.32	0.72 (0.59,0.85)	< 0.05	1.3E−9
P. oleae	Du	100	0.069	1.700	0.04	0.91 (0.78,1.0)	< 0.05	2.6E−10
P. dunensis	Ol	100	0.249	1.700	0.15	0.94 (0.89,0.99)	< 0.05	3.4E−24
P. sp. 1	Fa	100	0.188	1.434	0.13	0.78 (0.64,0.92)	< 0.05	6.6E−9
P. sp. 2	Sp3	100	0.418	2.101	0.20	0.49 (0.34,0.64)	0.43	2.1E−6
P. sp. 3	Sp2	100	0.051	2.101	0.02	0.58 (0.43,0.73)	< 0.05	2.2E−6
P. sp. 4	Fa	100	0.072	0.367	0.20	0.80 (0.68,0.93)	< 0.05	0.07

brachyurus (2n = 30–32) as opposed to the sexually reproducing *P. penetrans* (n = 5), *P. vulnus* (n = 6) and *P. coffeae* (n = 7). As the parthenogenetic *P. brachyurus* (2n = 30–32) is positioned as the early-branching sister taxon of the *Penetrans* group (Palomares-Rius et al., 2014), it is likely that *Pratylenchus* sp. 2, *Pratylenchus* sp. 3, *P. oleae* and *P. pinguicaudatus* are also associated with higher chromosome numbers given their asexual mode of reproduction. This indicates that a parthenogenetic reproduction mode might be triggered by a change in ploidy level through a genome duplication or hybridization and a consequently loss of meiosis as was previously suggested to be the case for root-knot nematodes (Janssen et al., 2017 b; Janssen et al., 2016; Lunt et al., 2014), and is also the case in many different metazoan taxa (Comai, 2005). In this study we tried to confirm this by karyotyping these *Pratylenchus* species, however, staining with propionic orcein and fluorescent dyes consistently failed. As a consequence this hypothesis remains to be confirmed, probably genome sequencing in combination with gene copy number analysis could provide a solution as already shown for root-knot nematodes (Lunt et al., 2014). Interestingly, *Pratylenchus bolivianus* was recently shown to have both amphimictic and parthenogenetic populations (Troccoli et al., 2016), also there the biological reason for change in reproductive strategy remains to be investigated.

Our phylogenetic analyses indicate that the *Penetrans* group might originate from Africa. The two earliest branching species of the *Penetrans* group, *Pratylenchus* sp. 2 and *Pratylenchus* sp. 3 are recovered from Africa, and the other early-branching asexual species *P. oleae* was found in North Africa and at the Mediterranean Sea. Also, *P. brachyurus*, early-branching sister taxon of the *Penetrans* group, albeit with poor branch support, is widely distributed in Africa (Castillo and Vovlas, 2007; Palomares-Rius et al., 2014). In this study *P. fallax* was found to be widely distributed in The Netherlands, parasitizing grasses, *Malus pumila*, *Prunus domestica*, *Prunus avium, Pyrus* sp. and *Vitis vinifera*, which confirms the previously suggested distribution pattern (Seinhorst, 1968, 1977). However, based on morphological identifications, *P. fallax* has been reported to be widely distributed in Europe, and present in Russia, India, Japan and the USA (Castillo and Vovlas, 2007), associated with a variety of crops. Ideally these morphological identifications should be confirmed using molecular data. While intraspecific variability was low for *P. fallax* within the Netherlands, Dutch *P. penetrans* populations showed high intraspecific variability (Fig. 9). Interestingly, this intraspecific variability was mainly confined to the third codon position of the *COI* gene. As a consequence this nucleotide variation was not associated with variations in amino acid sequences, indicating that this variability is biologically insignificant and confirming that different *P. penetrans* haplotypes are most likely conspecific. Recently, a similar variability pattern was discovered in *Longidorus orientalis*, displaying 15.5% intraspecific *COI* variability, which translated in only 1% intraspecific amino acid variation (Subbotin et al., 2015).

Despite the large intraspecific variability recovered in *P. penetrans*, identical *P. penetrans* haplotypes were found to be geographically widespread. This wide distribution of haplotypes indicates that *P. penetrans* populations are not reproductively isolated, indicating that *P. penetrans* could have been spread anthropogenically through agricultural development and crop exchange. While little is known about the natural dispersal capacities of plan-parasitic nematodes, anthropogenic distribution was previously suggested to explain the distribution patterns of other plant-parasitic nematodes, namely in root-knot nematodes (Castagnone-Sereno et al., 2013; Janssen et al., 2016) and cyst nematodes (Boucher et al., 2013; Plantard et al., 2008).

In this study, it has been demonstrated that morphometric characteristics are not useful for species diagnosis within the *Penetrans* group because of the large intraspecific morphological variability. In contrast to morphometrics, morphology can be used in some cases to identify species within the *Penetrans* group. However, morphological diagnostic characteristics are scarce and phenotypic plasticity is rampant as previously shown by Tarte and Mai (1976). For example, tail morphology is useful in some cases but cannot be used to differentiate all species of the *Penetrans* group, for example; *Pratylenchus penetrans* can be differentiated from *P. fallax* based on a larger hyaline tail tip region, which is proportionally less crenate in a given population (Seinhorst, 1968). However, based on tail morphology *P. penetrans* is hard to differentiate from *Pratylenchus* sp. 1 and *Pratylenchus* sp. 4, while *P. fallax* is difficult to differentiate from *P. convallariae* (Castillo

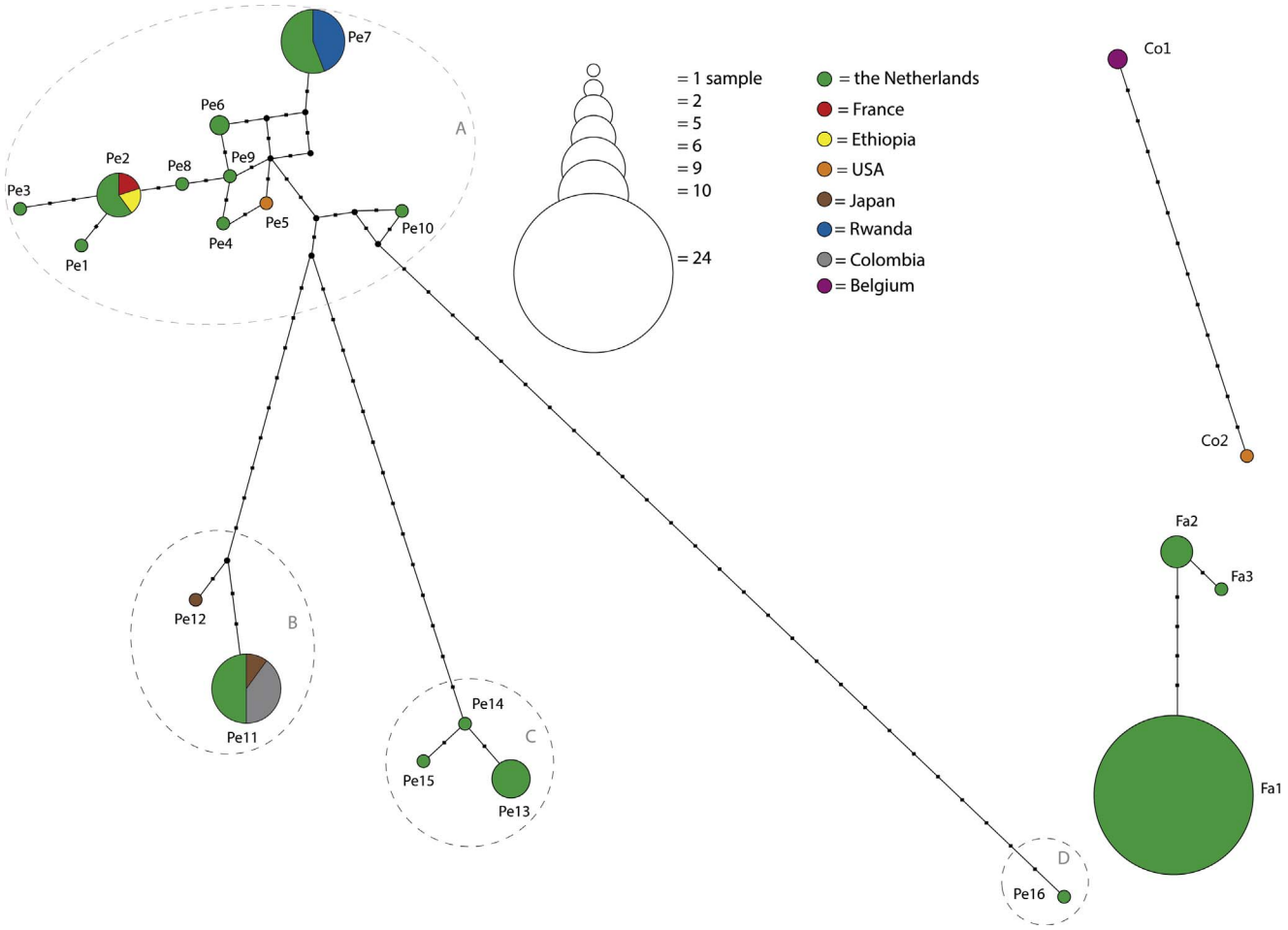

Fig. 9. *COI* haplotype network of *Pratylenchus penetrans* (left), *Pratylenchus convallariae* (upper right) and *Pratylenchus fallax* (lower right). The haplotype network shows the relationships between different haplotypes, circle size is equivalent to the number of studied specimen and branch length is equivalent to the number of mutations (shown as black squares). Different haplotypes are displayed according to their *COI* haplotype name as summarized in Table 1. Different geographic origins are visualized by different colors, median vectors are shown as black circles. Within *Pratylenchus penetrans*, different haplotype groups (A, B, C and D) are highlighted by a dashed circle.

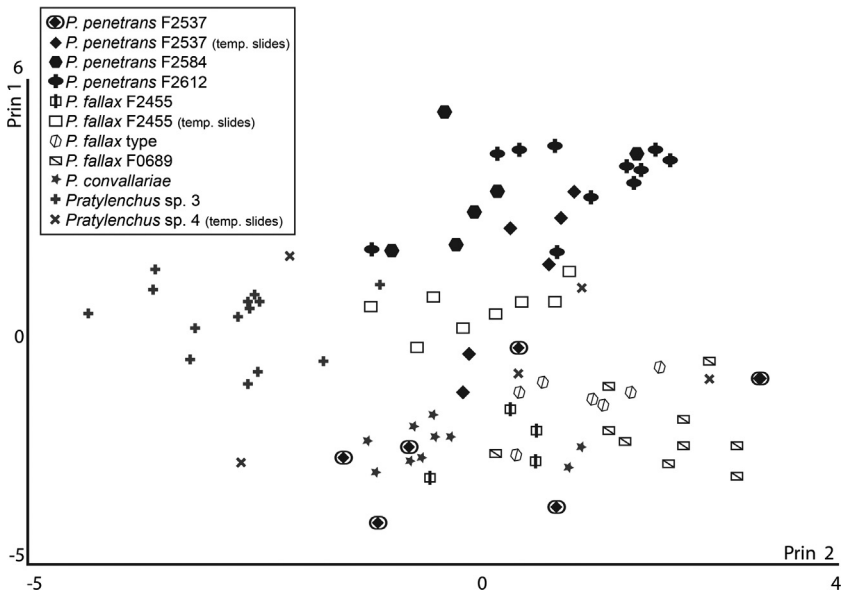

Fig. 10. PCA plot of penetrans group species based on the female morphometric values, including L, V, a, b, c, c′, stylet length, body width, post-uterine sac length, tail length, and position of metacorpus, cardia, pharyngeal lobe, excretion pore, vulva in respect to anterior end.

and Vovlas, 2007). The proportional spacing of lines in the lateral field was proposed as a way to differentiate *P. dunensis* from *P. penetrans* by de la Pena et al. (2006) and this is confirmed in our study. Nevertheless, other morphological characters associated with the lateral field are highly variable. Phenotypic plasticity within a single species includes areolation, striations and oblique lines within the lateral field and a large number of specimens must be examined in order to accurately diagnose the species of the *Penetrans* species complex. Especially difficult to distinguish are the asexually reproducing species, *Pratylenchus* sp. 2, *Pratylenchus* sp. 3, *Pratylenchus oleae* and *Pratylenchus pinguicaudatus*, which share most of their morphological characteristics and are almost indistinguishable using morphology. Although *Pratylenchus* sp. 3 appears to have a more variable tail tip, also *Pratylenchus* sp. 2 and *Pratylenchus oleae* have a variable crenated tail tip (Palomares-Rius et al., 2014), while *P. pinguicaudatus* was described as having a rounded tail tip (Corbett, 1969).

A further complication to morphological identification is the presence of sympatric species. For example, in this study, one sample form St. Oedenrode (the Netherlands) contained a mix of *P. penetrans* and *P. fallax*. Moreover, recently it has been noted that sexual and asexual populations of *Pratylenchus bolivianus* can be associated with differential morphotypes (Troccoli et al., 2016). These findings need to be accounted for as reproduction modes are highly variable within the *Penetrans* group, and diagnostic characters related to the reproductive system may vary depending on the reproductive state of the population. As demonstrated by the numerous morphologically misidentified species on GenBank, morphological identification of species within the *Penetrans* group can only be reliably done by specialists with years of experience and only then in combination with high quality reference material. Furthermore, traditional morphological identification should be supplemented with molecular analysis in order to robustly diagnose populations.

Augmenting the complexity of the species diagnosis problem is the presence of several morphospecies that are morphologically similar to species in the *Penetrans* group but which remain uncharacterized by molecular data. For example, *P. pseudofallax* and *P. subpenetrans* were differentiated form *P. fallax* and *P. penetrans* by morphometric characteristics (body length, length of the post uterine sac, stylet length, position of the vulva), the presence of males, the shape of the spermatheca and the shape of tail terminus (Café-Filho and Huang, 1989; Taylor and Jenkins, 1957). However, in reality differentiating *P. pseudofallax* and *P. subpenetrans* is difficult for the following main reasons: i) morphometric and morphological differentiating characters fall within the range of species within the *Penetrans* group complex as displayed by the ranges of morphometric variables in our study; ii) the presence of males and shape of the spermatheca might dependent on the reproductive phase of the population (Troccoli et al., 2016); iii) the tail shape can be highly variable within species of the *Penetrans* group; and iv) the reported hosts of *P. pseudofallax* (*Malus silvestris*) and *P. subpenetrans* (*Chrysanthemum* and ginseng) are also shown to be good hosts for *P. penetrans* (Castillo and Vovlas, 2007). Consequently, confirming the taxonomic status of *P. pseudofallax* and *P. subpenetrans* will require sequencing of topotype material as collecting topotype material has often proved to be the only way to confidently connect DNA sequences to formerly described morphospecies (De Luca et al., 2010; Inserra et al., 2007; Troccoli et al., 2016).

DNA barcoding has been proposed as a successful diagnostic strategy in many organisms (Hebert et al., 2003). DNA barcoding is especially useful in taxa that lack diagnostic morphological features (Hebert et al., 2003). In this regard, DNA barcoding was explored for many nematode taxa including marine nematodes (Derycke et al., 2010), root-knot nematodes (Janssen et al., 2016), Aphelenchoididae (Sánchez-Monge et al., 2017). In the current study, the *COI* gene of the mitochondrial genome was explored as a barcode marker for *Pratylenchus*. Our results indicate *COI* to be a reliable barcode marker as indicated by strict P(ID) values, indicating that the mean probability of making a correct identification of an unknown specimen based on its placement in a tree is high when using *COI* as a barcode marker (Masters et al., 2011). Although DNA barcoding was shown to be a reliable diagnostic strategy it is highly important to safeguard the link between DNA barcodes, morphology and sequences from topotype locations, as exemplified by the high number of misidentified species present on GenBank.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.07.027.

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