A study of the systematics and implications of the presence of the testa nematode, *Aphelenchoides arachidis* Bos, 1977 in South Africa

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Abstract

An introduction to nematode systematics is provided which deals broadly with the history of the classification of nematodes, the controversial usage of the Phylum names Nemata Cobb, 1919 and Nematoda (Rudolphi, 1808) Lankester, 1877 and the reason why the name Nematoda was used in the present study. The classification, diagnosis and bionomics of the genus *Aphelenchoides* Fischer, 1894, the genus to which *A. arachidis* Bos, 1977 belongs is discussed. The section on bionomics is included to capture the astounding ability of this group of organisms to adapt to different trophic levels, a concept that is used to attempt an explanation for the ability of a supposedly African nematode, *A. arachidis*, to infest an alien crop species (groundnut). The ability of *Aphelenchoides* spp. to adapt to different host plant species is discussed, as well as the ability of the groundnut plant to mature its pods underground, a characteristic that predisposes these plants to a host of pathogens. The damage caused by two of the most important endoparasitic nematode species on groundnut, *A. arachidis* and *Ditylenchus africanus* Wendt, Swart, Vrain & Webster, 1995 were compared with each other. The South African population of *A. arachidis* was found predominantly in the shells of groundnut, whereas they were found in the shells, roots, hypocotyls and testas of the groundnut plants in Nigeria. The present study showed that *A. arachidis* and *D. africanus* occur together in groundnut in South Africa with *D. africanus* usually being the dominant species. In only one instance, at Bullhill (Vaalharts Irrigation Scheme, Northern Cape), the groundnut shells and testas were infested by *A. arachidis* alone. The importance of plant quarantine in South Africa is dealt with and the aims and principles of quarantine, as well as the different guidelines that have to be adhered to when deciding on the quarantine status of an organism are explained. Descriptions are provided of the methods used to prepare specimens for viewing with the light microscope (LM) and the scanning electron microscope (SEM), as well as the procedures of the molecular study. A morphological and morphometrical description of *A. arachidis* specimens from South Africa, as well as a comparison with specimens from Nigeria was done. Differences between the South African and Nigerian populations included, respectively, a lower b-value (7 – 11 *vs* 10 – 18), more lateral lines (2 – 4 *vs* 2), a slightly shorter stylet (8 - 10 µm *vs* 10 - 12 µm) and a longer length of the post-uterine sac as a percentage of the

distance from vulva to anus (41 – 96 % *vs* ± 50 %). Scanning electron micrographs of this species are presented for the first time and shows the morphology of the lip region and lateral lines. Since both *A. arachidis* and *A. blastophthorus* were detected in the pods, a study was done to evaluate a PCR-based diagnostic method for the identification of these species and to compare the results with those reported in literature. Restriction fragment length polymorphisms (RFLPs) in the rDNA fragment were used to compare and differentiate between nematode species. The differences encountered within the South African population (morphological, morphometrical and molecular) warrant a study of more specimens from more localities. Through this it could be ascertained whether the South African population is a subspecies of *A. arachidis* or if this species just differs widely between localities. Future research should focus on a survey of the groundnut producing areas in South Africa to determine the distribution and economic impact of *A. arachidis*. The incidence of *A. arachidis* on other agricultural crops, especially those used in rotation with groundnut, also needs to be determined. The next issue to address is what enables a supposedly endemic species to Africa, *A. arachidis*, to parasitize an alien plant species (groundnut) from South America. Screening of the endemic bean family (Fabaceae) in Africa for the presence of *A. arachidis*, could hold the answer to this question.

Key words: *A. arachidis, A. blastophthorus, A. fragariae, Aphelenchoides*, description, groundnut, new record, Nigeria, PCR, SEM, South Africa, taxonomy.

Uittreksel

'n Algemene inleiding tot die sistematiek van die Nematoda wordgegee. In hierdie inleiding word die geskiedenis van die klassifikasie van nematodes word gegee behandel asook die teenstrydige gebruik van die filumname Nemata Cobb, 1919 en Nematoda (Rudolphi, 1808) Lankester, 1877. Die rede waarom Nematoda in hierdie studie gebruik word, word verskaf. Die klassifikasie, diagnose en ekologie van die genus *Aphelenchoides* Fischer, 1894 waartoe die onderwerpspesie *A. arachidis* Bos, 1977 behoort, word bespreek. Die gedeelte oor die ekologie van die spesies is ingesluit om die uitsonderlike vermoë van hierdie groep om aan te pas by verskillende trofiese vlakke, aan te dui. Hierdie agtergrond is dan ook gebruik om die verbintenis met 'n uitheemse gewas (grondboon) deur 'n waarskynlik inheemse Afrikanematode, *A. arachidis*, te verduidelik. Die aanpasbaarheid van *Aphelenchoides* spp. op verskillende gasheerplantspesies is bespreek, asook die vermoë van die grondboonplant om peule ondergronds te laat ryp word, 'n eienskap wat plante kwesbaar maak vir verskeie patogene en parasiete. Die skade wat veroorsaak word deur twee van die mees belangrike endoparasitiese nematodespesies van grondbone, nl. *A. arachidis* and *Ditylenchus africanus* Wendt, Swart, Vrain & Webster, 1995, is met mekaar vergelyk. Die Suid-Afrikaanse bevolking van *A. arachidis* is hoofsaaklik in grondboondoppe gevind terwyl dié van die Nigeriese bevolking in doppe, wortels, hipokotiele en testas van grondboonplante voorkom. Hierdie studie het getoon dat *A. arachidis* en *D. africanus* in Suid-Afrika gelyktydig op grondbone mag voorkom. In slegs een geval, te Bullhill (Vaalharts besproeiingskema, Noord-Kaap) was grondboondoppe en testas slegs deur *A. arachidis* besmet. Die belangrikheid van plantkwarantyn in Suid-Afrika is bespreek en die beginsels, asook die verskillende riglyne waarby gehou moet word wanneer op die kwarantynstatus van 'n organisme besluit word, is verduidelik. Die metodes wat gebruik is om eksamplare voor te berei vir ondersoek met 'n ligmikroskoop en skandeerelektronmikroskoop is verskaf, asook die metodes wat in die molekulêre studies gebruik is. Morfologiese en morfometriese beskrywings van *A. arachidis*-eksemplare van Suid-Afrika is gedoen en met eksemplare vanuit Nigerië vergelyk. Verskille tussen die Suid-Afrikaanse en Nigeriese eksemplare het onderskeidelik ingesluit 'n laer b-waarde (7 – 11 *vs* 10 – 18),

meer laterale lyne (2 – 4 *vs* 2), 'n effens korter stilet (8 - 10 µm *vs* 10 - 12 µm) en 'n langer post-uteriene sak as persentasie van die afstand vanaf die vulva tot by die anus (41 – 96 % *vs* ± 50 %). Skandeerelektronmikrograwe wat vir die eerste keer die morfologie van die lip-streek en laterale lyne van hierdie spesie aantoon, is ontwikkel. Aangesien beide *A. arachidis* en *A. blastophthorus* in peule aangetref is, is 'n studie gedoen om 'n PKR-gebasseerde diagnostiese metode vir die identifikasie van hierdie spesies te evalueer en resultate te vergelyk met beskrywings in die litertuur. Restriksie fragment lengte polimorfismes (RFLPs) in die rDNA-fragment is gebruik om te vergelyk en te onderskei tussen nematodespesies. Die verskille wat waargeneem is binne die Suid-Afrikaanse bevolking (morfologies, morfometries en molekulêr) regverdig 'n studie van meer eksemplare vanaf bykomende lokaliteite. Daardeur sal vasgestel kan word of die Suid-Afrikaanse bevolking 'n subspesie van *A. arachidis* is en of hierdie spesie net grootliks varieer tussen lokalitiete. Toekomstige navorsing behoort te fokus op 'n opname in die grondboonproduksiegebiede van Suid-Afrika om die verspreiding en ekonomiese impak van *A. arachidis* vas te stel. Die voorkoms van *A. arachidis* op ander landbougewasse, veral die wat in wisselbou met grondbone gebruik word, moet bepaal word. Die volgende aspek wat aangespreek behoort te word, is die vermoë wat 'n sogenaamde inheemse spesie van Afrika, *A. arachidis* besit wat dit in staat stel om 'n uitheemse plantspesie (grondbone) vanuit Suid-Amerika te parasiteer. Evaluering van die boonfamilie (Fabaceae) in Afrika vir die teenwoordigheid van *A. arachidis* sal moontlik die antwoord tot hierdie vraag bied.

Sleutelwoorde: *A. arachidis, A. blastophthorus, A. fragariae, Aphelenchoides*, beskrywing, grondbone, nuwe rekord, Nigerië, PCR, SEM, Suid-Afrika, taksonomie.

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CHAPTER 1

Introduction and literature review

1. Introduction

The groundnut, *Arachis hypogaea* L., belongs to the bean family Fabaceae. According to Duke (1983) it is endemic to South America and unlike other beans the groundnut plant is a single bush that matures its pods underground. In summer the plant starts to blossom and within a few days a unique floral structure, called a peg, forms and begins to grow downward towards the soil. The peg consists of the ovary with 1-5 ovules. It grows out from the floral bracts, bearing with it the dried petals, calyx lobes and hypanthium (Duke, 1983). In a few weeks the peg burrows its way into the soil and grows in a horizontal direction. At the tip, the peg with its ovules develops into a pod (Duke, 1983). By late summer the pod develops a hard shell, while on the inside the seeds or groundnuts are taking shape and maturing (Allen & Allen, 2002).

The agriculturally important testa nematode, *Aphelenchoides arachidis* Bos, 1977 is reported for the first time from South Africa and for the first time outside Nigeria in this study. Initially it was found in the groundnut-producing Vaalharts area of the Northern Cape Province during a survey conducted by the National Department of Agriculture in 2002. After that, *A. arachidis* has been found in Uganda (only juveniles) in 2004, and in 2005 at the farm Bullhill, Vaalharts Irrigation Scheme. The Uganda specimens were associated with maize roots and the Bullhill specimens with groundnut shells.

1.1. Importance of *Aphelenchoides arachidis*

Bos (1977) originally described the testa nematode, *A. arachidis* from groundnut in northern Nigeria where shriveled seeds were symptomatic of infested pods. It was the

first record of a seed-borne nematode infestation of groundnut (Bridge *et al.*, 1977). According to Bos, (1977, Cited by Minton & Baujard, 1990), there may be two biotypes of *A. arachidis* in Nigeria, one occurring on cereals and one occurring on both groundnut and cereals. According to Bridge *et al.* (1977), *A. arachidis* is a parasite of pods, testae, roots and hypocotyls but not the cotyledons, embryos or other parts of the groundnut plant. Nematodes are found mainly in the subepidermal parenchymatous layer and around the tracheids of the testa. They can survive desiccation in stored groundnut pods for up to 12 months. The nematode predisposes seeds to an invasion by fungi that may lead to reduced seed emergence. Although *A. arachidis* devalues confectionary peanut, no actual yield reduction due to the presence of this nematode has so far been reported (Dickson & De Waele, 2005). Because of its limited distribution in Nigeria *A. arachidis* has not caused major economic losses, but if it should become established in other groundnut-producing regions of the world, it could become a major economic pest (Dickson & De Waele, 2005). *A. arachidis* can also be disseminated in infested seeds increasing its potential as a worldwide pest (Dickson & De Waele, 2005). Khan & Misari (1992) did a survey of the four ecological zones of Nigeria and found 22 genera of plant-parasitic nematodes in the rhizosphere, roots and pods of groundnut. They postulated that due to the high frequency of occurrence, high populations and direct and indirect evidence of its pathogenicity, *A. arachidis* could be considered as one of the more important nematode pests of groundnut in Africa. The Commonwealth Agricultural Bureaux International and European Mediterranean Plant Protection Organisation (CABI/EPPO) Crop Protection Compendium (2004) documented Nigeria as the only country where this nematode species occurs and reports maize, sorghum, pearl millet, sugar cane and rice as alternative hosts of *A. arachidis*. *A. arachidis* is labelled a quarantine species by countries such as India where it is registered as a Schedule-V organism, i.e. post-entry quarantine for a period of six weeks and only decorticated seeds are permitted (India: Ministry of Agriculture, 2003).

1.2. A brief historical review of the classification of nematodes

As the present study includes nematode systematics and taxonomy, it is necessary to give a brief historical overview of the subject. During the past 200 years, nematodes have been assigned to four different phyla and were related to rotifers, gastrotrichs, kinorhynchs and nematomorphs (Maggenti, 1981). Even lately, the relationship between nematodes and other organisms remains an area of debate (Lorenzen, 1983; Hodda, 2006). Linnaeus (1758, Cited by Chitwood, 1958) placed nematodes in the Class Vermes, which, according to current knowledge, contained many unrelated organisms. Rudolphi (1808, Cited by Chitwood, 1958) proposed the Class Helmintha and subdivided it into five orders: Trematoda, Cestoidea, Cystica, Nematoidea (to host the nematode worms) and Acanthocephala. Rudolphi, unfortunately, omitted all free-living and plant-parasitic nematode species from the Nematoidea. Leuckart and also Von Siebold (1843, Cited by Chitwood, 1958) emended the spelling of Nematoidea to that of Nematodes and included horse-hair worms in this order. Diesing (1851, Cited by Chitwood, 1958) further emended the Order Nematodes to the Order Nematoda and included horse-hair worms, free-living nematodes and plant-parasitic nematodes into this order. Cobb (1917, Cited by Chitwood, 1958) separated the nematodes from all other organisms and placed them in a phylum of their own, the Phylum Nemates, later emended to Nemata by Pearse (1936, Cited by Chitwood, 1958). Potts (1932, Cited by Chitwood, 1958) promoted the Order Nematoda to the rank of phylum, the Phylum Nematoda.

1.3. Notes on the usage of the Phylum names Nemata and Nematoda

Since these early years, the name of the phylum remained a debate among nematode taxonomists (Maggenti, 1981; Anon., 2006a) and both names, Nemata Cobb, 1919 and Nematoda (Rudolphi, 1808) Lankester, 1877, are recognised by different workers in the field. Decraemer (2000) urged nematologists to decide on the use of one name for the phylum and suggested that as Nematoda is the name most commonly used, nematologists

should accept it. Since the Systema Naturae 2000 (Anon., 2006b) endorses the Phylum Nematoda this was the name that was adhered to in this study. According to Systema Naturae 2000 the classification of the Phylum Nematoda is as follows:

Biota
Domain: Eukaryota
Kingdom: Animalia
Subkingdom: Bilateria
Branch: Protostomia Grobben
Infrakingdom: Ecdysozoa Aguinaldo
Superphylum: Aschelminthes
Phylum: Nematoda (Rudolphi, 1808) Lankester, 1877
Class: Adenophorea Brusca & Brusca, 1990
Class: Secernentea Von Linstow, 1905

1.4. Systematic position of the genus *Aphelenchoides* Fischer, 1894

All living organisms are organized into a hierarchy of groups called taxa. This structure is based primarily on the degrees of similarity among members of the same group and also shows the contrasts among members of different taxa. The development of the light microscope (LM) and scanning-electron microscope (SEM) made it possible to classify nematodes on the basis of morphology. Recently the study of nematode biochemistry and DNA have changed or further defined taxonomic groupings (De Ley & Blaxter, 2002). Molecular techniques have been developed to provide a genetic basis of phylogeny (biological classification concerned with the history of speciation), referred to as molecular phylogenetics. Molecular approaches for distinguishing nematodes and examining genetic diversity are evolving rapidly and traditional methods such as morphology are rapidly augmented with molecular techniques (De Ley & Blaxter, 2002; Hooper *et al.*, 2005). This approach, a combination of LM, SEM and molecular techniques has been used during this study. The results are presented in Chapter 3.

According to Andrássy (1976), Luc *et al.* (1987), Maggenti *et al.* (1987), Nickle and Hooper (1991), Cited by Hunt, 1993), the majority of classification outlines for the aphelenchs regard them as members of the suborder Aphelenchina Geraert 1966 and under the order Tylenchida Thorne, 1949. Hunt (1993), however, saw the group as sufficiently distinct to warrant their classification in their own order, the Aphelenchida. Hunt's (1993) reason for his decision seems morphologically sound, compelling the author to use it in the present study. The outline classification of the genus *Aphelenchoides* according to Hunt (1993) is as follows:

Class: Secernentea Von Linstow, 1905
Order: Aphelenchida Siddiqi, 1980
Superfamily: Aphelenchoidoidea (Skarbilovich, 1947) Siddiqi, 1980
Family: Aphelenchoididae (Skarbilovich, 1947) Paramonov, 1953
Subfamily: Aphelenchoidinae Skarbilovich, 1947
Genus: *Aphelenchoides* Fischer, 1894

1.5. Systematic position of the genus *Ditylenchus* Filipjev, 1936

In point 1.8 of this chapter the damage caused by *A. arachidis* and *Ditylenchus africanus* on groundnut in South Africa is compared to each other. Although these two species share the same host they belong to different orders, viz. Aphelenchida and Tylenchida, respectively. Compared to that of *Aphelenchoides*, the outline classification of the genus *Ditylenchus* according to Siddiqi (2000) is as follows:

Class: Secernentea Von Linstow, 1905
Order: Tylenchida Siddiqi, 1980
Superfamily: Anguinoidea Nicoll, 1935
Family: Anguinidae Nicoll, 1935
Subfamily: Anguininae Nicoll, 1935
Genus: *Ditylenchus* Filipjev, 1936

1.6. Diagnosis of the genus *Aphelenchoides* according to Hunt, 1993 and Shahina, 1996

The body is short to long, between 0.4 to 1.2 mm in length. The cuticle is finely annulated and the lateral fields often with four incisures but sometimes with two or three. The cephalic region is usually rounded in form and slightly offset. With SEM Hooper and Clark (1980) saw that the cephalic region has a basic hexaradiate pattern. Anteriorly is a cephalic plate with two subdorsal, two lateral and two subventral lip sectors. The cuticle of the cephalic region behind the cephalic plate shows fine annules. The amphidial apertures are in the lateral sectors, just dorsal of the mid-line at the edge of the cephalic plate. Each of the other four lip sectors bears a prominent cephalic papilla on its outer margin. An important variable characteristic of the lip region is the extent to which the lips are demarcated from the rest of the cephalic region, e.g. by a groove so that a distinct labial disc is present, as in *A. besseyi* (Hooper & Ibrahim, 1994). The cephalic skeleton is weak, the stylet is slender and on average 10 – 12 µm long, but usually less than 20 µm in length. The stylet has well-defined basal knobs or swellings. The procorpus is cylindrical, leading to a well-developed, ovoid or spherical median bulb with central valve plates. The oesophageal gland lobe is well developed and positioned dorsal to the intestine. The nerve ring and excretory pore is situated posterior to the median bulb although the excretory pore may be anterior or posterior to the nerve ring. The tail is conoid with a variable terminus, which may be bluntly or finely rounded, digitate, bifurcate or with a ventral projection. One or more mucrons of various shapes may be present.

Female: Heat-relaxed females die straight to ventrally arcuate. The vulva is situated postmedian, usually at between 60 – 75 % of the body length. Only very exceptionally are they situated more posterior. The genital tract is monoprodelphic, typically outstretched but may reflex. The developing oocytes are arranged in one or more rows. The post-uterine sac is usually present, often containing spermatozoa.

Male: Heat-relaxed males assume a 'walking-stick' shape with the tail region sharply or strongly curved or hooked ventrally. The tail is conoid in shape and tapering to a variable

terminus. The spicules are rose-thorn shaped, paired and separate. With SEM the shaft of each spicule comprises a curved dorsal and ventral limb, which are connected by a thin scleroprotein membrane and joined proximally by a prominent transverse bar with a dorsal apex and ventral rostrum. The rostrum and apex are usually well developed but may be almost absent. Typically there are three pairs of caudal papillae, one pair adanal, one pair subterminal and the other in between. A bursa is absent.

1.7. Bionomics

The majority of *Aphelenchoides* species appear to be free-living. They are found world-wide in various habitats in the soil, decaying plant material and galleries of wood-boring beetles where they probably find a source of fungi on which they live (Hunt, 1993). A small number of important plant-parasitic *Aphelenchoides* species live both ecto- and endoparasitically on above-ground parts of plants (Hunt, 1993). One of these is *Aphelenchoides arachidis* that feeds endoparasitically and ectoparasitically on groundnut roots and on the two fungi *Macrophomina phaseolina* (Tassi) Goidànich and *Botrytis cinerea* Persoonia, that have been associated with seeds on agar plates (Dickson & De Waele, 2005). The ability to enter anhydrobiosis to survive desiccation has been reported in the genus (Hunt, 1993), explaining their ability to survive in dry plant tissue and seed.

1.8. Perspectives on the origin of the infestation of groundnut in Africa by the testa nematode *A. arachidis*

According to National Geographic News of 28 June 2007, archaeologists found the earliest evidence of groundnut as a food crop in the 8 500 year-old floor of an ancient hut in the Ñanchoc Valley, Peru (Inman, 2007). This support the supposition that groundnut is endemic to the Amazon Basin of South America and that its cultivation originated there (The Peanut Van, 2002-2007).

A possible scenario for the association of *A. arachidis* with groundnut could be as follows: During the 1500's South America was invaded by the Spanish and the Portuguese who introduced groundnut plants to Spain and Portugal. These plants did not survive the harsh winters of Europe but thrived in Africa where it was introduced by the Portuguese during slave-trading missions. Groundnut was ideal for the African climate and became an everyday food, especially in West Africa. During the 1700's groundnut was ground to a paste by the people of Nigeria (Allen & Allen, 2002). From West Africa this crop spread throughout Africa where it was used both as food and as medicine. In Zimbabwe for example, groundnut is used in folk remedies to cure plantar warts (Duke, 1983). The fact that *A. arachidis* invades groundnut only in Africa and not in South America or in any other part of the world, implies that this nematode is endemic to Africa and has "learned" to recognize the alien groundnut as a suitable host in a relatively short time.

As *Aphelenchoides* species feed on both fungus mycelia and higher plants (Hunt, 1993) it is suggested that this ability could lie at the centre of its parasitism of groundnut. The soil-maturing pods of the groundnut plants could predispose them to soil fungi, attracting all kinds of fungus-feeding nematodes, among them an *Aphelenchoides* species. This species could have invaded the peg and maturing pods when it followed its fungus host and became enclosed in the structures and organs of the pod (Bridge *et al.*, 1977). Because of its abilities to feed on higher plant tissues, the nematode then started to feed on the maturing groundnut pods, thereby establishing themselves as a parasite of this crop. One can also argue that the groundnut, as a member of the bean family, might have attracted the nematode because it already had an endemic species of the same family as a host. The fact that *A. arachidis* is also associated with cereals in Africa, points however, to a more common host, such as a fungus. Of interest and supporting the above hypothesis is the suggestion by Bridge *et al.* (1977) that the many fungi that invade groundnut might serve as a food source for *A. arachidis*.

1.9. Comparison between the damage caused by *A. arachidis* and *D. africanus* on groundnut in South Africa

A. arachidis is a facultative endoparasite of the seed testa, pod shell, roots and hypocotyl of groundnut and also feeds ectoparasitically on the roots (Bridge *et al.*, 1977). According to Jones and De Waele (1990), *A. arachidis* occurred in greater numbers in roots than does *D. africanus* and rapidly invades the developing pods. Although no quantitative yield reduction due to the presence of *A. arachidis* has so far been reported (Dickson & De Waele, 2005), severe infestations have an adverse effect on the appearance and size of the seed and devalues confectionary groundnut (Bridge *et al.*, 1977). It also predisposes seeds to invasion by the many fungi associated with groundnut, for example *Rhizoctonia solani*, *Sclerotium rolfsii*, *Macrophomina phaseoli* and *Fusarium* spp., which may lead to reduced seed emergence (McDonald *et al.*, 1979). Bridge *et al.* (1977) suggested that these fungi might be a source of food for the nematode. According to Bos (1977, Cited by Minton & Baujard, 1990), there may be two biotypes of *A. arachidis* in Nigeria, one occurring on cereals and one occurring both on groundnuts and cereals.

De Waele *et al.* (1989) showed that *D. africanus* was not only found in discoloured pods and hulls but also in the seeds. *D. africanus* was also detected inside roots (Venter *et al.*, 1991) and pegs (De Waele *et al.*, 1990), the testa and embryo of groundnut seeds, but not the cotyledons (Basson *et al.*, 1992). While *A. arachidis* was found only in the tissue of the testa, mainly in the sub-epidermal parenchymatous layer and around the tracheides (Bridge *et al.*, 1977), *D. africanus* invades the parenchymatous region of the hull exocarp and endocarp and eventually the seed testa (Dickson & De Waele, 2005). Hulls infested by *D. africanus* have brown necrotic tissue at the point of the connection with pegs and the longitudinal veins become black, similar to symptoms caused by *A. arachidis*. Seeds infested either by *D. africanus* or *A. arachidis* are shrunken or wrinkled, darker brown than normal seeds and testae and embryos become yellow, brown, or darker, than normal testas, (Bridge *et al.*, 1977; Jones & De Waele, 1990). The infestation of seeds by *D. africanus* stimulate second-generation germination by up to 25 % of the seeds, seed mass decreases by up to 20 – 25 %, sprouting of harvested seed is caused (Venter *et al.*, 1991;

Venter *et al.*, 1995) and seed yield is suppressed (Venter *et al.*, 1993; Venter *et al.*, 1995). As in *A. arachidis, D. africanus* can enter a state of anhydrobiosis to survive desiccation (Venter *et al.*, 1995; Hunt, 1993). Jones and De Waele (1990) observed *D. africanus* in a coiled position on the surface of a cotyledon of a mature groundnut seed. The study of Venter *et al.* (1995) showed that one third of *D. africanus* become active after rehydration to invade hulls and seeds of a new crop.

According to Jones and De Waele (1990) the histopathology of *D. africanus* on groundnut closely resemble that of *A. arachidis*. There is, however, a difference in the time of peg invasion between the two species, which might account for the different tissues of the pod infested. According to Bridge *et al.* (1977), *A. arachidis* invades the pod approximately ten days after the peg had penetrated the soil. The nematodes do not increase rapidly until after 30 days, with the greatest number present after about 60 days. All stages of the *A. arachidis* were found in the testa but at the end of the growing season heavily infested testas of mature seed contain mainly juvenile stages (Minton & Baujard, 1990). Testas showing no external symptoms contains mostly adults and eggs, which are often arranged along the vascular bundles of the testa (Minton & Baujard, 1990). According to a communication by Ms. S. Steenkamp (2007), *D. africanus*, on the other hand, invades the peg as soon as it touches the soil and therefore about ten days earlier than *A. arachidis*. *Ditylenchus africanus* infests the shells, testas, roots, hypocotyls and embryos of the pods, a wider range of organs in the groundnut pod. It is therefore, suggested that the earlier the peg is invaded by nematodes, the more organs of the pod were infected. With *D. africanus* the peg is invaded early and shells, testas, hypocotyls and embryo were infested (Venter *et al.*, 1991; De Waele *et al.*, 1990 and Basson *et al.*, 1992). The above statements indicate that *A. arachidis* on the other hand, invades the peg almost ten days after *D. africanus*, infesting only the shells, testa, root and hypocotyls because the cotyledons and embryos are already developed and therefore covered by other developing organs.

1.10. Conclusions

During the present study *A. arachidis* was found in the testas and shells of groundnut, together with high infestation levels of *D. africanus* (see Fig. 1.1). Both species feed endoparasitically on tissues of the roots, pegs, hulls and most occur in seeds (Jones & De Waele, 1990; De Waele *et al.*, 1990; Venter *et al.*, 1992). Morphologically *D. africanus* and *A. arachidis* are completely different from each other but systematically they belong to the same Class (Secernentea). This confirms Nickle's 1970 finding that due to their ability to adapt to a wide range of ecological relationships, aphelenchoid nematodes have their ecological counterparts in the Tylenchida, in this instance, *D. africanus*. This means that both species occupy the same niche and their infestation of groundnut will cause the same symptoms. The severity of the symptoms will depend on the population numbers of the two species.

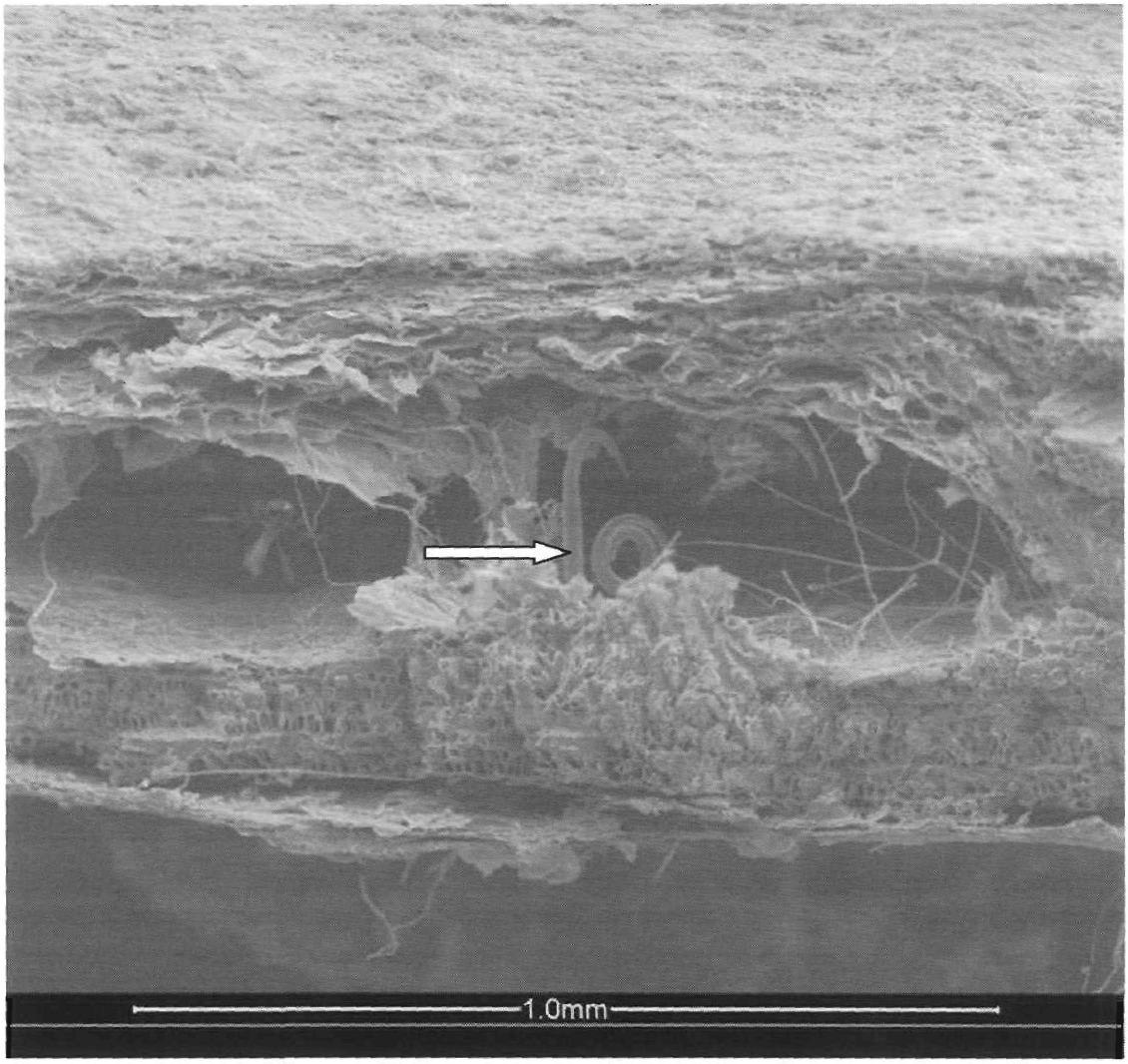

Fig. 1.1. *Aphelenchoides arachidis* and *Ditylenchus africanus* in a groundnut shell

1.11. Plant quarantine

Plant quarantine is a crop protection strategy enforced by legislation that is promulgated by governments or groups of governments under the auspices of the International Plant Protection Convention (IPPC), founded in Rome 1951 at a special meeting of the Food and Agricultural Organisation (FAO) of the United Nations. The International Plant Protection Convention is a multilateral treaty for cooperation in plant protection. Plant protection strategies are most effective when countries or regions co-ordinate their activities. The Republic of South Africa became a contracting member of the IPPC on the

21st of September 1956. In ratifying the Convention, the South African Government in effect agreed to provide legislation by which effective phytosanitary procedures can be established in order to prevent the introduction and spread of harmful organisms across national and international boundaries (IPPC, 2006). The Directorates of Plant Health (PH) and Agricultural Plant Inspection Services (APIS) of the Department of Agriculture are responsible for the administration of the Agricultural Pests Act, 1983 (Act 36 of 1983) to this end. The purpose of this Act is to prevent the introduction of agricultural pests which are not present or are of restricted presence in the country, to provide for measures whereby agricultural pests already in a country can be controlled or combatted and provide for facilities to import and export goods, subject to conditions (Act 36/1983).

1.11.1. Aims and principles of plant quarantine

Plant quarantine restrict the entry of plants, plant products, soil, cultures of living organisms, packing materials and commodities as well as their containers and means of conveyance. The aim is to protect agriculture and the environment from avoidable damage from hazardous organisms inadvertently introduced by man. Almost all countries regulate the importation of germplasm because of the pest and pathogen risk (IPPC, 2006; Kahn, 1977).

1.11.2. The effective implementation of the Articles of the IPPC is based on the plant importation permit and the phytosanitary certificate

1.11.2.1. Import permit

An import permit is an official document authorizing importation of a commodity in accordance with specific phytosanitary requirements. They are the passports to facilitate the entry of plant materials. Pest risk can be reduced by specifying in the permit, as a

condition of entry, that the importer must meet certain entry requirements (IPPC, 2006; Kahn, 1977).

1.11.2.2. Phytosanitary certificate

Under national and international plant protection and quarantine regulations, many kinds of plants and plant products must be accompanied by phytosanitary certificates. To comply with the specific requirements of the IPPC inspection shall be carried out and certificates issued only by or under authority of technically qualified and duly authorized officers and in such circumstances and with such knowledge and information available to those officers that the authorities of importing countries may accept such therefore be properly trained, with extensive knowledge of plant pests and with information available about the requirements of importing countries. It confirms that internationally acceptable standard procedures have been used in establishing the presence or absence of the specified pests in the growing crop or in samples of the plant material (IPPC, 2006; Taylor, 1977).

1.11.3. Pest Risk Analysis – PRA (Quarantine pests)

Pest risk analysis is the process of evaluating biological or other scientific and economic evidence to determine whether a pest should be regulated and the strength of any phytosanitary measures to be taken against it. It evaluates scientific evidence to determine whether an organism is a pest. These processes may be triggered by situations when a request is made to consider a pathway that may require phytosanitary measures, a pest is identified that may justify phytosanitary measures, a decision is made to review or revise phytosanitary measures or policies or a request is made to determine whether an organism is a pest (IPPC, 2006).

1.11.3.1. Quarantine status of *A. arachidis*

Initially *A. arachidis* was absent from the pest risk analysis (PRA) area South Africa, satisfying the need to be listed as a quarantine pest. As a result it was listed under import conditions for groundnut (seeds for planting and human consumption purpose).

The PRA for *A. arachidis* was triggered when it was first detected from groundnut samples obtained from Vaalharts silos in the Northern Cape Province as a new pest. A follow-up survey was conducted in the Vaalharts groundnut-growing area to verify their occurrence. More specimens were also detected and their identification as *A. arachidis* was confirmed. After that it was taken off the quarantine list in 2006 because its occurrence in the country had been confirmed (IPPC, 2006).

1.11.4. Discussion

A proper PRA on *A. arachidis* has not been conducted since its detection on groundnut in South Africa. First of all a survey of all the groundnut-producing areas needs to be done to establish its quarantine status and at the same time determine how wide spread this species is. To do such a survey, the groundnut industry needs to become involved, especially where funding is concerned. In a recent survey of groundnut in South Africa, the focus was on *D. africanus* so that all groundnut producing areas are well documented. Because of this, a survey of *A. arachidis* should be much easier to plan and execute.

Of grave concern is also the fact that infested groundnut seed could have entered South Africa without detection. This could have been accomplished by unsuspecting tourists entering South Africa with a packet of groundnut seed, or it could have been brought in illegally.

1.12. Objectives of this study

1.12.1. General objectives

The general objectives were to study the South African population of *A. arachidis* and compare this population morphologically, morphometrically and, if possible, molecularly with that of the Nigerian population of *A. arachidis*.

1.12.2. Specific objectives of this study were:

- to compare the damage caused by *D. africanus* and *A. arachidis* on groundnut by doing a literature review.
- to evaluate the quarantine status of *A. arachidis* in South Africa by doing a literature review.
- to compare the morphology and morphometrics of the South African and Nigerian population of *A. arachidis*.
- to evaluate the PCR-based diagnostic method for the identification of *A. arachidis* and *A. blastophthorus* and compare the results with those of Ibrahim *et al.*, (1994).

CHAPTER 2

Materials and Methods

2.1. Nematode populations

Populations of the genus *Aphelenchoides* were obtained from groundnut samples collected from Vaalharts (Northern Cape Province). A soil auger and garden trowel were used to collect soil samples from a depth of 20-30 cm (Kleynhans, 1999). Groundnut shells, seeds and soil were collected for the extraction of adult nematodes because they are required for taxonomical studies. The groundnuts were ±150 days old and ready for harvesting. Samples were taken systematically (Sardanelli & Ellison, 2005; Coyne, *et al.*, 2007). The plots were divided into 4 ha or less and the X-shaped sampling pattern was followed to take 80 core - samples/4 ha or ±20 samples/ha. Samples were taken from the root zone at 10-20 cm from the stem. Samples were thoroughly mixed in a plastic bag on site, from which a 2 kg sub-sample was drawn to analyse for nematodes. The 2 kg samples were packed in cooler boxes and immediately taken to the laboratory (Sardanelli & Ellison, 2005; Coyne *et al.*, 2007).

Nematodes were extracted from soil (250 mℓ) by the centrifugal flotation method. The 250 mℓ soil was washed through a coarse-meshed 2 mm aperture sieve into a 5 litre bucket. Then water was added to the bucket to increase the suspension to 5 litres. The suspension was stirred, and then allowed to settle for 30 seconds. The suspension was poured through a 45 µm-aperture sieve. The procedure was repeated with the soil in the bucket two more times, but the settling time was shortened to 20 and 10 seconds respectively. Then the residue was transferred from the 45 µm-aperture sieve to four 50 mℓ centrifuge tubes and centrifuged for 7 minutes at 1 750 rpm. The supernatant was decanted from the tubes and discarded. A sugar solution (450g/ℓ water) was added to the tubes, thoroughly mixed and centrifuged for 3 minutes at 1 750 rpm. The suspension was poured through the 45 µm sieve and the residue rinsed from the sieve for examination

(Jenkins, 1964; Kleynhans, 1999). Nematodes were extracted from 5 g dried and fresh pod shells, seeds and seed testas by soaking the tissues in shallow water in Petri dishes for 48 hours at room temperature (Bolton, *et al.*, 1990). Live adult nematodes of both *A. arachidis* and *A. blastophthorus* Franklin, 1952 were extracted from these and prepared for light and scanning electron microscopy, as well as molecular studies. Two paratypes of *A. arachidis* (Courtesy, Janet Rowe, Rothamsted Research, U.K.) were obtained for establishing the number of lateral lines on different parts of the body to compare with those of the South African species.

2.2. A morphological and morphometrical study of a South African population of *Aphelenchoides arachidis*

2.2.1. Scanning electron microscopy (SEM)

Five adult nematodes were fixed in TAF (distilled water, 40% formalin and triethanolamine), processed to 100% ethanol, critical-point dried with carbon dioxide as intermediate fluid and mounted on a SEM stub with double-sided tape. The infected groundnut shells were cut into 10-mm pieces, fixed in 70% ethanol, processed through an acetone series (70%, 80%, 90%, 100% and 100%) and critical-point dried. To examine the shells internally, each dried piece was broken and affixed to a SEM–stub with double–sided carbon tape.

These studies were done at the Laboratory for Electron Microscopy, North–West University, Potchefstroom Campus, Potchefstroom under the guidance of Dr. L.R. Tiedt. The material was coated with gold – palladium (27 nm) and examined with a Philips XL 30 stereoscan microscope at 10 kV.

2.2.2. Light microscopy (LM)

Fourteen specimens of *A. arachidis* that emerged from the shells and testae, were killed by gentle heat, fixed in FPG (distilled water, 40% formalin, propionic acid, glycerine and picric acid) as described by Netscher & Seinhorst (1969), processed into glycerine (Hooper & Evans, 1993) and mounted in anhydrous glycerine between coverslip slides. Measurements and drawings of the mounted specimens were done by means of a Nikon Labophot-2 Research Microscope equipped with a drawing tube at the Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI), Rietondale Campus, Pretoria. Abbreviations and symbols of De Man (1884) and Fortuner (1984) are used in the descriptions. Measurements of curved structures were made along the median line.

2.2.3. Morphometrical terms used in this study

n = number of specimens
L = total body length in mm or µm
a = body length ÷ greatest body width
b = body length ÷ distance from anterior end to junction of oesophagus and intestine
b′ = body length ÷ distance from anterior end to posterior end of oesophageal glands (used when glands overlap intestine)
c = body length ÷ tail length (anus or cloaca to tail terminus)
c′ = tail length ÷ body width at anus or cloaca
V = distance from anterior end to vulva ÷ body length × 100
T = distance from anterior end to anteriormost part of testis ÷ body length × 100

2.3. Molecular study

The objective of the molecular study was to evaluate a PCR-based diagnostic method for the identification of *A. arachidis* and *A. blastophthorus* and to compare the results with those of Ibrahim *et al.* (1994). *Aphelenchoides blastophthorus* was included in the

molecular study because it was extracted together with *A. arachidis* at Vaalharts, South Africa from the same groundnut pods. Specimens of both species were transferred to two Eppendorf tubes and prepared for molecular studies.

2.3.1. Preparation of DNA templates

Individual adults of *A. blastophthorus* (10 individuals) and *A. arachidis* (20 individuals) were handpicked from an Eppendorf tube under the stereo microscope and each was placed in a 5 - µl drop of 1× polymerase chain reaction (PCR) reaction buffer (16mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20) containing 60 µg/ml proteinase K in a sterile PCR tube. Each nematode was then cut into small pieces with a sterile needle of an insulin syringe, under the stereo microscope. Tubes were placed on ice to avoid denaturing of DNA by DNAses released from the ruptured nematodes at room temperature. The tube was kept at -80 °C for a minimum of 10 minutes, because DNAses released from the ruptured nematode will rapidly cut up the DNA at room temperature. The tube was then incubated at 60 °C for 15 minutes and a further 5 minutes at 95 °C. An initial denaturation step (95 °C for 5 minutes) is recommended to ensure complete denaturation of the template DNA.

2.3.2. Polymerase chain reaction (PCR)

2.3.2.1. Experiment A

The objective of this experiment was to evaluate a PCR-based diagnostic method for the identification of *A. arachidis* and *A. blastophthorus*, targeting the first rDNA internal transcribed spacer region ITS1. Two PCR amplification primers that amplify the ITS1 region, partial rDNA operon, as well as short parts of the 18 S and 5.8 S ribosomal genes were used. The 18s primer (5'-TTGATTACGTCCCTGCCCTTT -3') has been described

by Vrain *et al.* (1992) and the 5.8s primer (5'-ACGAGCCGAGTGATCCACCG–3') was designed by comparative sequence alignments of various nematode species by Szalanski *et al.* (1997). Primers were synthesized by Genosys Biotechnologies Ltd in The United Kingdom (UK).

PCR amplifications were carried out in tubes containing 5 µl of nematode lysate together with 0.5 µM of each primer and 12.5 µl GoTaq® Green Master Mix (dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 1 × GoTaq® reaction buffer , 1.5 mM $MgCl_2$ and GoTaq® DNA polymerase). The final reaction volume was 25 µl. Amplifications were performed on a Hybaid PCR Sprint thermal cycler. The cycling conditions were as follows: denaturation at 94 °C for 20 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 45 seconds, repeated for 25 cycles. A two-minute incubation period at 72 °C followed the last cycle in order to complete any partially synthesized strands.

2.3.2.2. Experiment B

The objective of this experiment was to evaluate a PCR-based diagnostic method for the identification of *A. arachidis* and *A. blastophthorus*, targeting the rDNA internal transcribed spacer region ITS. Two PCR amplification primers that amplify the ITS region as well as short parts of the 18 S and 26 S ribosomal genes were used. The 18s primer (5'-TTGATTACGTCCCTGCCCTTT -3') and 26s primer (5'-TTTCACTCGCCGTTACTAAGG–3') has been described by Vrain *et al.* (1992). Primers were synthesized by Genosys Biotechnologies Ltd in the UK.

PCR amplifications were carried out in tubes containing 5 µl of nematode lysate together with 0.5 µM of each primer and A Ready-To-Go PCR bead (Amersham Pharmacia Biotech), containing dATP, dCTP, dGTP and dTTP each at 200 µM final concentration, 10 mM Tris-HCl (pH 9), 50 mM KCl , 1.5 mM $MgCl_2$ and 1.5 units of DNA

polymerase). The final reaction volume of 25 µl was obtained by adding 15 µl water. Amplifications were performed on a Hybaid PCR Sprint thermal cycler. The cycling conditions were as follows: denaturation at 94 °C for 20 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 45 seconds, repeated for 25 cycles. A 2-minute incubation period at 72 °C followed the last cycle in order to complete any partially synthesized strands.

2.3.2.3. Experiment C

The objective of this experiment was to evaluate a RFLP-PCR-based diagnostic method for the identification of *A. arachidis*, utilising digestion patterns of the ITS region. Ten µl of each of the PCR products were digested with 10 U of a restriction enzyme in the appropriate buffer according to the manufacturer's instructions in a total volume of 20 µl. The amplified fragments of *Aphelenchoides blastophtorus* and *A. arachidis* were digested with *Alu*I, *Cfo*I, *Dra*I, *Eco*RI, *Hae*II, *Hinf*I, *Msp*I and *Ssp*I. The digested DNA was loaded on a 2 % agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. A 100 bp DNA ladder was used as a size marker.

2.3.2.4. DNA sequencing

The objective of this experiment was to compare the products of the ITS1 region of *A. arachidis* with any *Aphelenchoides* spp. products available in the Genbank (only *A. fragariae* was available). PCR products of the ITS1 region of the different populations were cleaned up and sequenced by the University of Stellenbosch, using an AB3100 Genetic analyser.

The ITS1 and ITS were targeted in the above experiments and sequencing because the ITS, located between the repeating array of nuclear 18S and 28S ribosomal DNA genes, is a versatile genetic marker. Among eukaryotes ITS data have been used in constructing

phylogenetic trees, estimating genetic population structures, evaluating population-level evolutionary processes and determining taxonomic identity. The structure of the rDNA cistron contributes to its wide applicability. The rDNA cistron is divided into domains that evolve at different rates; thus this region can be used to address diagnostic and evolutionary problems at different levels of divergence. The rDNA is a component of the middle repetitive family of the nuclear DNA genome, and the presence of multiple copies of these genes in the genome facilitate PCR amplification from single juvenile and adult nematodes. Universal amplification coupled with the ability to amplify ITS from individual nematodes suggests that any species, population, or ecological community of nematodes can be analysed using a molecular approach based on the rDNA ITS region. A standardised taxonomic marker would be particularly useful when populations contain a large number of juvenile stages, when sexes are dimorphic, or when unfamiliar nematodes are encountered, Powers, *et al.*, (1997).

CHAPTER 3

Results and Discussion

3.1. Morphological and morphometrical data

3.1.1. Light microscopy (LM)

3.1.1.1. Description

***Aphelenchoides arachidis* Bos, 1977**

(Fig. 3.1: South African population of *Aphelenchoides arachidis* Bos, 1977 & 3.2: *Aphelenchoides arachidis* Bos, 1977 (female)

Measurements: See Table 3.1. Comparative morphometrical data of *Aphelenchoides arachidis* specimens from Nigeria and South Africa (all measurements in µm).

Females: The morphology of the population in the present study compares well with that in the original description of *A. arachidis* Bos, 1977. In this population the female body is slender (± 15µm wide at the position of the vulva, the widest part of the body) with narrow annules, approximately 0.9 – 1.2 µm wide at the vulva. The lateral field is narrow (1.3 – 2.0 µm wide at the vulva) with either two or four lines/incisures. The lip region is 2.6 (2.5 – 3) µm high, 5.9 (5 – 6.5) µm wide and varying from slightly narrower to continuous with the adjacent body. The lip region is flattened anteriorly. The tail is short and conical with a bluntly pointed tip, adorned with a single mucro. The stylet has distinct basal knobs. The nerve ring is situated at approximately one body width, or slightly less, behind the median bulb. The excretory pore is situated at 10 – 15 % of the total body length or 1 – 3 body widths behind the median bulb. The oesophago-intestinal junction was difficult to see in some specimens, but in others it was situated 76.3 (60 – 93.5) µm behind the median bulb. The distance between the vulva and anus is 172.7 ±

27.1 (142 – 233) µm and the postvulval uterine sac (PUS) extends posteriad for approximately half to almost the whole vulva-anus distance.

Males: The male morphology is close to that of the female except for the following: The posterior end of body is curved ventrad and three pairs of ventro-submedian caudal papillae are situated on the tail. The tail is short and usually more pointed than in the female. A small terminal mucro is situated on the tail. The spicules are smoothly curved, rose-thorn shaped, with a prominent apex and rostrum. The rostrum is pointed.

3.1.2. Scanning electron microscopy (SEM)

When searching for the lateral lines with the LM, two clearly demarcated lateral lines could be seen, but in some specimens, two additional, evenly spaced, fine lines were seen in parts of the body of several individuals with SEM, two to four lines could clearly be discerned (Fig. 3.2 C & D) along the lateral body length of the same specimen. The two paratypes of *A. arachidis* were slightly shrunk and the lateral lines could not be seen properly.

When viewing the lip region with the SEM, about five very faint head annules could be discerned (Fig. 3.2 A). The SEM *en face* view shows the typical hexaradiate pattern of the cephalic region of the genus, with six equally sized sectors. The part of the labial disc not obscured by exudates seems to be clearly demarcated (Fig. 3.2 B) from the rest of the lip region.

3.1.3. Discussion

Comparing the South African and Nigerian populations of *A. arachidis*, the following morphometric differences stand out: Number of lateral lines (2 – 4 *vs* 2), lower b – value (7 – 11 *vs* 11 – 18), slightly shorter stylet (8 – 10 µm *vs* 10 -12 µm), length of PUS as

percentage of distance from vulva to anus (41 – 96 *vs* 50) and slightly shorter spicules (12 – 18 µm *vs* 15 – 25 µm). The slight differences in b - value, PUS – value, stylet length and spicule length might be attributed to the availability of food. Poinar (1983) stated that nourishment is important in determining final size, as well as qualitative and quantitative morphological characters of nematode specimens within species. The number of lateral lines might have been wrongly counted by Bos (1977), the author of *A. arachidis*. He had the use of only a LM whilst the present study was done with the aid of both the LM and SEM. *A. arachidis* is also a very slender nematode (see description), making the counting of faint lines very difficult. The males in the South African population have longer tails than the females (See Table 3.1), a fact not mentioned in the original description of *A. arachidis*. The morphology of the two populations are otherwise in close agreement.

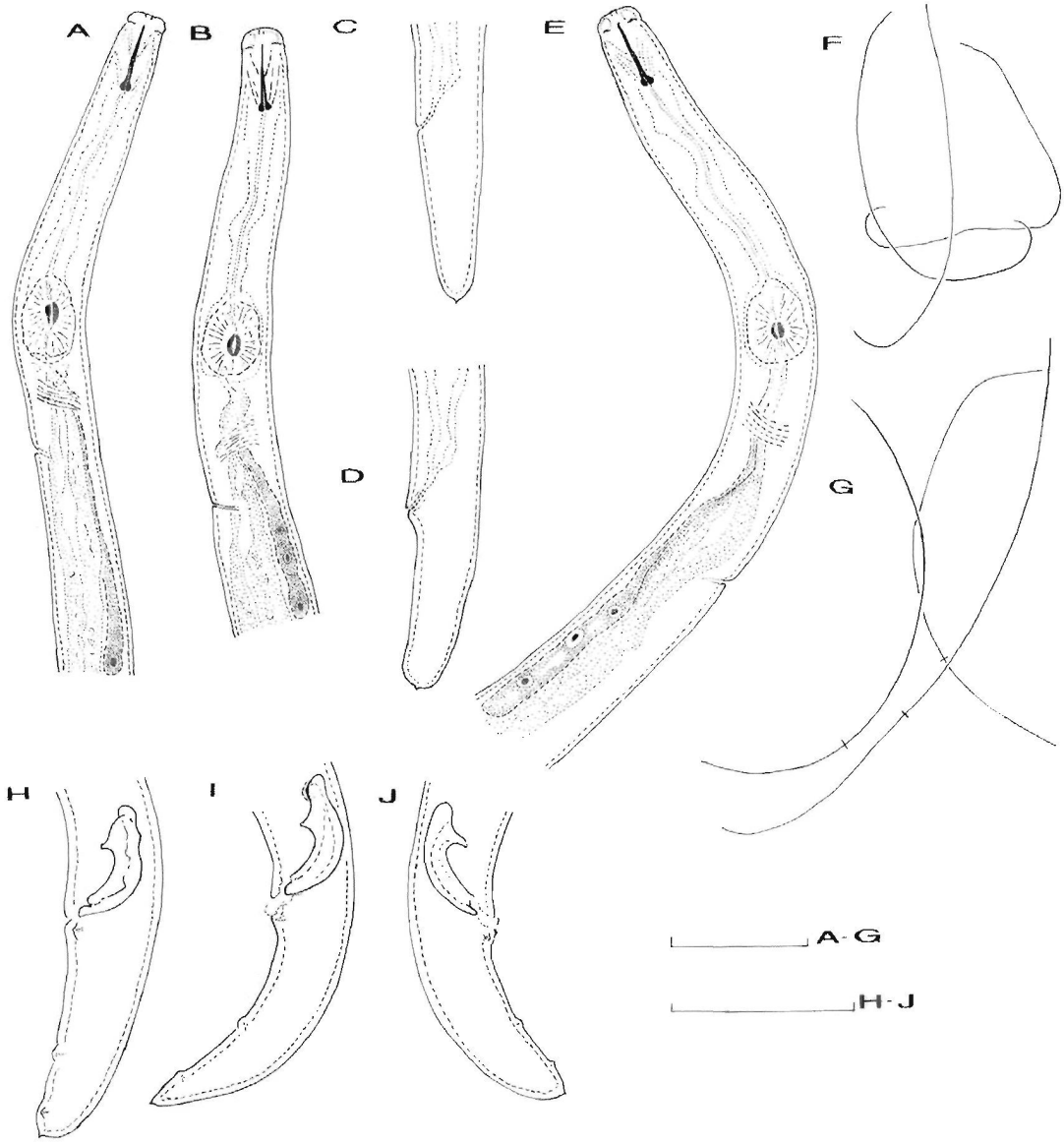

Fig. 3.1: South African population of *Aphelenchoides arachidis* Bos, 1977: A – B: Anterior region of different females. C – D: Tails of different females. E: Anterior region of male. F: Habitus of different males. G: Habitus of different females. H – J: Tails of different males.

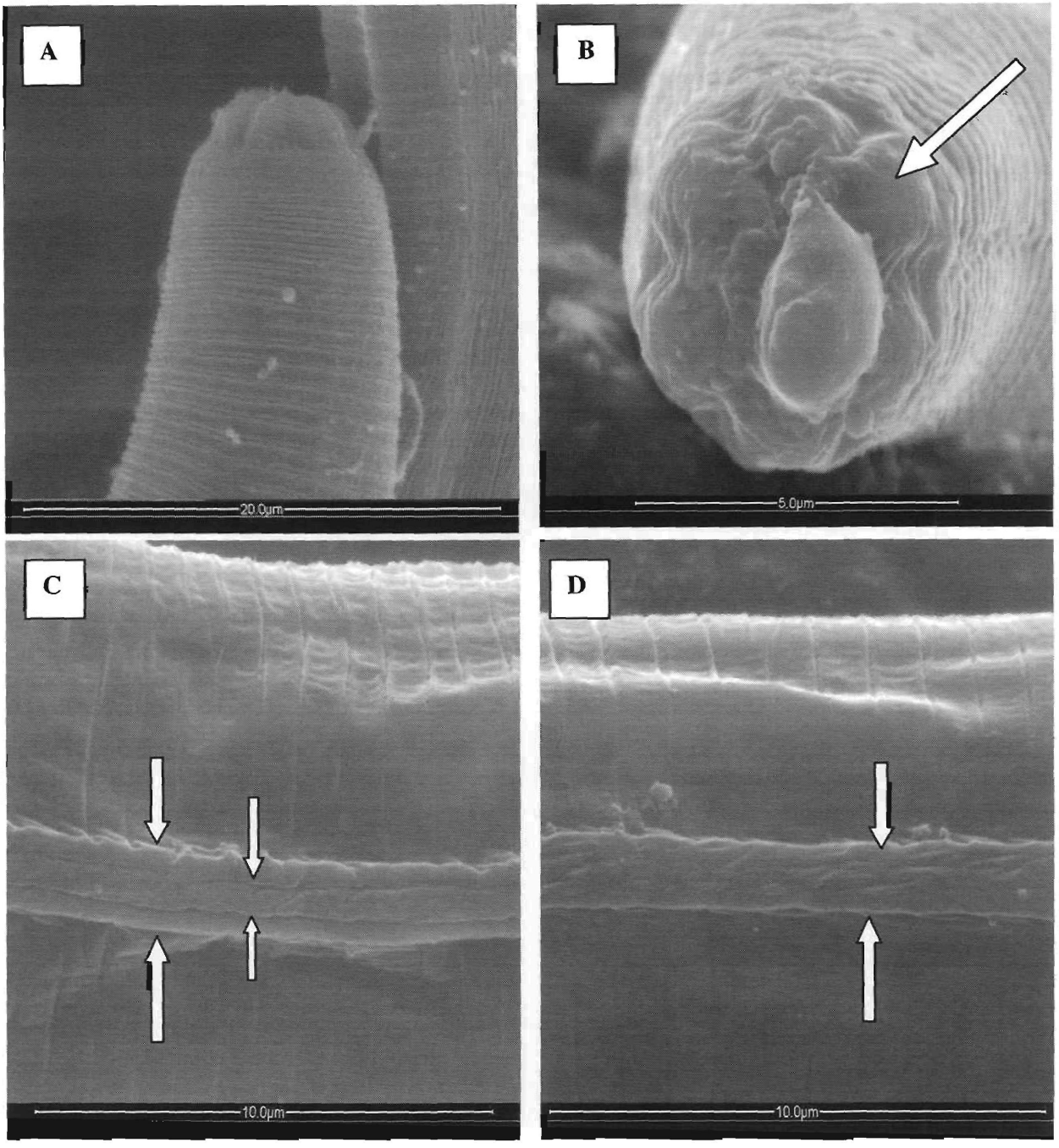

Fig. 3.2: *Aphelenchoides arachidis* Bos, 1977 female: A: Head, side view. B: Head, frontal view, showing the six sectors of the hexaradiate pattern of the cephalic region (arrow). C & D: Lateral lines at mid-body of the same female, arrows indicating four lines in C and two in D.

Table 3.1: Comparative morphometrical data of *Aphelenchoides arachidis* specimens from Nigeria and South Africa (all measurements in µm).

	1. Type population (Nigeria)		2. South African Population	
n	20 ♀♀	20 ♂♂	11 ♀♀	3 ♂♂
Character				
L	751 (510 –1000)	795 (560 – 1040)	694.7 ±119.2 (568 – 984)	631.7 ± 44.7 (601 – 683)
a	43.4 (39 – 50)	47.0 (37 – 60)	44.5 ± 3.4 (38 – 49)	55.7 ± 6.1 (49 – 61)
b	14.1 (11 – 18)	14.2 (10 – 18)	8.4 ± 1.2 (7 – 11)	8.7 ± 0.5 (8 – 9)
b′	6.6 (5 – 8)	7.0 (5 – 9)	6.9 ± 0.9 (6 –9)	7.3 ± 1.0 (6 – 8)
c	30.2 (25 – 42)	27.4 (20 – 39)	27.1 ± 3.7 (20 – 34)	19 ± 2 (17 – 21)
c′	2.5 (2 – 3)	2.4 (2 – 3)	2.6 ± 0.5 (2 – 3)	3
V/T (%)	71.7 (67 – 74)	60.4 (40 – 84)	71.2 ± 1.4 (69 – 73)	47 ±1.4 (46 – 48)
PUS (%)	± 50	-	73.9 ± 27.4 (41 – 96)	-
Tail length	25.3 (21.6 – 27.6)	-	25.9 ± 2.5 (22 – 30)	33.7 ± 5.6 (29 – 40)
Lateral lines (number)	2	2	2 – 4	2 – 4
Stylet length	11 – 12	10 – 11	9.8 ± 0.4 (9 – 10)	8.5 ± 0.7 (8 – 9)
Spiculum length (dorsal limb)	-	15 – 25		15.0 ± 4.2 (12 – 18)

1. Type population (Nigeria) according to Bos, 1977.

2. South African population obtained from Vaalharts.

3.2. Molecular data

3.2.1. Amplification

3.2.1.1. Experiment A

Figure 3.3 shows the different sizes of amplification products obtained from each of the *Aphelenchoides* species. The amplification product of *A. blastophthorus* was approximately 600 base pairs (bp) in size and the amplification products of *A. arachidis* were approximately 500 bp in size. Two amplification products per individual were observed for *A. arachidis*.

3.2.1.2. Experiment B

Figure 3.4 shows the different sizes of amplification products obtained from each of the *Aphelenchoides* species. The amplification products of *A. blastophthorus* was approximately 1000 base pairs in size and the amplification products of *A. arachidis* were approximately 900 bp in size.

3.2.1.3. Experiment C

3.2.1.3.1. Restriction fragment length polymorphisms (RFLP)

Eight endonucleases were used to digest the PCR product of the ITS region of *Aphelenchoides* spp. from experiment B. *Alu*I, *Hinf*I, *Cfo*I and *Msp*I cut the amplification products into fragments, but no restriction digest took place with *Eco*RI, *Hae*II, *Dra*I and *Ssp*I. The restriction patterns are shown in Figure 3.5 *Alu*I cut the product from *A. arachidis* into fragments of approximately 550, 230 and 120 base pairs. *Hinf*I cut the product into fragments of approximately 600 and 220 base pairs. *Cfo*I cut the product into fragments of approximately 520 and 380 base pairs.

3.2.1.3.2. DNA sequencing

The ITS1 and ITS2 region, including parts of the 18s and 26s gene and the whole 5.8s gene was amplified from *A. arachidis* in Experiment B and sequenced. After alignment of sequences from three individuals, a consensus sequence was derived. The consensus sequences from *A. arachidis* were compared to sequences of *A. fragariae* (AF119049) which was the only *Aphelenchoides* sp. available on Genbank. Apart from the 18s, 5.8s and 26s gene regions which were similar, the ITS1 and ITS2 regions from *A. arachidis* differed indeed significantly from that of *A. fragariae*.

3.2.2. Discussion

One amplification product with rDNA primers from Experiment B was observed from the present results (*Aphelenchoides* spp.) as compared to two amplification products by Ibrahim *et al.* (1994). However, when the ITS1 region in Experiment A of the present study was amplified, two products could be observed for *A. blastophthorus* and only a thick band for *A. arachidis*, probably indicating the possibility of two products.

The results of restriction enzyme digests (Experiment C) were difficult to compare with those of Ibrahim *et al.* (1994) because he provided no fragment sizes. His published photographs could also not be used to accurately estimate the fragment sizes. However, the data (Experiment C) do suggest that the amplification products in the present study do not have the same restriction sites than those described in the literature (Ibrahim *et al.*, 1994). Moreover, no digest with *Hae*II and different fragments with *Alu*I were obtained in the present study. Analysis of the sequence that was obtained confirmed the results in Experiment C.

Ibrahim *et al.* (1994) suggested that the 5.8S/ITS region in *Aphelenchoides* spp. accumulated a lot of sequence variation and could be readily used for differentiating between species. It was further suggested by Ibrahim *et al.* (1994) that the technique may be useful for finding new species because three undescribed populations of *Aphelenchoides* were clearly differentiated from each other and from the named species (*A. nechaleos* Hooper & Ibrahim, 1994; *A. paranechaleos* Hooper & Ibrahim, 1994) by the numbers and positions of the restriction fragments. The sequence obtained in the study was compared to other available *Aphelenchoides* sequences and clear differences were found. Further testing and sequencing of *Aphelenchoides* spp. will have to be performed to design a diagnostic test and do phylogenetic studies because limited data are available on Genbank (only *A. fragariae*) and it seems that the South African isolates of *Aphelenchoides* spp. may exhibit different ITS sequences than those used by Ibrahim *et al.* (1994).

The morphological and morphometrical differences between the South African and Nigerian population seem to be minimal and I am therefore convinced that they are conspecific.

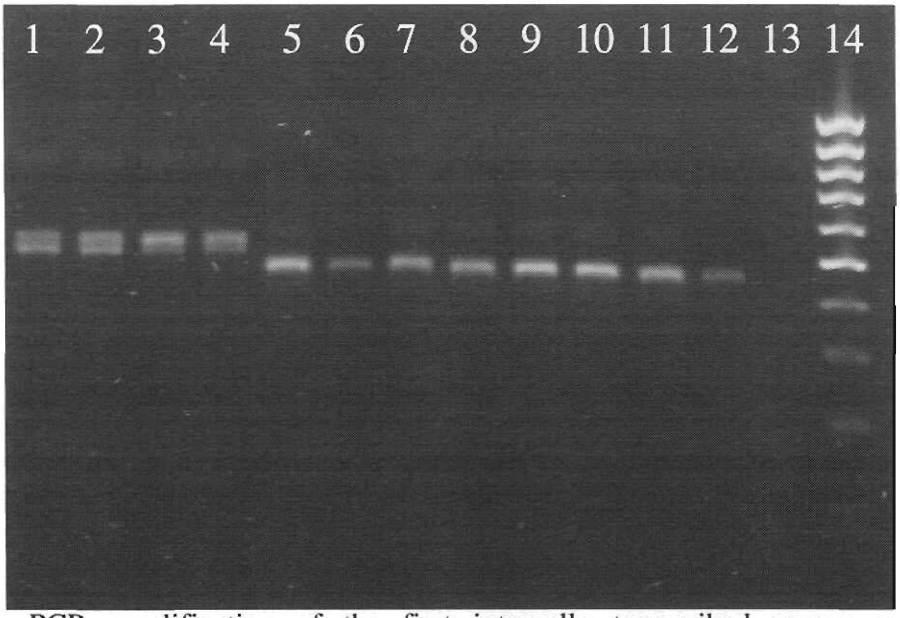

Fig. 3.3: PCR amplification of the first internally transcribed spacer regions of *Aphelenchoides blastophthorus* (lanes 1-4) and *A. arachidis* (lanes 5-12). 13 = Negative control. 14 = 100 bp Marker.

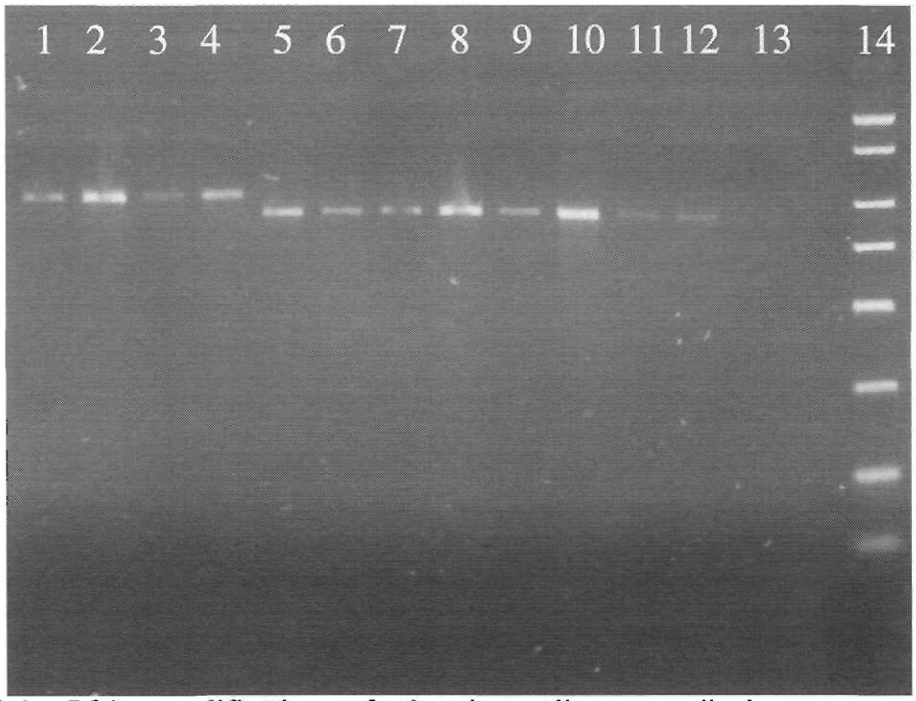

Fig. 3.4: PCR amplification of the internally transcribed spacer regions of *Aphelenchoides blastophthorus* (lanes 1-4) and *A. arachidis* (lanes 5-12). 13 = Negative control. 14 = PCR.

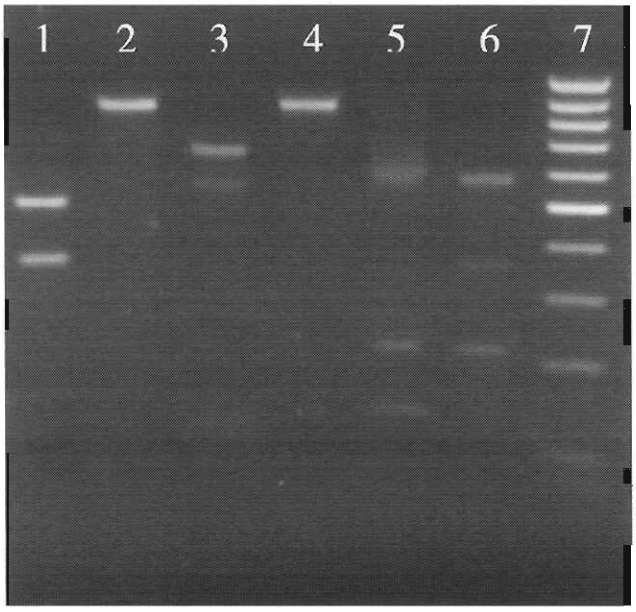

Fig. 3.5: Digestion patterns of the ITS region from *Aphelenchoides arachidis*. 1 = *Cfo*I; 2 = *Dra*I; 3 = *Msp*I; 4 = *Ssp*I; 5 = *Alu*I; 6 = *Hinf*I; 7 = 100 bp Marker.

CHAPTER 4

Conclusions

A morphological and morphometrical study was conducted in order to compare the South African population of *A. arachidis* with that of the Nigerian one as described by Bos in 1977. The observed differences in b - value, PUS – value, stylet length, spicule length, number of lateral lines and the fact that the males in the South African population have longer tails than the females (not mentioned in the original description of *A. arachidis*), warrant a follow-up study of more specimens from more localities. Such an extended study should ascertain if the South African population is a subspecies of *A. arachidis* or whether this species just differs widely from one locality to the next and from the Nigerian one. The importance of identifying species correctly cannot be emphasized enough as no sound recommendation on, for example, control can be made before the species, biotype or race is known. A biotype of *A. arachidis* that occurs on cereals could, for instance, become a major problem in South Africa, especially in maize-producing areas.

The objective of the molecular study was to evaluate a PCR-based diagnostic method for the identification of *A. arachidis* and *A. blastophthorus* and to compare the results with those of Ibrahim *et al.* (1994). The results from the present study emphasize the importance of further sequencing of *Aphelenchoides* spp. to design a diagnostic test for these species. Further phylogenetic studies should also be done because limited data are available on Genbank (data from only *A. fragariae* are available). The fact that the South African isolates of *Aphelenchoides* spp. exhibit different ITS sequences than those used by Ibrahim *et al.* (1994) also warrants further investigation.

From the literature review, the histopathology of *A. arachidis* on groundnut seems to closely resemble that of *D. africanus*. There is, however, a difference in the time of invasion of the peg between the two species, *D. africanus* being first to invade and *A. arachidis* about ten days later. Because *D. africanus* infests the peg and therefore the

pods for a longer period, the damage caused by *D. africanus* could be far worse than that of *A. arachidis*. The pathogenicity of the two species (both apart from each other and combined) can only be determined by conducting future host studies where both nematode species and their host are involved.

A question that also begged answering during the present study was why a supposedly endemic species to Africa, *A. arachidis*, was able to parasitize an alien plant (groundnut) from South America. A future screening of the entire endemic bean family of Africa for the presence of *A. arachidis* among its roots or in its pods is recommended and could hold the answer to this problem. If one of the members of the African bean families proves to be a host for *A. arachidis*, it follows that groundnut could also be parasitized by this species. This is indeed the mission of the science of taxonomy and systematics - to discover and describe biological diversity to broaden our understanding of natural patterns and processes that are linked to it.

The fact that *A. arachidis* was detected in groundnuts in South Africa (2002 - 2005) and on maize from Uganda (2004), suggests that *A. arachidis* is either spreading in Africa or is more widely spread than previously thought. This raises a challenge to African countries who claim not to have the organism, to start with a survey to determine the incidence of *A. arachidis*, especially in groundnut producing areas. A proper pest risk analysis on *A. arachidis* in South Africa has not been conducted since its detection on groundnut. This implies that a survey of all the groundnut producing areas needs to be done to determine its quarantine status. If such a survey should show that *A. arachidis* is already wide spread, no quarantine measures are necessary, but if the nematode proves to be localized, quarantine measures should be taken immediately. To summarize, a survey of the groundnut producing areas in South Africa must be done, the economic impact of *A. arachidis* on groundnut in South Africa needs to be established and the incidence of *A. arachidis* in other agricultural crops, especially those used in rotation with groundnut, needs to be determined.

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