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Identification of three *Meloidogyne* species from Cameroon by the polymerase chain reaction (PCR)

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ABSTRACT

A study was conducted to identify some Cameroonian *Meloidogyne* species by the polymerase chain reaction (PCR). *Meloidogyne* isolates from various host crops in fifteen localities of the South West, West, and North West Provinces of Cameroon, were cultured on susceptible tomato in a greenhouse in Münster, Germany. DNA was extracted from *Meloidogyne* second-stage juveniles extracted from the tomato roots, and used in the PCR as template. The PCR product was loaded onto a 2% agarose gel, and the DNA bands on the gel visualised under short-wave (254 nm) ultraviolet transillumination. The *Meloidogyne* species were identified by comparing their DNA fragment sizes with the molecular weight marker in the same gel. Three sizes of DNA amplification products were obtained: 1200 base pairs (bp), 1500 bp, and 420 bp corresponding, respectively, to *M. incognita, M. hapla,* and *M. arenaria.* No DNA amplification was observed using the *M. javanica* specific primers. *Meloidogyne incognita* was the most common species; *M. hapla* was found in areas of high altitudes such as Pastoral, Djutitsa (West Province) and Bambui Upper Farm (North West Province); while *M. arenaria* was identified only in peanut (*A. hypogaea*), its principal host.

Key words: DNA amplification, DNA-based identification, *Meloidogyne* spp., Polymerase Chain Reaction

RESUME

Une étude a été menée pour identifier quelques espèces camerounaises de *Meloidogyne* à l'aide de l'amplification de l'ADN par la technique de « PCR » (i.e. « polymerase chain reaction »). Les isolats de *Meloidogyne* provenant de diverses plantes hôtes provenant de quinze localités dans les provinces du Sud-Ouest, de l'Ouest, et du Nord-Ouest au Cameroun, ont été élevés sur la tomate sensible dans une serre à Münster, Allemagne. L'ADN a été extrait des larves de deuxième stade de *Meloidogyne* obtenues des racines de la tomate, et utilisé dans la « PCR » comme matrice. Le produit de l'amplification a été analysé sur gel d'agarose 2%, et les bandes de l'ADN visualisées sous trans-illumination ultraviolette à 254 nm. Les espèces de *Meloidogyne* ont été identifiées en comparant la taille (en paires de base, bp) des fragments de l'ADN avec les marqueurs de poids moléculaires dans le même gel. Trois produits d'amplification de l'ADN ont été obtenues : les fragments de 1200 paires de bases (pb), 1500 pb, et 420 pb, correspondant respectivement, à *M. incognita, M. hapla,* et *M. arenaria.* Aucune amplification d'ADN n'était observée lorsque les amorces specifiques à *M. javanica* étaient utilisées. *Meloidogyne incognita* était l'espèce la plus répandue ; *M. hapla* était réncontrée dans les zones de de hautes altitudes telle que Pastoral, Djutitsa et Bambui Upper Farm ; tandis que *M. arenaria* n'était identifiée que dans l'arachide (*A. hypogaea*), son hôte principal.

Mots clés: Amplification de l'ADN, identification basée sur l'ADN, les espèces *Meloidogyne*, Polymerase Chain Reaction.

INTRODUCTION

Species identification in *Meloidogyne* has been a major component of taxonomic research in nematology. Although there are about seventy described species of *Meloidogyne* [1], most taxonomic attention has focused on less than the dozen that are typically associated with diseases of agronomically important plant species. *Meloidogyne incognita, M. javanica, M. arenaria,* and *M. hapla* [2] account for most of the crop losses caused by *Meloidogyne* species [3]. The four species are widely distributed, but the first three are generally limited to warm tropical regions, while *M. hapla* is more common in cold temperate regions and in higher elevations of the tropics [4].

Traditional techniques for identification of *Meloidogyne* species have relied on morphological characters [5, 6], host range tests [7, 8], and isozyme analysis [9]. However, these methods have limitations. Identification based on morphological characters requires a lot of skill and is often inconclusive for individuals because the characters vary considerably within a population [6]. Isozyme patterns are phenotypic characters that are subject to developmentally- or environmentally-induced variation.

In recent years, identification of *Meloidogyne* species by use of the polymerase chain reaction (PCR) has provided an attractive alternative to all previous identification methods. DNA-based methods do not rely on the expressed products of the genome and are, therefore, independent of environmental influence and of the developmental stage of the nematode. Direct analysis of the genome by detection of differences in DNA fragment sizes enables precise species identification based directly on the genome [10, 11, 12, 13, 14].

Meloidogyne species occur widely in Cameroon in different agro-ecological zones. They thrive in the hot, humid, coastal lowlands of the East, Centre, South, Littoral, and South West Provinces with mean annual temperatures estimated at between 19.7 and 31.1°C. They are equally successful parasites in the cold highland regions of the West and North West Provinces where the mean annual temperatures range from 15.4 to 28.0°C. However, no studies have been carried out to accurately identify the species present.

The objective of this work was, therefore, to identify Cameroonian *Meloidogyne* species by the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Meloidogyne isolates

Meloidogyne isolates were collected from fifteen localities in Cameroon: five in the South West Province, four in the West Province, and six in the North West Province (Table 1). The sites and crops for sample collection were selected at random, knowing that

Meloidogyne species are widely distributed and parasitise almost all major crops. In each locality and using fine forceps, ten *Meloidogyne* egg masses were carefully picked from infected roots of a selected crop, after cautiously digging the roots out of the soil. The egg masses were collected in 25-ml sample bottles containing 0.3M sodium chloride solution to prevent the eggs from hatching. The egg masses from each of the fifteen localities in Table 1 represented one *Meloidogyne* isolate, giving a total of fifteen isolates. The isolates were numbered consecutively from 1 to 15 (Table 1), and transported to the Institute of Nematology and Vertebrate Research in Münster, Germany.

Table 1: Sources of *Meloidogyne* isolates characterised by the polymerase chain reaction (PCR).

		Region and locality
Isolate	**Host plant**	**South West Province**
1	Tomato (*Lycopersicon esculentum*)	Kumba
2	Pumpkin (*Cucurbita pepo*)	Muyuka
3	Plantain (*Musa paradisiaca*)	Small Ekombe
4	Soybean (*Glycine max*)	Weme (30 km from Kumba on the road to Mamfe)
5	Sugarcane (*Saccharum officinarum*)	Ekona
		West Province
6	Potato (*Solanum tuberosum*)	Pastoral, Djutitsa
7	Peanut (*Arachis hypogaea*)	Bansoa (25 km from Dschang on the road to Bafoussam)
8	Maize (*Zea mays*)	Foumbot
9	Peanut (*Arachis hypogaea*)	Dschang, IRAD Farm
		North West Province
10	Potato (*Solanum tuberosum*)	Bambui Upper Farm
11	Soybean (*Glycine max*)	Bamunka, Ndop
12	Soybean (*Glycine max*)	Santa Mbei, Mezam Division
13	Pineapple (*Ananas comosus*)	Mfonta (7 km NW of Bambui)
14	Peanut (*Arachis hypogaea*)	Babungo (19 km from Ndop on the road to Kumbo)
15	Plantain (*Musa paradisiaca*)	Bali (17 km from Bamenda on the road to Batibo)

Culturing of *Meloidogyne* in the greenhouse

Four-week-old susceptible tomato (*Lycopersicon esculentum* cv. Moneymaker) seedlings grown in 15-cm diameter plastic pots were inoculated with *Meloidogyne* eggs of each of the fifteen isolates. Three holes, each about 2.5 cm deep, were made in the soil around the base of the stem of each seedling. *Meloidogyne* eggs were poured into each hole and covered with soil. Two tomato seedlings were inoculated with *Meloidogyne* eggs of each of the fifteen isolates. The soil was watered and the plants transferred to the greenhouse maintained at 25-27°C, and at an average relative humidity of 65%. The plants were watered once every two days.

Extraction of *Meloidogyne* DNA

Meloidogyne second-stage juveniles (J2) were extracted from the roots of eight-week-old greenhouse tomato cultures by the mistifier extraction technique [15]. DNA was extracted from single *Meloidogyne* second-stage juveniles (J2) using proteinase K with worm lysis buffer [16].

Polymerase Chain Reaction (PCR)

The PCR was performed using DNA extracted from single second-stage juveniles of each of the fifteen *Meloidogyne* populations. Owing to the small volumes of components required in the PCR mixture, a "master mix" of components for twelve PCR samples of 30 µl each, without the DNA, was prepared (Table 2). Twenty microlitres (µl) of *Meloidogyne* DNA solution were added to 30 µl of the master mix in a PCR tube to give a final volume of 50 µl. Twelve tubes were prepared for a fourteen-well gel: ten tubes containing the unidentified *Meloidogyne* DNA samples (template DNA), one (tube 11) a negative control without template DNA to avoid misinterpretations of the DNA bands due to artifacts, and one (tube 12) a positive control with template DNA of a known *Meloidogyne* species. The remaining two wells in the gel contained 5 µl each of the molecular weight marker. The PCR was performed in a thermocycler (Perkin Elmer GeneAmp PCR System 2400) with the following hot-start thermal profile: an initial heating at 94°C for 2 min 30 sec; DNA denaturation at 94°C for 1 min; annealing at 55-56°C for 1 min, and primer extension at 72°C for 2 min. The denaturation, annealing, and primer extension cycle was repeated 30 times. A 5-min incubation period at 72°C followed the last cycle to complete any partially synthesised second strand and ended with a soak and storage file at 4°C. Two runs, each with the species-specific primer pair for each of the four *Meloidogyne* species: *M. incognita, M. javanica, M. arenaria*, and *M. hapla* were carried out for each of the fifteen Cameroonian *Meloidogyne* isolates.

Identification of *Meloidogyne* species from the PCR amplification products

A 10-µl sample of the PCR product was loaded, along with a molecular weight marker ("DNA ladder" 100-5000 bp), onto a 2% agarose gel.

Table 2: Composition of the PCR mixture for one sample and the "master mix" for twelve samples.

Component	Volume (µl) required for 1 sample of 50 µl	Volume (µl) for 12 samples of 50 µl each
Double distilled water	21.2	254.4
10 x PCR buffer	5	60
$MgCl_2$ solution*	1	12
dNTPs (mixture)**	2	24
Finc, Far, Fjav, or Fhap***	0.3	3.6
Rinc, Rar, Rjav, or Rhap***	0.3	3.6
T aq polymerase****	0.2	2.4
Total	30	360
DNA solution	20	-

* Qiagen *T aq* core kit together with *T aq* polymerase, 10 x buffer, dNTPs; ** A mixture of deoxyribonucleoside triphosphates (dNTPs) containing deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP); *** Species-specific primer pairs (F: the forward primer; R: the reverse primer) developed for *M. incognita* (Finc/Rinc), *M. arenaria* (Far/Rar), and *M. javanica* (Fjav/Rjav) [14] and for *M. hapla* (Fhap/Rhap) [10]; **** Heat-stable DNA polymerase extracted from the thermophilic bacteria, *Thermus aquaticus.*

The gel was placed in an electrophoresis chamber and run at 55V for 2h 30 min. The gel was then stained with the fluorescent dye, ethidium bromide, for 15-20 min, and the DNA bands on the gel were visualised under short-wave (254 nm) ultraviolet transillumination. A picture of the gel was taken and the sizes of the *Meloidogyne* DNA fragments were compared with the positive control *Meoidogyne* DNA bands and the molecular weight markers ("DNA ladder") in the same gel.

RESULTS

Using total DNA of single second-stage juveniles from each of the fifteen Cameroonian *Meloidogyne* isolates as the template with species-specific primers in PCR, three sizes of DNA amplification products were obtained (Figs 1, 2, 3). In Fig. 1 the gel wells 1-10 each contained the same PCR mixture including the DNA template of the unidentified *Meloidogyne* species and the species-specific primer pair Finc/Rinc. The amplification product was a DNA fragment of 1200 bp (Fig. 1: lanes 4-7, 9, and 10), corresponding to *M. incognita* [14]. However, the PCR in gel wells 1-3 and 8 failed and produced no bands. Lane 11 showed no band: there was no DNA template, the mixture serving as the negative control.

The PCR mixture in gel well 12 (the positive control) contained the Finc/Rinc primers and *M. incognita* DNA template, but there was no band: the PCR failed. This might have been due to contamination of the template DNA by foreign particles during storage. The 1200 bp DNA fragment size was the most common, obtained from ten of the fifteen *Meloidogyne* isolates. These isolates were those collected from tomato (*L. esculentum*), pumpkin (*C. pepo*), plantain (*M. paradisiaca*) soybean (*G. max*), sugarcane (*S. officinarum*), maize (*Z. mays*), and pineapple (*A. comosus*) in the South West, West, and North West Provinces (Table 1).

The DNA template of two unidentified *Meloidogyne* isolates and the species-specific primer pair Fhap/Rhap produced DNA fragments of 1500 bp (Fig. 2: lanes 1-3 and 5-10), corresponding to *M. hapla* [10]. In lane 4 the PCR failed, and no bands were produced. Lane 11 served as the negative control without DNA template, while the band in lane 12 was the positive control with DNA template of *M. hapla*. The two *M. hapla* isolates were collected from potato (*S. tuberosum*) in Pastoral Djutitsa and Bambui Upper Farm, respectively, in the West and North West Provinces.

A DNA fragment size of 420 bp was obtained using the DNA template of three unidentified *Meloidogyne* isolates with the Far/Rar species-specific primer pair (Fig. 3: lanes 5-8). These bands correspond to *M. arenaria* [14]. However, the PCR products in gel wells 1-4, 9, and 10 failed and no bands were produced. No band was obtained in lane 11, the negative control without DNA template, while in lane 12, the positive control with *M. arenaria* template, the PCR equally failed and there was no band produced, most probably due to contamination of the *M. arenaria* DNA during storage. The *M. arenaria* isolates were collected from peanut (*A. hypogaea*) in the West and North West Provinces. No DNA amplification was observed when the Fjav/Rjav primer pair was used with template DNA of any of the fifteen Cameroonian *Meloidogyne* isolates.

DISCUSSION

The Polymerase Chain Reaction (PCR) is an enzymatic reaction which allows *in vitro* amplification of a specific DNA segment up to a billionfold, by the simultaneous extension of primers to form complementary strands of DNA in a test tube. By amplifying micro quantities of DNA from second-stage juveniles of Cameroonian *Meloidogyne* isolates using the PCR, followed by analysis of the amplification products by agarose gel electrophoresis, three of the four most common *Meloidogyne* species were identified.

The sizes of the amplified DNA fragments obtained were typical for *M. incognita* (1200 bp), *M. hapla* (1500 bp), and *M. arenaria* (420 bp). Thus, the appearance of these DNA fragments allowed the precise identification of the source of the amplified DNA – the three *Meloidogyne* species. Similar results had previously been reported by other researchers [14, 10].

The absence of amplified DNA fragments with the Fjav/Rjav primer set indicates that there was no *M. javanica* in any of the Cameroonian isolates studied. On the other hand, the failure of the PCR in some of the mixtures of species-specific primer pairs and template DNA of *M. incognita, M. hapla,* and *M. arenaria*, might have been due to contamination of the DNA in those particular mixtures.

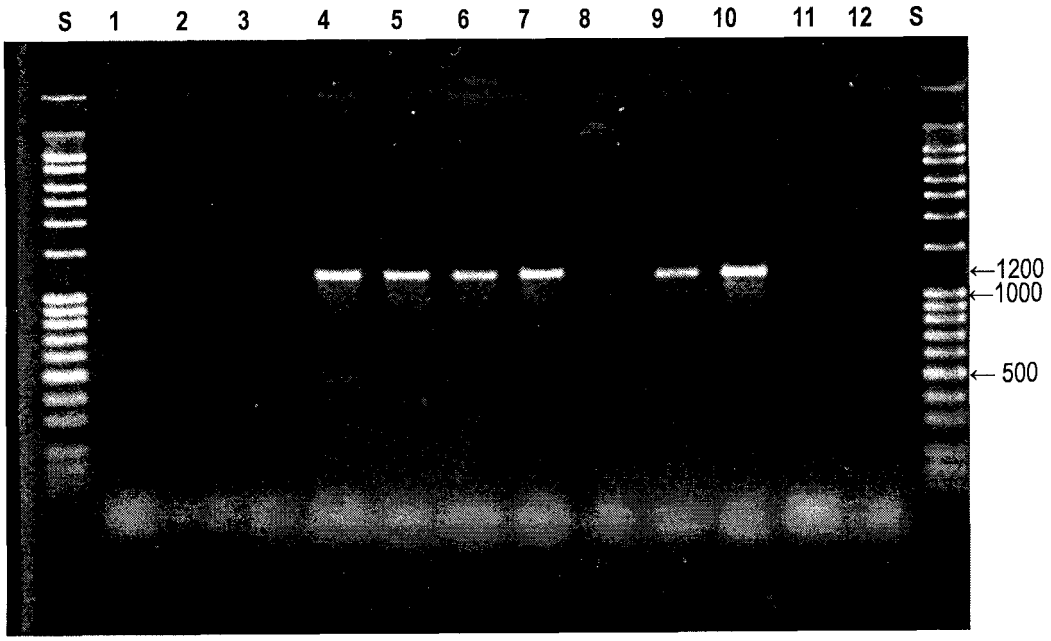

Figure 1: Photograph of an agarose gel showing typical amplification products of PCR using the species-specific primer pair Finc/Rinc and template of total DNA of single second-stage juveniles from ten of the fifteen Cameroonian *Meloidogyne* isolates.

S: molecular weight marker (running from 100 bp to 5000 bp). Amplified DNA fragment size = 1200 bp. Lanes 1-3 and 8: failed PCR; lane 11: negative control without template DNA; lane 12: failed positive control with *M. incognita* template.

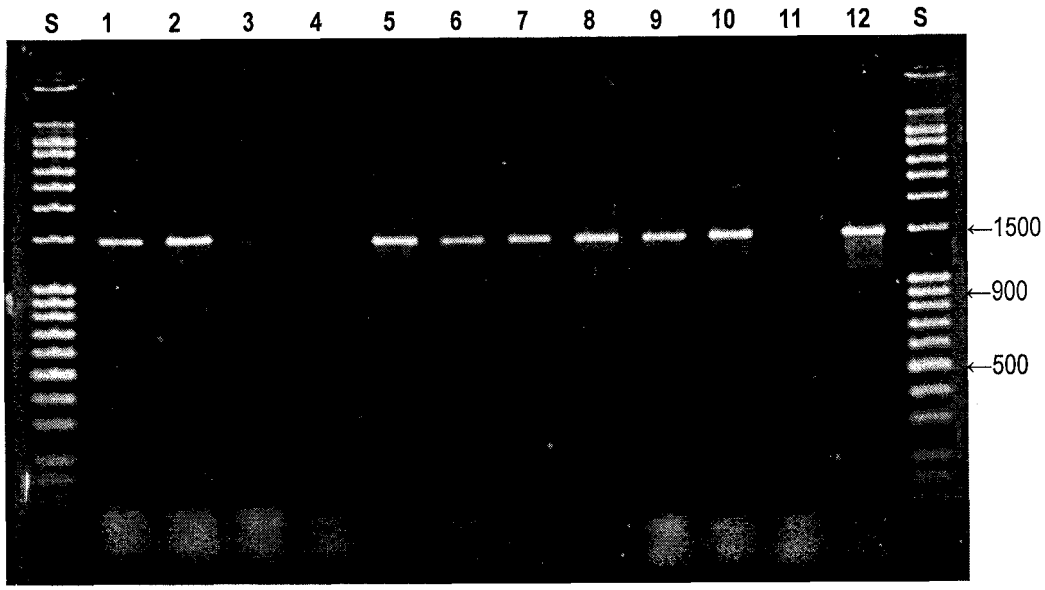

Figure 2: Photograph of an agarose gel showing typical amplification products of PCR using the species-specific primer pair Fhap/Rhap and template of total DNA of single second-stage juveniles from two of the fifteen Cameroonian *Meloidogyne* isolates.

S: molecular weight marker (running from 100 bp to 5000 bp). Amplified DNA fragment size = 1500 bp. Lane 4: failed PCR; lane 11: negative control without template DNA; lane 12: positive control with *M. hapla* template DNA.

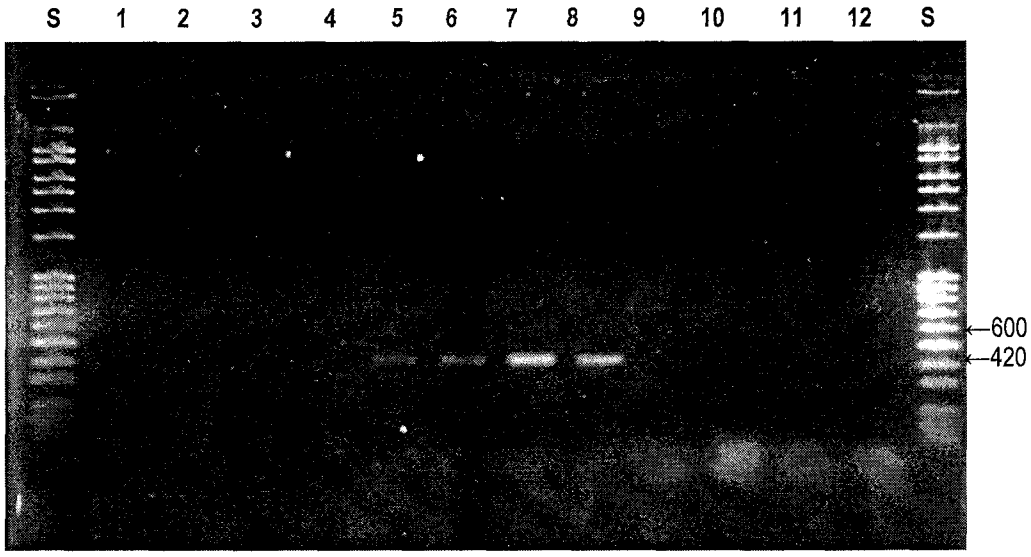

Figure 3: Photograph of an agarose gel showing typical amplification products of PCR using the species-specific primer pair Far/Rar and template of total DNA of single second-stage juveniles from three of the fifteen Cameroonian *Meloidogyne* isolates.

S: molecular weight marker DNA (running from 100 to 5000 bp). Amplified DNA fragment size = 420 bp. Lanes 1-4, 9, and 10: failed PCR; lane 11: negative control without template DNA; lane 12: failed positive control with *M. arenaria* template.

The ten isolates which were identified as *M. incognita* were collected from a variety of host plants in the South West, West, and North West Provinces of Cameroon. *Meloidogyne incognita* is generally the most common *Meloidogyne* species in warm tropical regions and has a wide host range [17]. *Meloidogyne arenaria* was identified in isolates collected from its principal host in tropical and subtropical regions, peanut (*A. hypogaea*), in the West and Noth West Provinces. The two *Meloidogyne* isolates, which were identified as *M. hapla* were collected from potatoes (*S. tuberosum*) grown at an altitude of 2000 m above sea level in Pastoral Djutitsa and Bambui Upper Farm, in the West and North West Provinces, respectively. Although *M. hapla* occurs mostly in cold temperate regions, it is known that the species equally thrives at high altitudes in the tropics [17].

Worldwide, *Meloidogyne* is the most important plant-parasitic nematode genus, attacking almost all major crops. Accurate identification of these economically important pests is an increasingly essential component of plant protection. *Meloidogyne incognita, M. javanica,* and *M. arenaria* have the same geographical distribution, but somewhat different host ranges. This enables them to be controlled by crop rotation and resistance management, which require accurate identification.

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