Electrophoretic Patterns of Some Populations of *Meloidogyne Arenaria* and *M. javanica*

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Abstract

Root-knot nematodes (*Meloidogyne* spp.) are important polyphagous plant parasites worldwide. Intraspecific variation and the occurrence of physiological races have necessitated the development of taxonomic techniques enabling differentiation and classification of races related to major *Meloidogyne* spp. Accurate identification of their races and populations is essential for determining appropriate management approaches and benefits from further characterization and understanding of the variability within a single nematode species. Electrophoretic focusing of malic dehydrogenase, α-glycerophosphate dehydrogenase and esterase for seven populations of *Meloidogyne* spp. by polyacrylamide gels was tested. These populations included 1, 2 and 4 populations of *M. javanica* and *M. arenaria* race I and II, respectively. Malic Dehydrogenase profiles obtained by polyacrylamide-gel electrophoresis indicated that differences between *Meloidogyne arenaria* and *M. javanica* were more pronounced than were differences between races or populations of *M. arenaria.* The enzyme patterns of the two species varied even though the nematodes were propagated on the same host plant (Rutgers tomato).

Key words: Biochemical markers, *Meloidogyne arenaria*, *M. javanica*, root-knot nematode, molecular taxonomy.

Introduction

It is well-established that the variability of many morphological characters and the presence of numerous physiological races within the same nematode species, are two of the most important problems associated with the taxonomy of plant-parasitic nematodes **(Allen and Sher, 1967; Osman *et al*., 1985; Abd-Elgawad and Askary, 2015).** These problems have prompted the search for other approaches not based entirely on anatomy and morphology, as other tools for the identification and characterization of species and races of nematodes. Among these approaches, biochemical systematics is one which has provided accurate and helpful information about nematodes and their phylogenetic relationships, complementing and extending the information provided by classical morphologically-based taxonomy **(Hussey, 1979, 1990).**

Root-knot nematodes, *Meloidogyne* spp., have been grouped among the major plant pathogens affecting many economically important crops (**Perry *et al*., 2009)**. The increasing development and use of cultivars resistant to species of *Meloidogyne* make accurate identification of this species necessary for effective management procedures to be developed and implemented **(Lawson *et al*., 1984).** Clearly, proteins are an expression of the sequence of the nucleotides in a gene, and the analysis of these molecules may provide an approach for comparing the genotypes of nematodes. In general, electrophoretic focusing of isozymes, proteins with different molecular forms and identical or similar substates, obtained from mass homogenates of a population, may enable us to compare and identify different populations **(Nei and Chakraborty, 1973; Siciliano and Shaw, 1976).**

The taxonomic value of electrophoresis has dearly been established for invertebrates and vertebrates. Electrophoretic data are particularly valuable in separating species, especially sibling species, but can discriminate between subspecies only when the organisms have undergone an exceptional amount of divergence **(**Ibrahim and Rowe, 1995). However, **Ayala (1978)** stated that the theoretical considerations of the genetic code and of electrical properties of amino acids suggested that only about one-third of all amino acid replacements are detectable by electrophoresis. **Blok and Powers (2009)** presented a taxonomic overview of root-nematodes (*Meloidogyne* spp.) with relevant historical and recent studies, including methodology used in chemotaxonomy, morphotaxonomy and molecular taxonomy. **García and Sánchez-Puerta (2012)** reported morphometric, morphological, biochemical, reproductive, molecular, and host range characterization of a root-knot nematode species from Argentina. Even after gathering morphological and morphometric data of this population and partially sequencing its rRNA, an unequivocal taxonomic assignment could not be achieved. The most decisive data was provided by esterase phenotyping and molecular methods using SCARs. These results highlight the importance of taking a multidimensional approach for *Meloidogyne* spp. diagnosis. This study contributes to the understanding of the variability of morphological, reproductive and molecular traits of *M. arenaria*, and provides data on the identification of root-knot nematodes on tomato cultivars. **Aydinli and Mennan (2016)** identified 90 populations of root-knot nematodes collected from different greenhouses in the Middle Black Sea Region, Turkey using not only morphological (perineal pattern morphology) but also biochemical (esterase phenotype) and molecular (PCR with species-specific primers) techniques. They confirmed that the E3 esterase phenotype is a useful character for distinguishing *M. ethiopica* from other *Meloidogyne* species.

Some complications in the preparation of nematodes for electrophoretic study were raised by **Chow and Pasternak (1969)** and **Dickson *et al*. (1971)** who showed changes in the isozymes patterns with the development of nematodes. **Hussey (1971)** reported a technique defined for obtaining appropriate quantities of

living *Meloidogyne* females usable for biochemical studies. Various means of homogenizing nematodes, including glass tissue homogenizing have been reported **(Dickson *et al*., 1971). Platzer (1981),** in a survey about electrophoretic studies, indicated that most investigators of nematode proteins used polyacrylamide-gel electrophoresis at an alkaline pH.

Starting from the 1970's isozyme patterns of root-knot nematodes were largely investigated and used in the separation of these nematodes **(e.g., Dickson *et al*., 1970 and 1971; Dalmasso and Berg, 1978; Hussey *et al*., 1979; Osman and Dickson, 1984). Ishibashi (1970) and Hussey *et al.* (1972)** reported that certain enzyme patterns of *M. incognita* and *M. arenaria* varied, depending on the host plant used for propagating the nematodes. However, **Dickson *et al.* (1971)** found that the host plant used for propagating the nematodes did not affect the enzyme patterns related to the same nematode population. Conflict or non-significant results of electrophoretic analyses of nematode proteins can occur for many different reasons **(Hussey *et al*., 1972; Lawson *et al*., 1984; Lopez, 1984).** The primary problem is determining which chemical characters will be most valuable in providing information for nematode taxonomy **(Hussey, 1979).** He reported that esterase, malate dehydrogenase, and α-glycerophosphate dehydrogenase patterns were distinctly characteristic for *M. incognita* and *M. arenaria*. In 1981, **Lawson *et al.* (1984)** found that *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica* were distinguishable from each other by isoelectric focusing of nematode egg proteins. **Lopez (1984)** reported that intraspecific differences were noted in patterns of five enzymes between two populations of *M. hapla* and of four enzymes between two populations of *M. exigua*.

Materials and Methods

1. **Nematode populations:** Six populations of *Meloidogyne arenaria* and one population of *M. javanica* were investigated.The designation, host race, and source of populations of *M. arenaria* and *M. javanica* were: *M. arenaria* (183), Race II, Suwannee Co., FL; *M. arenaria* (186), Race I Jackson Co., FL; *M. arenaria* (201), Race I, Jackson Co., FL; *M. arenaria* (204), Race II, NC; *M. arenaria* (206), Race II, TX; *M. arenaria* (207), Race II, CA; and *M. javanica* (167), Suwannee Co., FL. The nematode populations were multiplied on tomato, cv. Rutgers growing in Arredondo fine sand top soil treated with steam at 100 ^{0}C for 24 hours before filling the pots. Each pot was fertilized twice a week during the first five weeks of growth with 100 ml of a 1% solution of Nitrisol® (12 - 10 - 20). The pots of each population were placed randomly on a greenhouse bench and kept separated from other populations by plastic dividers to avoid splashes and contamination.

2. **Sample preparation:** Sixty days after inoculation, the root systems were cut into sections approximately 3 cm long and treated as described by **Hussey (1971),**

with some modifications. These modifications consisted of agitating the flasks containing chopped roots covered by Pectinol® 59 L, at 150 oscillations per minute for 18-34 hours, the use of a 1.0 M sucrose solution for centrifuging the suspension of females, and transferring females directly from the 60-mesh sieve to a beaker containing a 1% Na Cl solution **(Lopez, 1984).** The females were collected free of debris with a Pasteur pipette. Twenty-five adult females of each nematode population were placed in polyethylene microcentrifuge tubes (7 x 30 mm, 250 Ml), the saline solution was removed and replaced with 20 µl of a 0.1 MK_2 HPO_4 buffer with 0.8% NaCl and 0.001 MMg $C1_2$ **(Hussey *et al*., 1972).** Samples were stored at - 85 ^{0}C for no more than two months until proteins were extracted and processed electrophoretically.

3. **Electrophoresis:** Stock solutions, reservoir buffer, and wash-solution for destaining and storing gels were prepared and processed for polyacrylamide-gel electrophoresis as reported by **Davis (1964).** Electrophoresis was conducted in a Polyanalyst® (Buchler Instruments Division, Fort Lee, N,J., U.S.A.) at 1.5 mA/column in an anionic system for 15 minutes then at 3 mA/column and terminated when the maker dye, bromophenol blue, migrated about 50 mm into the separating gel. A cooling system provided a temperature of 4 °C for the outer walls of the apparatus during the operation. The nematode protein was prepared as described by **Huettel *et al*. (1983),** except that sucrose (60 mg/ml of sample) was added to increase the density of the solution which was layered on top of the spacer gel; a sample for each glass column of 5 mm inner diameter (i.d.).

4. **Enzyme reaction mixture:** Sites of enzyme activity were detected following electrophoretic separation by immersing the polyacrylamide gels in reaction mixtures until the appearance of bands. Malic Dehydrogenase (MDH) and α-glycerophosphate dehydrogenase (GDH) activities were tested on polyacrylamide gels by nitro blue tetrazolium (NBT) deposition according to **Gilbert and Goldbery (1966).** Gels to be tested for esterase activity were first placed in 0.2 M phosphate buffer (pH 7.0) as published by **Dickson *et al*. (1971).** Half of this solution was decanted before adding the substrate solution which included fast blue RR as the staining dye. Both DH buffer and esterase buffer were prepared as published by **Bush and Huettel (1972).** A light diffuser was used for focusing the bands and estimating their Electrophoretic focusing (Ef).

Results

Multiple bands of MDH were detected for all populations of *Meloidogyne* spp. (Fig. 1). The number of bands and the intensity of the staining reaction on the gel surface related to enzymatic activity were the same for all replicates; each nematode population was replicated twice. *M. javanica* was distinguished from populations of *M. arenaria* by the intensity of two slow moving bands. Additional distinguishable characteristics of *M. javanica* were the slowest MDH migration for

the last band, an Ef of 0.19, and the relatively light intensity of four of its bands. Differences between the two races of *M. arenaria* were noted. The slowest migrating MDH for the race I had an average Electrophoretic focusing (Ef) of 0.22 whereas that average of the race II was 0.29 but the fastest migrating MDH had an average Ef of 0.57 for both races. Differences in the number of bands, migration rates, and enzymatic intensity of MDH were recorded among the different populations of *M. arenaria*. These differences were much more pronounced among *Meloidogyne* spp. tested than they were among either the races or the populations of *M. arenaria*. No activity was detected for GDH and no discrete bands were observed for esterase, although some smearing was evident on all gels indicating enzymatic activity.

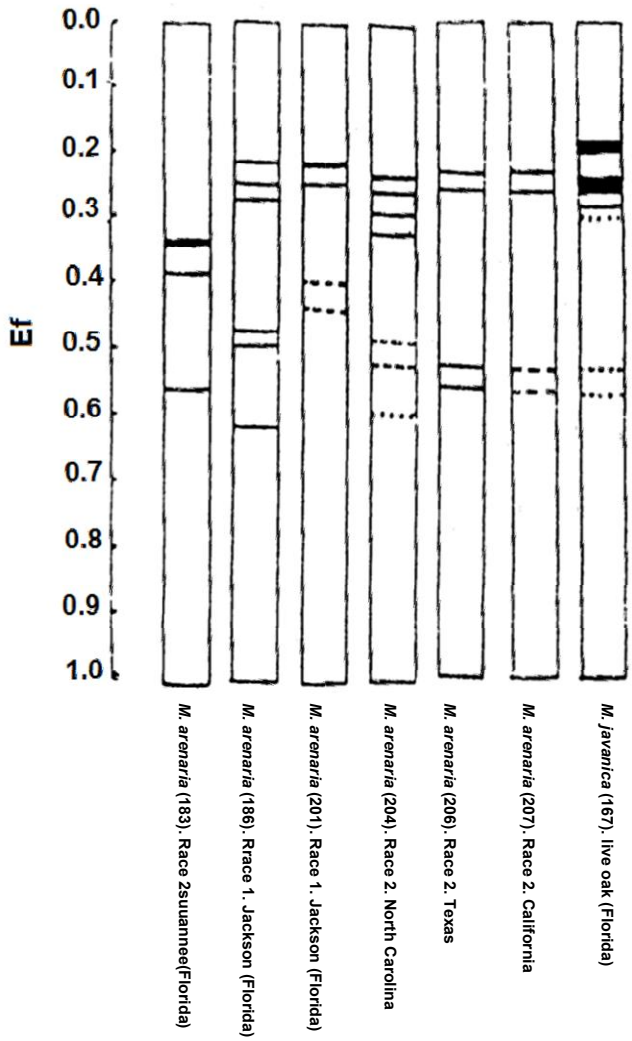

Figure (1): Electrophoretic focusing of malic dehydrogenase for seven populations of *Meloidogyne* spp. by polyacrylamide gels.

Discussion

Results of the electrophoretic study confirmed earlier reports that polyacrylamide-gel electrophoresis of proteins can be used to identify some specific enzymes and aid in the taxonomy of nematodes as published by **Platzer (1981).** The obtained data agreed completely with **Ayala's (1978)** report that theoretical considerations of the genetic code and of electrical properties of amino acids suggested that only about one-third of all amino acid replacements are detectable by electrophoresis. However, other reports, fortunately, could get more detectable isozymes than ours **(Dickson *et al*., 1971; Hussey *et al*., 1972)**. Several factors possibly have contributed to the failure to detect enzymatic activity of GDH and for obtaining only smears instead of bands in the esterase gels. The method of culturing, stage of development, physiological state of the nematodes, protein extraction procedures, storage conditions of the protein extract and the method of protein analyses are factors that can induce variability in electrophoretic analyses of nematode proteins as reported by **Hussey *et al*. (1972). Lopez (1984)** suggested such a proposal for three non-detectable enzymes that he encountered in electrophoretic studies of adult females of *Meloidogyne* spp.

The study of MDH indicated that differences between the two species of root-knot nematodes were more pronounced than were differences between races or populations of *Meloidogyne arenaria* tested. This coincided with the morphological systematics that variations among the species are more distinct than they are among the subspecies. However, differences among different populations for each of the two races of *M. arenaria* were indicated in our results, too. These are possibly due to the variation in the aggressiveness or virulence of these populations since this enzyme is useful for the metabolism of nematodes. The variations are promising to assist future investigators in comparative studies along with the host plants for a better understanding of host-parasite relationship.

Results indicated that enzyme patterns of MDH for *M. javanica* and *M. arenaria* varied even though the nematodes were propagated on the same host plant, i.e., Rutgers tomato. Similar results were reported by **Dickson *et al*. (1971).** This suggests that the differences might be more attributed to the genetic constitution of the nematode rather than to the environmental conditions; especially the host plants.

Our present results highlight the importance of taking a multidimensional approach for *Meloidogyne* spp. diagnosis and provide further data on the identification of root-knot nematodes on tomato cultivars. Nevertheless, a further comparison of the results of electrophoretic study with those reported by others **(e.g. Lopez, 1984; Blok and Powers, 2009; Abd-Elgawad and Askary, 2015; Aydinli and Mennan, 2016)** for the root-knot nematodes indicates the need for an accurate standardization of procedures.

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References

Abd-Elgawad, M.M.M. and Askary, T.H. (2015). Impact of phytonematodes on agriculture economy. In: Askary, T.H. and Martinelli, P.R.P. (eds) Biocontrol Agents of Phytonematodes. Wallingford, CAB International, UK, pp. 3-49.

Allen, M.W. and Sher, S.A. (1967). Taxonomic problems concerning the phytoparasitic nematodes. *Annual Review of Phytopathology* 5: 247-264.

Avise, J.C. (1974). Systematic value of electrophoretic data. Systematic Zoology 23: 465-481.

Ayala, F.J. (1978). Chemical genetics and evolution. In: Rockstein, M. (ed.) Biochemistry of Insects. Academic Press, New York, pp. 579-616.

Aydinli, G. and Mennan, S. (2016). Identification of root-knot nematodes (*Meloidogyne* spp.) from greenhouses in the Middle Black Sea Region of Turkey. *Turkish Journal of Zoology* doi:10.3906/zoo-1508-19.

Blok, V.C. and Powers, T.O. (2009). Biochemical and molecular identification. In: Perry, R.N., Moens, M. and Starr, J.L. (eds) *Root-knot nematodes*. St. Albans, UK, CABI Publishing, pp. 98-112.

Bush, G.L. and Huettel, R.N. (1972). Starch gel electrophoresis of tephritid proteins: A manual of techniques, Int. Biol, Program, Working Group on Fruit Flies. Population Genetics Project. Phase I. 56 p.

Chow, H.H. and Pasternak, J. (1969). Protein changes during maturation of the free-living nematode, *Panagrellus silusiae*. *Journal of Experimental Zoology* 170: 77-84.

Dalmasso, A. and Berg, J.B. (1978). Molecular polymorphism and phytogenetic relationship in some *Meloidogyne* spp.: application to the taxonomy of *Meloidogyne*. *Journal of Nematology* 10: 323-332.

Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* 121: 404-427.

Dickson, D.W., Sasser, J.N. and Huisingh, D. (1970). Comparative disc-electrophoretic protein analysis of selected *Meloidogyne*, *Ditylenchus*, *Heterodera* and *Aphelenchus* spp. *Journal of Nematology* 2: 286-293.

Dickson, D.W., Huisingh, D. and Sasser, J.N. (1971). Dehydrogenases, acid and alkalinephosphatases, and esterases for chemotaxonomy of selected *Meloidogyne*, *Ditylenchus*, *Heterodera* and *Aphelenchus* spp. *Journal of Nematology* 3:1-16.

García, L.E. and Sánchez-Puerta, M.V. (2012). Characterization of a root-knot nematode population of *Meloidogyne arenaria* from Tupungato (Mendoza, Argentina). *Journal of Nematology* 44(3): 291-301.

Gilbert, L.I. and Goldberg, E. (1966). Metabolic relationships in the cockroach, *Leucopheae moderae*. II. Electrophoretic analysis of dehydrogenase activity in tissue extracts. *Journal of Insect Physiology* 12: 53-63.

Huettel, R.N., Dickson, D.W. and Kaplan, D.T. (1983). Biochemical identification of thetwo races of Radopholus similis by starch gel electrophoresis. *Journal of Nematology* 15: 338-344.

Hussey, R.S. (1971). A technique for obtaining quantities of living *Meloidogyne* females. *Journal of Nematology* 3: 99-100.

Hussey, R.S. (1979). Biochemical systematics of nematodes: a review. *Helminthological Abstracts, Series B, Plant Nematology* 48: 141-148.

Hussey, R.S. (1990). Biochemical and molecular methods of identifying *Meloidogyne* species: symposium introduction. *Journal of Nematology* 22(1): 8-9.

Hussey, R.S., Sasser, J.N. and Huisingh, D. (1972). Disc-electrophoretic studies of soluble proteins and enzymes of *Meloidogyne incognita* and *M. arenaria*. *Journal of Nematology* 4: 183-189.

Ibrahim, S.K. and Rowe, J. (1995). Use of isoelectric focusing and polyacrylamide gel electrophoresis of nonspecific esterase phenotypes for the identification of cyst nematodes, *Heterodera* species. *Fundamental and Applied Nematology* 18: 189-196.

Ishibashi, N. (1970). Variations of the electrophoretic protein patterns of Heteroderidae(Nematoda: Tylenchida) depending on the developmental stages of the nematode andon the growing conditions of the host plants. *Applied Entomology* and *Zoology* 5: 23-32.

Lawson, E.C., Carter, G.E. Jr., and Lewis, S.A. (1984). Application of isoelectric focusing to the taxonomic identification of *Meloidogyne* spp. *Journal of Nematology* 16: 91-96.

Lopez, R. (1984). Differential plant responses, morphometrics and electrophoretic patterns of some *Meloidogyne* spp. from Costa Rica and Florida, U.S.A. and description of *Meloidogyne salasi sp. n*. Ph. D. thesis, Dept Entomology and Nematology, University of Florida, pp. 124.

Nei, M. and Chakraborty, R. (1973). Genetic distance and electrophoretic identity of proteins between taxa. *Journal of Molecular Evolution* 2: 323-328.

Osman, A. Hamida and D.W. Dickson (1984). Physiological studies on three species of *Meloidogyne arenaria* races, and 2 *M. incognita, and M. javanica*. Al-Azhar Agric. Res., J. (15), 22-30.

Osman, A. Hamida, D.W. Dickson and G.C. Smart Jr. (1984). Morphological comparisons of host races one and two of *Meloidogyne arenaria* from Florida. Journal of Nematology, 17(3):279-285.

Perry, R.N., Moens, M. and Starr, J.L. (Eds) (2009). *Root-knot Nematodes*. St. Albans, UK, CABI Publishing.

Platzer, E.G. (1981). Potential use of protein and DNA nucleotide sequences in nematode taxonomy. In: Zuckerman, B.M. and Rohde, R.A. (eds) Plant parasitic nematodes, vol. 3. London and New York: Academic Press, 1-21.

Siciliano, M. and Shaw, C.R. (1976). Separationand visualization of enzymes on gels. In: Smith, I. (ed.). *Chromatographic and electrophoretic Techniques, 4th edn, vol. 2*. Chicago: Yearbook Medical Publishers, pp. 185-209.

الملخص العربي

استخدام طريقة العزل الكهربائي لتحديد أنماط بعض عشائر نيماتودا ميلودوجين أرنريا وميلودوجين جافنيكا

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تشكل نيماتودا تعقد الجذور مجموعة من الطفيليات الهامة متعددة العوائل في جميع أنحاء العالم. استلزم وجود اختلافات مورفولوجية وحدوث فروق فسيولوجية بين عشائر النوع الواحد لهذه النيماتودا تطوير تقنيات تصنيفها لتمكين التمييز بين عشائرها وتصنيف السلالات التابعة للأنواع الرئيسة من هذه النيماتودا فالتحديد الدقيق لهذه السلالات والعشائر أمر ضروري لتحديد نهج الإدارة المناسبة لمكافحتها كما يضيف المزيد من توصيف وفهم التباين داخل هذه الأنواع من نيماتودا تعقد الجذور، لذا قمنا باستخدام طريقة العزل الكهربائي لإنزيمات ماليك ديهيدوجينيز، وجليسروفوسفات ديهيدوجينيز، واستريز لتمييز ومقارنة سبع عشائر من نيماتودا تعقد الجذور على جيل البولي أكريلاميد. شملت هذه الطريقة ١ و٢ و٤ عشائر تابعة للنوع ميلودوجين جافنيكا، وميلودوجين أرنريا سلالة أولى وثانية، على التوالي. أمكن باستخدام طريقة العزل الكهربائي لأنزيم ماليك ديهيدوجينيز التمييز بين نوعي النيماتودا *ميلودوجين أرنريا*، و*ميلودوجين جافنيكا*، وكذلك التمييز – لكن بدرجة أقل – بين سلالات وعشائر النيماتودا ميلودوجين أرنريا رغم أن تكاثرها في الصوبة الزجاجية كان علي نفس النوع النباتي حيث استخدم نبات الطماطم (صنف رتجرز) كعائل نباتي لها جميعا.