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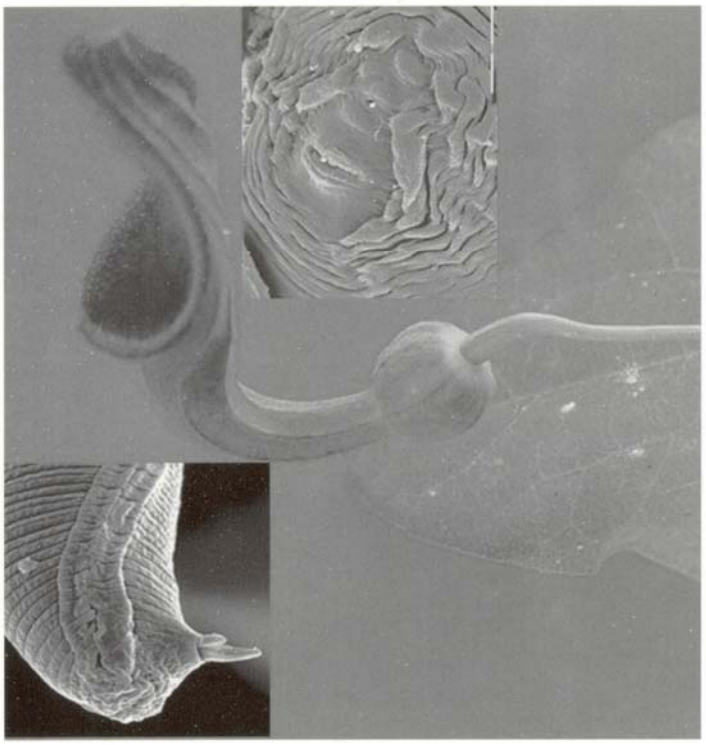

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SEM studies on the Mediterranean olive root-knot nematode, *Meloidogyne baetica*, and histopathology on two additional natural hosts

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Summary – SEM studies on a *Meloidogyne baetica* population provided additional details of the external morphology for female, male and second-stage juveniles. The labial disc in female and male specimens is fused with the medial lips forming a single structure. In second-stage juveniles the lateral lips are triangular with rounded margins. The amphidial opening for all life stages appears oval to rectangular in shape and is located between the labial disc and lateral lips. Lateral fields of male and second-stage juveniles have four incisures irregularly areolated along the entire body. The results of a host-range study for additional natural hosts of *M. baetica* conducted in wild olive communities growing at Vejer de la Frontera (Cádiz province) in southern Spain are also reported. Apart from the type host, *M. baetica* was found to infect two natural woody host plants, lentisc (*Pistacia lentiscus*) and *Aristolochia baetica*. Host-parasite relationships in these new hosts confirmed the typical susceptible reaction observed in wild and cultivated olives. Similarly, the reproductive fitness, evaluated as the number of eggs per egg mass, was not significantly different in all plant hosts. No infections or galled roots were observed in herbaceous plant species studied and *M. baetica* must therefore be considered as a parasite of woody plants.

Keywords – *Aristolochia baetica*, candilito, histopathology, host-parasite relationships, lentisc, mastic tree, *Pistacia lentiscus*, reproductive fitness, Spain.

Meloidogyne baetica Castillo, Vovlas, Subbotin & Troccoli, 2003 was recently described infecting wild olive at southern Spain (Castillo *et al.*, 2003). To date, its geographical distribution is limited to a small area of natural sandy soils at Vejer de la Frontera (Cádiz province) in southern Spain. Although the nematode type locality includes soil and plant communities referred to as Oleo-Lenticetum and Rosmarino-Ericion, otherwise known as 'matorral' in Spain (Rivas Martinez, 1982), information on the host-range of this root-knot nematode was limited to wild and cultivated (cvs Arbequina and Picual) olives (Castillo *et al.*, 2003). It is important to establish the natural hosts of this nematode and therefore a nematode survey was conducted in wild olive and associated plant communities growing at the type locality during the spring of 2003. In addition, although a complete morphological description, based on light microscopy, of the life stages of the nematode was presented in the original description, no scanning electron microscopy (SEM) studies were done. Consequently, the objectives of this study were: *i*) to describe the morphology of life stages of the nematode as seen by SEM; and *ii*) to study the natural hosts of *M. baetica* and describe their host-parasite relationships.

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Materials and methods

SEM STUDIES

For SEM studies, mature females were dissected from naturally-infected roots of wild olive, and migratory stages (including males and second-stage juveniles) were extracted from infested soils by the centrifugal flotation method (Coolen, 1979). Specimens were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid, and processed to pure glycerine using Seinhorst's method (Seinhorst, 1966). Fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold, and observed with a JEOL JSM-5800 microscope (Abolafia *et al.*, 2002).

HOST-RANGE STUDY

A nematode survey was conducted in wild olive communities growing at Vejer de la Frontera (Cádiz province) in southern Spain to obtain more information about the natural hosts of *M. baetica*. Thirty samples consisting of *ca* 3-4 kg soil and associated roots and plant parts were collected, placed in plastic bags, and stored at 7-10°C for up to 3-4 days before observation and nematode extraction. Soil was processed by the centrifugal flotation method (Coolen, 1979) in order to collect *M. baetica* second-stage juveniles and males. Roots and plant parts from soil samples infested with second-stage juveniles and males of *M. baetica* were separated according to plant species, washed, and examined with the aid of a stereomicroscope for nematode infection. Frequency of soil infestation and population density was determined. Frequency was calculated as the percentage of samples in which the nematode was found. For morphological and diagnostic studies, specimens were processed as above (Seinhorst, 1966). Measurements were done using a drawing tube attached to a light microscope.

In each natural host, including wild olive, the reproductive fitness of *M. baetica* was assessed by the number of eggs per egg mass. In each host, 20 mature egg masses of *M. baetica*-infected roots were individually separated, each egg mass being deposited in an individual 10 ml glass tube. Egg number per egg mass was determined after exposure to 1% NaOCl (Hussey & Barker, 1973) and vortexing for 5 min. Data of number of eggs per egg mass (X) were normalised before analysis by transforming them to log_{10} $(X + 1)$ (Gomez & Gomez, 1984). Analysis of variance (ANOVA) was carried out using Statistix 8.0 (NH Analytical Software, Roseville, MN, USA). Means values of number of eggs per egg mass were compared using Fisher's protected least significant difference test (LSD) at $P = 0.05$.

HISTOPATHOLOGY

Infected roots from naturally-infected host plants were gently washed free of adhering soil and debris and individual galls were selected together with healthy roots. Root tissues were fixed in formaldehyde chromo-acetic solution for 48 h, dehydrated in a tertiary butyl alcohol series (40-70-85-90-100%) and embedded in 58°C (melting point) paraffin wax for histopathological observations. Embedded tissues were sectioned with a rotary microtome. Sections 10-12 μm thick were placed on glass slides, stained with safranin and fast-green, mounted permanently in a 40% xylene solution of a polymethacrylic ester (Synocril 9122X), examined microscopically and photographed (Johansen, 1940).

Results

SEM STUDIES

Female

In face view, the labial disc is small, rounded, and slightly raised above the medial lips. The labial disc and medial lips are fused to form a narrow lip structure in face view. Amphidial openings are oval shaped and are located between the labial disc and lateral lips (Fig. 1A). The excretory pore is located near the lip region, six to eight annuli posterior to labial plate (Fig. 1B). Examination of the perineal pattern by SEM revealed the same features as with light microscopy (LM) studies, the pattern being typically formed from striae and cuticular ridges, the latter being more pronounced in the vicinity of the vulva and anus. The dorsal arch encloses the relatively distinct phasmids and the anus (Fig. 1C).

Male

Lip region usually marked by a short, incomplete annulation in lateral view. In SEM, the labial disc is oval and is fused with the elongate-oval medial lips forming a rectangular structure (Fig. 1D). The lateral lips are rounded-oval (Fig. 1D). The amphidial apertures are elongate slits located between the labial disc and the lateral lips (Fig. 1D). The lateral fields are marked by four incisures and are faintly and irregularly areolated at the mid-body and tail region (Fig. 1E, F).

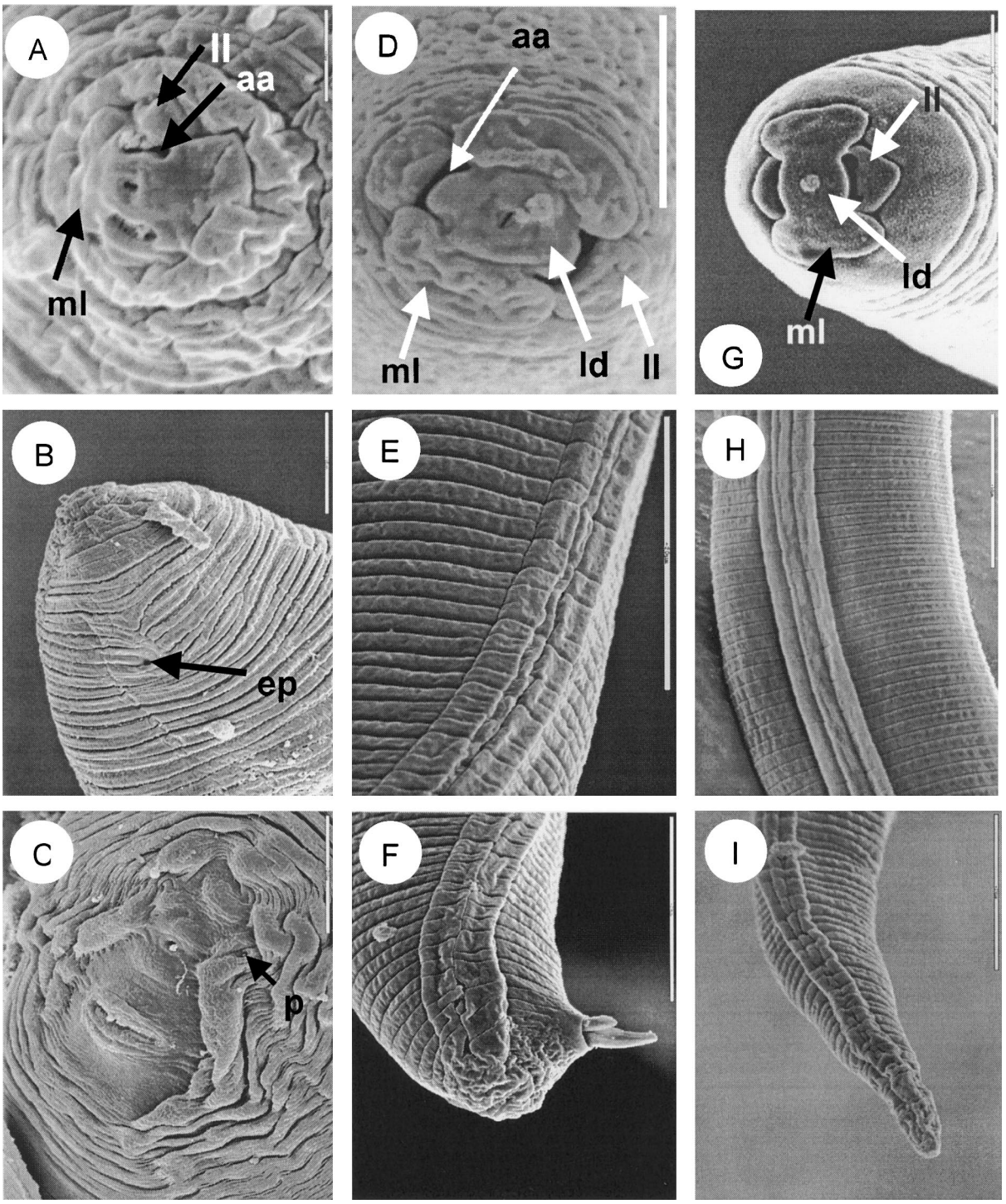

Fig. 1. *SEM micrographs of* Meloidogyne baetica*. Female (A-C). A: Face view; B: Anterior region showing excretory pore; C: Perineal pattern. Male (D-F); D: Face view; E: Lateral field at mid body; F: Tail region. Second-stage juvenile (G-I); G: Face view; H: Lateral fields at mid body; I: Tail region. Abbreviations: aa = amphidial aperture; ep = excretory pore; ld = labial disc; ll = lateral lip; ml = medial lips; p = phasmid. (Scale bars: A, G = 2 μm; D = 5 μm; B, H, I = 10 μm; C, E, F = 20 μm.)*

Table 1. *Plant species studied as potential hosts of* Meloidogyne baetica *Castillo* et al.*, 2003.*

Common name	Scientific name	Host (+) non-host (–)
wild garlic	*Allium ursinum* L.	–
'candilito'	*Aristolochia baetica* L.	+
white asparagus	*Asparagus albus* L.	–
asphodel	*Asphodelus albus* Mill.	–
daisy	*Bellis sylvestris* Cyr.	–
small pink bindweed	*Convolvulus althaeoides* L.	–
field bindweed	*Convolvulus arvensis* L.	–
wild artichoke	*Cynara cardunculus* L.	–
black broom	*Cytisus baeticus* (Webb) Steudel	–
lesser chickpea or vetch	*Lathyrus cicera* L.	–
wild olive	*Olea europaea* spp. *sylvestris* (Miller) Hegi	+
red or common poppy	*Papaver rhoeas* L.	–
Jerusalem sage	*Phlomis purpurea* L.	–
lentisc or mastic tree	*Pistacia lentiscus* L.	+
buttercup	*Ranunculus rupestris* Guss.	–
false sowthistle	*Reichardia tingitiana* (L.) Roth	–

Second-stage juvenile

In face view, labial disc and medial lips fused into one structure (Fig. 1G). Labial disc small and rounded, slightly elevated above medial lips (Fig. 1G). Medial lips elongate-oval, not indented medially with rounded margin. Amphidial apertures appearing as elongate slits between the labial disc and lateral lips (Fig. 1G). Lateral lips triangular with rounded margins. (Fig. 1H). Lip region smooth. (Fig. 1G). Lateral fields with four incisures, irregularly areolated along entire body (Fig. 1G-I).

Natural hosts

Meloidogyne baetica was detected in 14 out of the 30 soil samples (46.7%) collected in the type habitat locality, with population densities ranging between 128 and 1190 eggs and second-stage juveniles per 100 cm^3 of soil. *Meloidogyne baetica* infested soil samples were taken from around the herbaceous and woody plant species listed in Table 1, all of which were studied for potential infection with the nematode. Examination of root systems of these plant species indicated that the only natural hosts of *M. baetica* in the studied area, apart from wild olive, were the lentisc or mastic tree (*Pistacia lentiscus* L.) and 'candilito' (*Aristolochia baetica* L.), mature females with mature egg masses being observed on the root surface (Fig. 2A, D). No infections and no galled roots were detected in the other plant species studied (Table 1). Reproductive fitness, as determined by the number of eggs per egg mass, was not significantly ($P = 0.172$) different among natural-hosts of *M. baetica*, with 229 ± 80.8 (132-450) eggs in wild olive, 189 ± 73.5 (122-480) in lentisc, and 177 ± 72.9 (112-380) in *A. baetica*.

Histopathology

Root galls induced by *M. baetica* on lentisc and candilito varied in size and location, the majority of egg masses being observed on the root surface (Fig. 2A, D). Histological observations of *M. baetica*-infected lentisc and candilito roots revealed cellular alterations induced by the nematode in the cortex, endodermis, pericycle and vascular parenchyma (Fig. 2B-F). In the permanent feeding sites, nematode-induced, large, multinucleate, giant cells located adjacent to the vascular tissues were observed in both natural hosts (Fig. 2C, F). This formation led to the distortion and disruption of xylem elements and primary phloem cells (Fig. 2B, E). Nematode feeding sites comprised three to six giant cells surrounding the lip region of a single female (Fig. 2). Giant cells had a thickened cell wall and granular cytoplasm with four to ten hypertrophied nuclei and nucleoli (Fig. 2F). The histological modifications induced by *M. baetica* in roots of lentisc and candilito revealed a typical susceptible reaction to infection by the nematode.

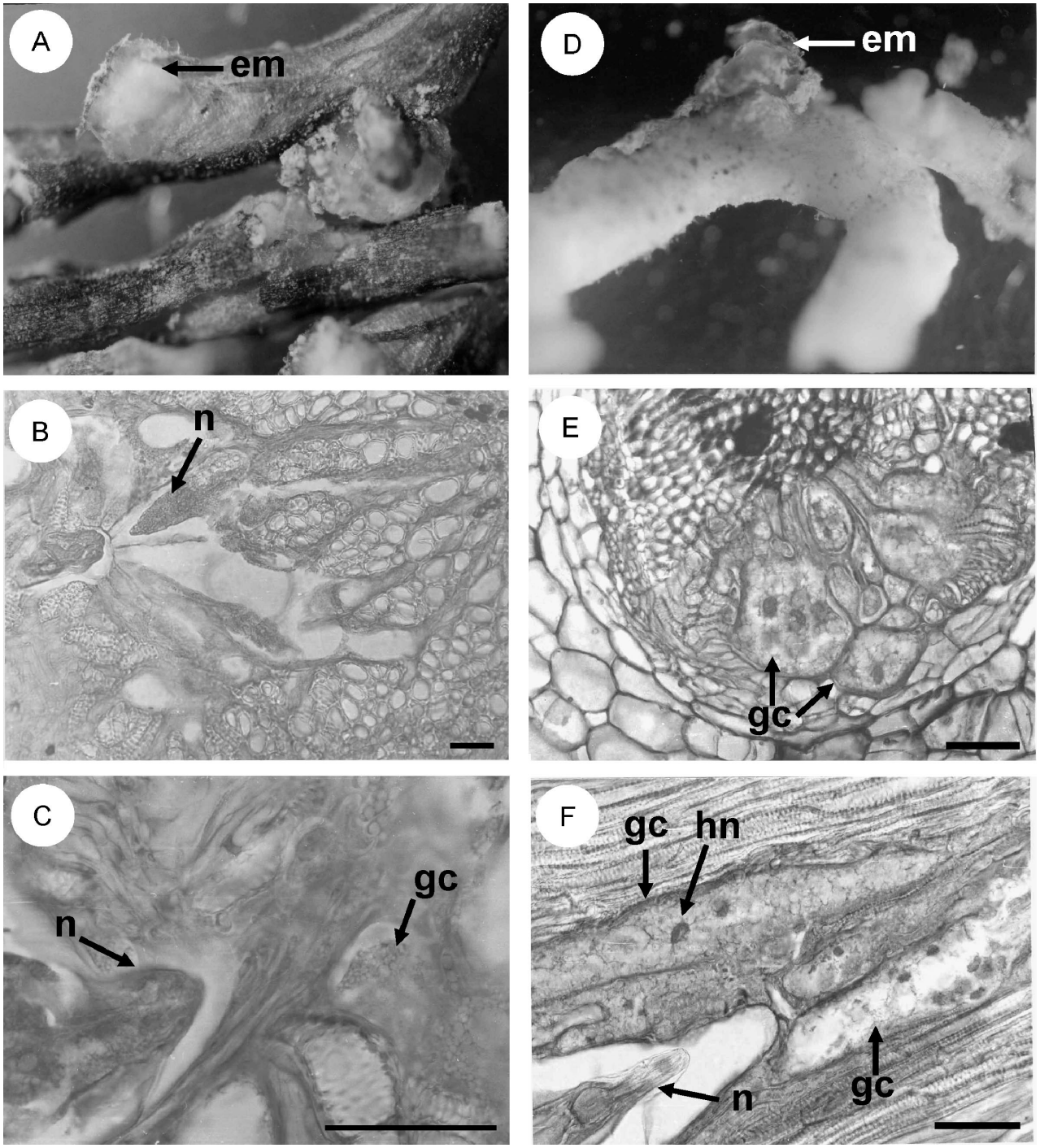

Fig. 2. *Infection and feeding site structures of* Meloidogyne baetica *in naturally-infected lentisc (A-C), and* Aristolochia baetica *roots (D-F). A, D: Infected root segment showing gall and egg mass (em); B, C: Transverse sections of lentisc roots showing nematode (n) and induced giant cells (gc) in feeding site; E, F: Transverse sections of* A. baetica *roots showing nematode (n) and distortion and disruption of cortex and xylem elements by expansion of giant cells (gc) with hypertrophied nuclei (hn) and nucleoli in feeding site. (Scale bars = 50 μm.)*

Discussion

SEM studies of life stages of *M. baetica* confirmed, but in greater detail, those features observed with LM in the original description (Castillo *et al.*, 2003). In addition, SEM studies revealed new data on the face view of female, male and second-stage juveniles, as well as some modifications to the original description, *i.e.* lateral fields of males and second-stage juveniles were revealed as faintly and irregularly areolated whereas they were described as non-areolated in the original description (Castillo *et al.*, 2003). As in most *Meloidogyne* species, the labial disc of females, males and second-stage juveniles of *M. baetica* is fused with the medial lips (Eisenback *et al.*, 1980; Jepson, 1987). Male and second-stage juvenile *en face* SEM views of *M. baetica* clearly differ from those of *M. artiellia* Franklin which were previously considered unique in the genus (Jepson, 1987). The following differences can be observed between both species in SEM studies: a) male labial disc of *M. baetica* is oval *vs* rounded in *M. artiellia*; b) male medial lips (elongate-oval not separated by constriction *vs* oval separated by a deep constriction); c) male lateral lips (rounded-oval *vs* rounded or almost semicircular); d) second-stage juvenile medial lips (not indented *vs* indented medially); and e) second-stage juvenile lateral lips (triangular *vs* rounded or almost semicircular).

Studies on the natural host status of herbaceous and woody plant species revealed that *M. baetica* has a narrow host-range including wild and cultivated (cvs Arbequina and Picual) olives (Castillo *et al.*, 2003) and the two new host plants lentisc and candilito. Data on nematode populations in soil indicate that *M. baetica* was found in small clumps of wild olive and the new woody hosts growing in the type locality. The morphological features of *M. baetica* specimens detected on these new natural hosts did no differ from those reported in the original description. These results, as well as those of the original description, demonstrate that *M. baetica* does not parasitise natural herbaceous plants at the type locality or cultivated herbaceous plants, such as tomato, pea and chickpea (Castillo *et al.*, 2003) and, therefore, must be considered as a parasite of woody plants. In addition, results on the reproductive fitness of the nematode in natural hosts, as well as those from histopathological studies indicate that all of these woody hosts showed a similar response to nematode infection and reproduction. The feeding behaviour of *M. baetica* and disease response on lentisc and candilito roots, was similar to that observed in wild and cultivated (cvs Arbequina and Picual) olives, as well as those of *Meloidogyne* spp. in cultivated olive planting stocks (Nico *et al.*, 2002).

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