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[1]DRAFT ANNEX TO ISPM 27: Mononychellus tanajoa (2018-006)

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[22]Consultation on technical level	 [23]The first draft of this protocol was written by: [24]Qing Hai FAN (NZ, Lead author) [25]Denise NAVIA (BR, Co-author) [26]Rachid HANNA (US, Co-author) [27]In addition, the draft has also been subject to expert review and the following international experts submitted comments: Frederic Beaulieu (CA), Sophie Peterson (AU) and Rajesh Ramarathnam (CA). 		
[28]Main discussion points during the development of the diagnostic protocol	[29]		
[30] Notes	[31]This is a draft document [32]2022-05 Edited		

[33]CONTENTS

[34][to be added later]

[35]Adoption

[36]This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in [Month 20--]. [to be completed after adoption]

[37] The annex is a prescriptive part of ISPM 27 (Diagnostic protocols for regulated pests).

1. [38]Pest information

[39]The cassava green mite, *Mononychellus tanajoa* (Bondar) (Acari: Tetranychidae), is one of the major pests of cassava *Manihot esculenta* (Euphorbiaceae) (Byrne *et al.*, 1982; Byrne, Belloti and Guerrero, 1983; Veiga, 1985) – the main staple crop for more than 11 percent of the world's population (FAO,

2013). It prefers to feed on the underside of young leaves of growing shoots of the cassava plant. Immature and adult mites feed by piercing plant tissues and sucking out the contents of cells, leading to leaf distortion and chlorotic mottling (Figure 1A, Figure 1B and Figure 1C). Severe mite damage can lead to defoliation of the upper parts of shoots, producing a "candlestick" appearance (Figure 1D) and resulting in 50–80% storage root yield loss (Shukla, 1976; Byrne, Belloti and Guerrero, 1983; Byrne *et al.*, 1982; Veiga, 1985; CABI, 2020). *M. tanajoa* has been on the A1¹ list of pests of the Asia and Pacific Plant Protection Commission since 1992 and the Pacific Plant Protection Organisation since 1993. The mite has also been on the A2² list of pests of East Africa and Southern Africa since 2001 and China since 1992 and has been a quarantine pest in the United States of America since 1989 (EPPO, 2020).

[42]*Mononychellus tanajoa* (Figure 2A) is considered to be a tropical and subtropical species. It was first described from *Manihot* spp. in South America in 1938 and is now widely distributed in South and Central America (Machi, *et al.* 2014; Vásquez-Ordóñez and Parsa, 2014; CABI, 2020; EPPO, 2020; Migeon and Dorkeld, 2021). In Africa, *M. tanajoa* was first reported in Uganda in 1971 (Lyon, 1973). From there, it rapidly spreads throughout the cassava-growing regions of the continent and is now established in over 30 countries (Byrne, Belloti and Guerrero, 1983; Gutierrez *et al.*, 1988; Yaninek, *et al.* 1989; Bolland, Gutierrez and Flechtmann, 1998; Vásquez-Ordóñez and Parsa, 2014; CABI, 2020; EPPO, 2020; Migeon and Dorkeld, 2021).

[43]This mite is mainly a pest of cultivated *M. esculenta*, although has also been recorded on other *Manihot* species (Bondar, 1938; Flechtmann and Baker, 1970). In addition, it occurs on several species in other plant families, including *Erythrina* sp., *Gliricidia maculata*, *Gliricidia sepium*, *Phaseolus vulgaris* and *Senna occidentalis* (Fabaceae) (Rossi Simons, 1961; Baker and Pritchard, 1962; Estebanes-Gonzalez and Baker, 1968; Andrews and Poe, 1980; Mendonça *et al.*, 2011); *Passiflora cincinnata* and *Passiflora edulis* (Passifloraceae) (Moraes, Moreira and Delalibera, 1995; Mendonça *et al.*, 2011); and *Typha domingensis* (Typhaceae) (Aguilar and Murillo, 2008; Moraes, Moreira and Delalibera, 1995; Migeon and Dorkeld, 2021).

[44]The life cycle of *M. tanajoa* consists of the egg and four active stages: six-legged larva, eight-legged protonymph, deutonymph and adult. An inactive (quiescent) stage is present between the active stages, during which moulting occurs. This species overwinters in temperate climates as eggs or adult females. It completes a generation in 24.7 days on *M. esculenta* at $24 \pm 2 \,^{\circ}C$, $65 \pm 10\%$ relative humidity (Moraes, Moreira and Delalibera, 1995). The developmental time can decrease substantially with increasing temperature; *M. tanajoa* needs only eight days to develop from egg to adult at 31 $^{\circ}C$. An adult female can lay about 58 eggs during its lifetime (Yaninek *et al.*, 1989). The highest population density of *M. tanajoa* occurs during the first half of the dry season (Yaninek *et al.*, 1989). In Brazil, severe damage is only observed in the dry areas of the northeast region, although the mite is widely distributed in the country (Moraes and Flechtmann, 2008).

[45]Mites in the family Phytoseiidae (Figure 2B) are considered to be the main natural enemies of spider mites (Tetranychidae). Among the natural enemies of *M. tanajoa*, more than 30 species of Phytoseiidae are found on *Manihot* spp. (Zannou *et al.*, 2005; Mutisya *et al.*, 2017; Demite *et al.*, 2021). Apart from the phytoseiid mites, the acaropathogenic fungus *Neozygites tanajoae* (Entomophthorales: Neozygitaceae) (Figure 2C) is a widespread host-specific pathogen of *M. tanajoa* in Brazil (Delalibera, Hajek and Humber, 2004). It was introduced into Benin in West Africa where it is presently widespread (Agboton, Hanna and Tiedmann, 2011) alongside the much less virulent *Neozygites floridana*), which has a much broader host range among tetranychid mites (Lopes Ribeiro *et al.*, 2009).

2. [46] Taxonomic information

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[47] **Name:** *Mononychellus tanajoa* (Bondar, 1938)

^{[40]&}lt;sup>1</sup> A1 pests are regulated as quarantine pests and are not present in the region

^{[41]&}lt;sup>2</sup> A2 pests are regulated as quarantine pests and are present in the region, with limited distribution

[48] Synonyms: Eotetranychus estradai Baker and Pritchard, 1962

[49] Tetranychus tanajoa Bondar, 1938

[50] Mononychus tanajoa (Bondar, 1938)

[51] Taxonomic position: Acari, Trombidiformes, Prostigmata, Tetranychidae

[52]Common names: cassava green mite, cassava mite

3. [53]Detection

[54]*Mononychellus tanajoa* may be first found on the underside of young leaves of the upper third of the cassava canopy (Yaninek *et al.*, 1989). With continuous feeding by a growing population of *M. tanajoa*, damage to the leaf expands to impact all interveinal leaf surfaces. Heavily damaged leaves eventually become desiccated and fall. As the mite prefers to feed on young leaves in the upper part of the cassava plant, severe damage can lead to shoot defoliation, giving the appearance of a "candlestick" (Figure 1D).

[55]In addition to plant foliage, *M. tanajoa* may be found on cassava stem cuttings, packaging, farm machinery, vehicles and tools, farm waste, workers' clothing, soil, and nearby plants that are in contact with the host. Because of the small size of *M. tanajoa*, it is extremely difficult to detect during the early stages of an infestation.

[56]While host symptoms are seen with the naked eye, all life stages of *M. tanajoa* require magnification with a hand lens or stereomicroscope. The uppermost, recently unfolded, mature leaves are the best to look at for detecting and monitoring *M. tanajoa* infestations. A hand (or head) lens of a minimum of $5 \times$ magnification (or preferably $10 \times$) can be used to detect *M. tanajoa* on leaf surfaces. Sampling programmes based on actual counts, classes of densities and binomial (presence/absence) sequential sampling have been developed (Yaninek *et al.*, 1989; Nachman *et al.*, 1993; Onzo *et al.*, 2005).

[57]In addition to *M. tanajoa*, other mites co-infest cassava in the Americas (principally several species in the *Mononychellus* genus) and in Africa (principally *Oligonychus gossypii* and rarely *Tetranychus urticae*. In Africa, *O. gossypii* is easily distinguishable from *M. tanajoa* by its larger size, reddish appearance, and colonization of older leaves – hence the common name "red spider mite". Adult females and males are used for identification with dichotomous keys (Flechtmann and de Queiroz, 2015).

[58]Mites can be directly collected from leaves with a fine brush (size 0) and put into small tubes containing 70% ethanol or Oudemans' fluid (5 parts glycerin, 87 parts 70% ethanol, 8 parts glacial acetic acid) for morphological identification, or 95%–100% ethanol for molecular analyses (Walter and Krantz, 2009).

[59]Beating the host plant is one of the most effective methods for collecting spider mites. A stick is used to vigorously beat the leaves and branches over a white tray from which the mites are picked up with a fine brush.

[60]The washing and sieving method can also be used to collect spider mites. First, leaves or branches are dipped in 0.2–0.3% household detergent or 50–70% ethanol in a large container and stirred for a few minutes to dislodge the mites. Next, the suspension is poured into a stack of three stainless-steel sieves: 1680 μ m, 600 μ m and 44 μ m aperture for the top, middle and bottom sieves, respectively (modified from de Lillo, 2009). Finally, mites on the bottom sieve are back-washed with 70% ethanol into a Petri dish and picked up with a fine brush. An alternative method is to simply immerse plant material in 50–70% ethanol, allowing the mites to sink to the bottom of the container, pipetting them into a small vial and storing.

[61]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4. [62]Identification

[63]Identification of spider mites in the *Mononychellus* genus has traditionally been based on microscopic morphological characters. Adult female and male specimens must be mounted on slides and examined using a high-power microscope (e.g., ×400–1000). Morphological characters are best observed with a compound microscope using either differential interference contrast or phase contrast. Features of the adult body are illustrated and labelled in Figures 3–8. Keys for the morphological identification of immature stages of *Mononychellus* are not available.

[64]Molecular methods can be used to identify all life stages of M. tanajoa. DNA sequencing of a barcoding fragment of the cytochrome c oxidase subunit I (*COI*) can support identification since sequences of *M. tanajoa* and of some closely related cassava *Mononychellus* species (*M. caribbeanae*, *M. progresivus* and *M. mcgregori*) are available in the GenBank public database (a National Center for Biotechnology Information database). In addition, a molecular method based on restriction fragment length polymorphism has been established to distinguish some tetranychid species associated with cassava, including *M. tanajoa*, *M. caribbeanae*, *M. mcgregori* and *T. urticae* (Ovalle *et al.*, 2020).

[65]4.1Preparation of specimens for microscopic examination

[66]Mites need to be cleared for morphological examination. Clearing can be accomplished with 85–92% lactic acid (suitable for recently collected specimens) or Nesbitt's fluid (chloral hydrate 40 g, concentrated HCl (12 M) 2.5 mL, distilled water 25 mL, suitable for old alcohol-preserved specimens). The clearing process varies from specimen to specimen. It is advisable to check occasionally until the specimens become translucent. Specimens are mounted in Hoyer's medium (chloral hydrate 200 g, crystalline gum arabic 30 g, glycerol 20 mL, distilled water 50 mL) or in Heinze-PVA medium (chloral hydrate 100 g, glycerol 10 mL, polyvinyl alcohol 10 g, distilled water 60 mL, 85-92% lactic acid 35 mL). Adult females are mounted dorsoventrally, but adult males should be mounted laterally to display the taxonomically informative characters of the aedeagus (male genitalia). The male specimens can be mounted as Henderson (2001) described or repositioned laterally by gently pushing the coverslip to one side. Slides are then labelled with the collection data (i.e., an accession number, locality, host, collector, and collection date) and then put on a hot plate at 70 °C for at least 20 minutes before identification. A longer heating time (24 hr) on the hot plate is required to ensure the slides are completely stable before using immersion objectives. The slides should be completely stable before using immersion objectives. If the specimens are to be retained following identification (see section 5), the identified specimens are placed in an oven at $45-50^{\circ}$ C for a few weeks until the medium is dry. For long-term storage, specimens mounted in Hoyer's medium on microscope slides should be sealed with a sealant. (i.e., Glyptal³ Glyceel or Euparol). Detailed methods for mite specimen preparation and mounting are available in Walter and Krantz (2009).

[68]4.2Morphological characters of the family Tetranychidae

[69]The spider mite family Tetranychidae includes more than 1 300 species in 86 genera (Migeon and Dorkeld, 2021). Various keys to separate Tetranychidae from other families are available in general acarological references (e.g. Walter *et al.*, 2009). Species of this family share the following characters:

- [70]dorsal propodosoma without bothridia (Figure 3);
- [71]hysterosoma with 4 or fewer pairs of setae in the *c* series (Figure 3);
- [72]chelicerae basally fused, forming a retractable stylophore (Figure 3);
- [73]stylophore without a ribbed collar basally (Figure 3 and Figure 6A);
- [74]movable cheliceral digits greatly elongate, whip-like;
- [75]palp with a thumb-claw structure (Figure 6B);

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- [76]tarsi I and II usually with 1–2 sets of duplex setae (Figure 7A and Figure 7B);

^{[67]&}lt;sup>3</sup> The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

- [77]solenidia on tarsi I and II setiform (Figure 7A and Figure 7B); and
- [78]tarsal claws usually with tenent hairs (Figure 9).

[79]4.3Key to genera of Tetranychidae on Manihot spp.

[80]In addition to *M. tanajoa*, 56 species in 12 genera of Tetranychidae have been recorded on *M. esculenta* and other *Manihot* species so far (Migeon and Dorkeld, 2021): *Allonychus* (3 species), *Aponychus* (1 species), *Atrichoproctus* (1 species), *Eotetranychus* (1 species), *Eutetranychus* (5 species), *Mononychellus* (7 species), *Neotetranychus* (1 species), *Oligonychus* (8 species), *Panonychus* (1 species), *Petrobia* (1 species), *Schizotetranychus* (1 species), and *Tetranychus* (26 species). These genera can be distinguished by morphological traits.

[81] This key can be used to distinguish the Tetranychidae genera encountered on *Manihot* spp. (modified from Meyer (1987) and Bolland, Gutierrez and Flechtmann (1998)).

[82]1. Empodium without tenent hairs (Figures 9B–9H); female with one or two pairs of pseudanal (ps) setae (Figures 3B and 6C); male with four pairs of setae (g and ps) on genito-anal valves (Figure 4A).				
[83]– Empodium with tenent hairs (Figure 9A); female with three pairs of pseudanal setae; male with five pairs of setae on genito-anal valves				
[84]2. Tarsus I without duplex setae or with one set of duplex setae; empodia I–IV in female and III–IV in male absent (Figure 9B) or vestigial				
[85]– Tarsus I with two sets of duplex setae (Figure 7A); empodia I–IV in female and III–IV in male uncinate (claw-like) (Figure 9C) or split into three pairs of proximoventral hairs (Figure 9D)				
[86]3. Female with one pair of pseudanal setae; hysterosomal setae <i>f1</i> inserted in marginal area, lateral to dorsocentral setae <i>c1</i> , <i>d1</i> and <i>e1</i> (Figure 4C)				
[87]– Female with two pairs of pseudanal setae; hysterosomal setae fl inserted in median area, approximately aligned with dorsocentral setae cl , dl and el (Figure 4D) <i>Eutetranychus</i> Banks				
[88]4. Female with two pairs of h setae ($h1$ missing) and one or two pairs of pseudanal setae				
[89]— Female with three pairs of h setae and two pairs of pseudanal setae (Figure 3B)7				
[90]5. Female with two pairs of pseudanal setae; tarsal duplex setae close to each other in distal half of segment, or well separated from each other with one in distal half and the other in basal half; empodium variable				
[91]- Female with one pair of pseudanal setae; tarsal duplex setae close to each other in distal half of segment; empodium uncinate				
[92]6. Duplex setae on tarsus I close to each other, in distal half; empodium claw-like, strong, not shorter than proximoventral hairs (Figure 9E); male with claw I bifid				
[93] – Duplex setae on tarsus I relatively distant from each other, one in distal half and other in basal half of segment; empodium variable (Figure 9D), if claw-like, much shorter than proximoventral hairs; male with claw I not bifid				
[94]7. Empodium claw-like (Figure 9C and Figure 9F)				
[95]– Empodium not claw-like, ending in a tuft of hairs (Figure 9D)10				
[96]8. Empodial claw ending in a single tip (Figure 9C)9				
[97]– Empodial claw bifurcate distally (Figure 9F)				

[99]– Dorsal idiosomal setae on strong tubercles; empodial claw longer than or as long as proximoventral hairs (Figure 9E).....*Panonychus* Yokoyama

[100]10. Hysterosomal striae forming a basket-weave pattern (Figure 4B) or varying from longitudinal to irregular between e1-e1; dorsal idiosomal setae usually on tubercles (Figure 4B).....11

[101]– Hysterosomal striae transverse; dorsal idiosomal setae not on tubercles*Eotetranychus* Oudemans

[103]- Hysterosomal striae forming a basket-weave pattern (Figure 4B).......... Neotetranychus Trägårdh

[104]4.4 Morphological characters of the genus *Mononychellus* Wainstein

[105]The genus includes 31 species worldwide, which can be recognized by the following characters: [106]Adult female:

- [107]prodorsum mostly striated (Figures 3A, 10A, 10B, 10D, 10F) or medially finely reticulate and dotted (Figure 10C and Figure 10E);
- [108]dorsal idiosomal setae linear or slightly expanded apically or proximately, usually on tubercles (Figures 11C–E) (with exceptions, e.g. in *M. tanajoa*);
- [109]three pairs of *h* setae and two pairs of pseudanal (*ps*) setae present (Figure 3B);
- [110]striae between *e1–e1* variable, from oblique to longitudinal (Figure 11);
- [111]pregenital striae varying from procurved to transverse (Figure 12)
- [112]palpal terminal eupathidium (spinneret) varying from as long as wide to 2.5× as long as wide (Figure 13);
- [113]tarsus I with two sets of distal duplex setae adjacent to each other in distal part of segment, and four (rarely five) tactile setae and one solenidion (rarely none or three) in proximal part (Figure 7A);
- [114]tibia I with eight or nine (rarely 12) tactile setae and one solenidion (rarely four) (Figure 7A);
- [115]tarsus II with three tactile setae and one solenidion in proximal part (Figure 7B); and
- [116]tibia II with seven (rarely six) tactile setae (Figure 7B)

[117]Adult male:

- [118]genito-anal valves with four pairs of setae (g and ps) (Figure 4A);
- [119]aedeagus with shaft mostly straight, slender, bending upward or downward apically, forming a narrow neck, with a small, usually subtriangular knob (Figure 8 and Figure 14.
- [120]palp with terminal eupathidium slightly tapered;
- [121]tarsus I with three or four tactile setae and two to four solenidia in proximal part;
- [122]tibia I with eight or nine (rarely 12) tactile setae and three or four solenidia;
- [123]tarsus II with three tactile setae and one solenidion in proximal part; and
- [124]tibia II with seven tactile setae.

[125]4.5 Key to species of Mononychellus on Manihot spp. based on females

[126]Eight species of *Mononychellus* have been recorded from *Manihot* spp. (Flechtmann and de Queiroz, 2015; Migeon and Dorkeld, 2021) and can be differentiated by using the following key.

 [128]– Prodorsum mostly striated (Figures 10A, 10B, 10D, 10F); area surrounding base of hysterosomal setae merely striated, not reticulate (Figures 11A, 11B, 11D, 11F)......**3**

[135]5. Hysterosomal setae c1, d1 and e1 extending beyond or almost reaching bases of setae in next row (Figure 11A); c1, d1 and e1 longer than distances c1-c1, d1-d1 and e1-e1, respectively (Figure 11A)6

[136]– Hysterosomal setae cl, dl and el far from reaching bases of setae in next row; cl, dl and el much shorter than distances cl-cl, dl-dl and el-el, respectively**M.** progressivus Doreste, 1981

[141]4.6 Identification of Mononychellus tanajoa

[142]**4.6.1** Morphological identification

[143]Adult female

[144]Idiosoma: oval, 330–480 µm long and 275–335 µm wide, from greenish to yellowish in colour when alive (Figure 2A). Dorsal idiosomal setae stout, oblanceolate (Figure 3A and Figure 5); lobes of dorsal striae strong and rounded; prodorsum (Figure 3A and Figures 5A–C) striated; seta *sc1* not reaching base of *sc2*; hysterosomal setae *c1*, *d1* and *e1* short, not reaching half of distances to bases of setae in next row (Figure 3A and Figure 11F); *c1* shorter than half distance between *c1* and *c1*, *d1* about one-third distance between *d1* and *d1*, *e1* shorter than half distance between *e1* and *e1*; marginal setae *c2*, *d2* and *e2* about $1.4-1.8\times$ as long as *c1*, *d1* and *e1*, respectively; *f1* more than twice as long as *c1* or *d1*

(Figure 3A and Figure 11F); striae between el and el varying from oblique to longitudinal, area posterior to f2 simply striated (Figure 3 and Figures 5D–5F).

[145]Gnathosoma: palp with terminal eupathidium about $1.5 \times$ as long as wide (Figure 6B and Figure 13F). Peritreme usually distally straight, ending in a small bulb or sometimes a tiny hook (Figure 6A and Figure 12F).

[146]Legs: tarsus I (Figure 7A) with five tactile setae and one solenidion in proximal part; tibia I with nine tactile setae and one solenidion; tarsus II (Figure 7B) with three tactile setae and one solenidion in proximal part; tibia II with seven tactile setae; tarsi III (Figure 7C) and IV (Figure 7D) each with ten tactile setae and one solenidion; tibiae III and IV each with six tactile setae.

[147]Adult male

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[148]Idiosoma: tapered posteriorly (Figure 2A), 312 µm long and 167 µm wide, paler than adult female when alive. Aedeagus (Figure 8) with main shaft nearly straight, slightly curving ventrally, progressively tapering and forming a narrow neck before reaching aedeagal knob; knob with two sharp projections (Figure 8).

[149]Gnathosoma: palp with terminal eupathidium apically tapered, slightly longer than wide.

[150]Legs: tarsus I with five tactile setae and three solenidia in proximal part; tibia I with nine tactile setae and three solenidia; tarsus II with three tactile setae and one solenidion in proximal part; tibia II with seven tactile setae.

[151]4.6.2 Molecular identification

[152]All developmental stages of *M. tanajoa* can be identified through mitochondrial DNA sequencing.

[153]A molecular identification method for *M. tanajoa* using restriction fragment length polymorphism of the *COI* gene was reported by Ovalle *et al.* (2020) but is not described here as DNA sequencing of *COI* is used only for confirmatory diagnosis.

[154]4.6.2.1 Extraction of nucleic acid

[155]Genomic DNA should be extracted from a single specimen of any developmental stage, since infestation by more than one species on the same host plant (mixed infestation) is typical for spider mites. There are many different methods available for DNA extraction, such as the modified cetyltrimethylammonium bromide method (potassium acetate 2.5 M, pH 5.5) (Ovalle *et al.* 2020), the Qiagen DNeasy Blood and Tissue Kit⁴ (de Mendonça *et al.*, 2011; Li *et al.* 2015), and the PrepMan Ultra Sample Preparation Reagent⁴ (Matsuda *et al.*, 2013). Voucher specimens should be routinely preserved in ethanol (95%–100%) or on slides after non-destructive DNA extraction (as detailed in Mendonça *et al.*, 2011) for any clarification needed in future integrative taxonomic studies.

[157]4.6.2.2 Conventional PCR and sequencing

[158]The DNA extracted can be used for polymerase chain reaction (PCR) amplification of the DNA barcoding region as chosen by the Consortium for the Barcode of Life with the following primers presented by Folmer *et al.* (1994):

[159]LCO1490 (forward): 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' [160]HCO2198 (reverse): 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'

[161]The above primer set may not always work for *Tetranychus* spp.; if this is the case, the following alternative primers can be used to amplify the *COI* gene (Li *et al.* 2015):

[162]CI J-1718F (forward): 5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3'

^{[156]&}lt;sup>4</sup> The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

[163]COIVERA (reverse): 5'-GAT AAA ACG TAA TGA AAA TGA GCT AC-3'

[164]Ovalle *et al.* (2020) provide an example for the amplification of *COI*, in a total volume of 20 μ L, as in Table1 below.

[165]**Table 1.** Master mix composition, cycling parameters and amplicons for PCR to distinguish *Mononychellus tanajoa* from *M. caribbeanae*, *M. mcgregori* and *Tetranychus urticae* (Ovalle *et al.* 2020)

[166]Reagents		[167]Final concentration
[168]PCR grade water		[169]_†
[170]PCR buffer		[171]]×
[172]MgCl2		[173]2.5 mM
[174]dNTPs		[175]2.5 mM of each
[176]Primer (forward)		[177]5 µM
[178]Primer (reverse)		[179]5 µM
[180]DNA polymerase		[181]0.25 U
[182]DNA sample		[183]20 ng
[184]Cycling parameters	[185]	
[186]Number of cycles		[187]5
[188]- Denaturation		[189]94 °C for 40 s
[190]- Annealing		[191]45 °C for 40 s
[192]- Elongation		[193]72 °C for 60 s
[194]Number of cycles		[195]35
[196]- Denaturation		[197]94 °C for 40 s
[198]- Annealing		[199]51 °C for 40 s
[200]- Elongation		[201]72 °C for 60 s
[202]Final elongation		[203]72 °C for 10 min
[204]Expected amplicons	[205]	
[206]Size		[207]600–650 bp [208](the amplicon size varies for species and individuals)

[209]⁺ For a final reaction volume of 30 μ l.

[210]bp, base pairs; PCR, polymerase chain reaction.

[211]4.6.2.3 Controls for molecular tests

[212]For the test result to be considered reliable, appropriate controls - which will depend on the type of test used and the level of certainty required- should be considered for each series of nucleic acid extractions and PCR amplifications of the target pest. As a minimum, a positive nucleic acid control and a negative amplification control (no template control) should be used.

[213]**Positive nucleic acid control.** This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA may be used.

[214]**Negative amplification control (no template control).** This control is necessary to rule out false positives resulting from contamination with other genetic material during the preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added in place of template DNA.

[215]**Negative extraction control.** This control is used to monitor contamination during nucleic acid extraction. This requires extraction blanks to be processed alongside the samples to be tested.

[216]4.6.2.4 Sequence edition and analyses

[217]The sequences are edited using specific software (e.g. open-source Staden Package, BioEdit). The quality of the sequences should be checked. A consensus sequence should be obtained using the sequence edition software by overlapping the forward and reverse sequences of the same DNA template. The edited sequences are compared with those available in the public DNA database GenBank using the Basic Local Alignment Search Tool (BLAST), available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). An identity with *M. tanajoa* equal to or higher than 97% based on a query cover higher than 90% is required to confirm species-level identification (Smith, Fisher and Hebert, 2005; Porter and Hajibabaei, 2020). One sequence of a 597 bp of the *COI* fragment is available in GenBank for an *M. tanajoa* haplotype (accession number MN913384.1) and also for the closely related species *M. mcgregori* (MN913383) and *M. caribbeanae* (MN913382.1).

[218]Additional work on the diagnostics of mites in the family Tetranychidae (Navajas *et al.*, 1992, 1998, 2000; Ros and Breeuwer, 2007; de Mendonça, *et al.* 2011; Matsuda *et al.*, 2013; Li *et al.*, 2015; Zélé, Weill and Magalhães, 2018) has not yet been adapted to the identification of *M. tanajoa* and consequently not included in this protocol.

5. [219]Records

[220]Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

[221]In cases where other contracting parties may be adversely affected by the diagnosis, the records and evidence of the results of the diagnosis (in particular, preserved or slide-mounted specimens, photographs of distinctive taxonomic structures, DNA extracts and photographs of gels, as appropriate), should be kept for at least one year.

6. [222]Contact points for further information

[223]Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, P.O. Box 2095, Auckland 1072, New Zealand (Qing-Hai Fan; email: <u>qinghai.fan@mpi.govt.nz</u>; tel.: (+64) 9 9095077; fax: (+64) 9 9095739).

[224]Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Centre de Biologie pour la Gestion des Populations (UMR CBGP), CS 30016, 34988 Montferrier sur Lez cedex, France (Denise Navia; email: <u>denise.navia@inrae.fr</u>).

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7. [226]Acknowledgements

[227]The first draft of this protocol was drafted by Qing-Hai Fan (Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand), Denise Navia (Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France) and Rachid Hanna (Center for Tropical Research, University of California, Los Angeles, USA).

[228]In addition, the following experts were significantly involved in the development of this protocol: Frederic Beaulieu (Agriculture & Agri-Food Canada), Jurgen Otto (Department of Agriculture, Water and the Environment, Victoria, Australia), Hasan Rahmani (Department of Agriculture, Water and the Environment, Victoria, Australia) and Karen Mclachlan-Hamilton (entomology–diagnostic biologist, Canada). [229]The following experts provided some references and specimens: Carlos Holger Wenzel Flechtmann, Gilberto José de Moraes and Raphael de Campos Castilho (Departamento de Entolomogia e Acarologia, Universidade de São Paulo, Brazil), and Renata Santos de Mendonça (Universidade de Brasília, Federal District, Brazil). Mite reference collections used for this protocol include those of the Department of Entomology and Acarology, Escola Superior de Agricultura Luiz de Queiroz/Universidade de São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil and Embrapa Recursos Genéticos e Biotecnologia, Brasilia, Federal District, Brazil.

8. [230]References

[231]The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <u>www.ippc.int/core-activities/standards-setting/ispms</u>.

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[284]9. Figures

[285]**Figure 1.** Progression of *Mononychellus tanajoa* damage to cassava plant: (A) stippled leaves; (B) distorted leaves; (C) chlorotic, mottled leaves with considerable reduction in leaf size; (D) death and fall of upper shoot leaves giving a "candlestick" appearance.

[286]Source: Photos courtesy of R. Hanna, Center for Tropical Research, University of California, Los Angeles, USA.

[287] **Figure 2.** *Mononychellus tanajoa* and its principal biocontrol agents: (A) adult and immature stages of *M. tanajoa*; (B) predatory mite *Typhlodromalus aripo*; (C) *Neozygites tanajaoe* infected *M. tanajoa*.

[288] Source: (A) J.S. Yaninek, (B & C) G. Goergen, International Institute of Tropical Agriculture.

[289] Figure 3. *Mononychellus tanajoa*, adult female: (A) dorsal view of idiosoma; (B) genital and anal region.

[290]Source: Line drawings courtesy of Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand and D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France.

[291]**Figure 4.** Hysterosoma: (A) male *Mononychellus*; (B) female *Neotetranychus*; (C) female *Aponychus*; (D) female *Eutetranychus*.

[292]Source: Photo and line drawings courtesy of Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand and D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France.

[293] Figure 5. Mononychellus tanajoa: variation of prodorsal striation in adult female.

[294]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

[295]**Figure 6.** *Mononychellus tanajoa*, adult female: (A) dorsal view of stylophore and peritremes; (B) infracapitulum and palp; (C) genital and anal region.

[296]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

[297]**Figure 7.** *Mononychellus tanajoa*, adult female: (A) tibia and tarsus I; (B) tibia and tarsus II; (C) tibia and tarsus III; (D) tibia and tarsus IV.

[298]Source: Line drawings courtesy of Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand and D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France.

[299]Figure 8. Mononychellus tanajoa, lateral view of aedeagus: (A) photograph; (B) schematic overview.

[300]Source: Photo and line drawings courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

[301]Figure 9. Types of pretarsi in Tetranychidae on *Manihot* spp.

[302]Source: Line drawings courtesy of Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand and D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France.

[303]**Figure 10.** Prodorsum of adult females of *Mononychellus* spp. (A) *M. bondari*; (B) *M. caribbeanae*; (C) *M. chemosetosus*; (D) *M. mcgregori*; (E) *M. planki*; (F) *M. tanajoa*.

[304]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

[305]**Figure 11.** Hysterosoma of adult females of *Mononychellus* spp. (A) *M. bondari*; (B) *M. caribbeanae*; (C) *M. chemosetosus*; (D) *M. mcgregori*; (E) *M. planki*; (F) *M. tanajoa.*

[306]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

[307]**Figure 12.** Genital and anal regions of adult females of *Mononychellus* spp.: (A) *M. bondari*; (B) *M. caribbeanae*; (C) *M. chemosetosus*; (D) *M. mcgregori*; (E) *M. planki*; (F) *M. tanajoa*.

[308]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand

[309]**Figure 13.** Infracapitulum and palpi of adult females of *Mononychellus* spp.: (A) *M. bondari*; (B) *M. caribbeanae*; (C) *M. chemosetosus*; (D) *M. mcgregori*; (E) *M. planki*; (F) *M. tanajoa*.

[310]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand

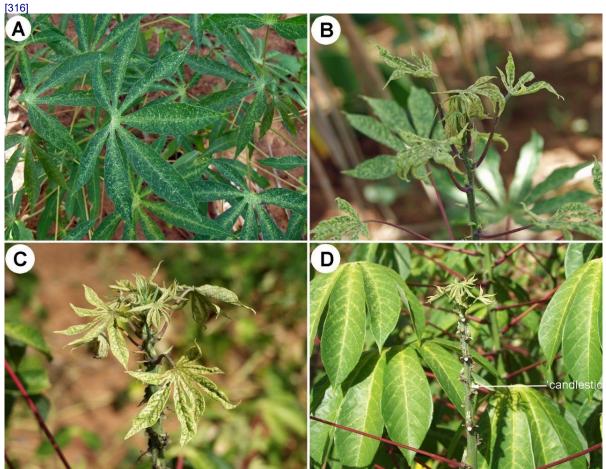
[311]**Figure 14.** Aedeagi of adult males of *Mononychellus* spp.: (A) *M. bondari*; (B) *M. chemosetosus*; (C) *M. mcgregori*; (D) *M. planki*.

[312]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

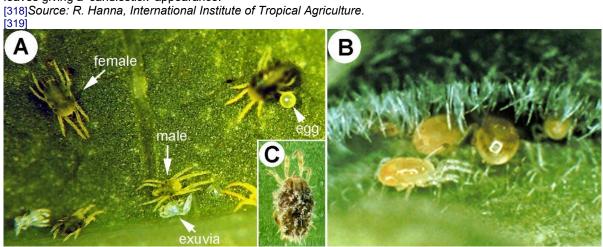
[313]**Figure 15.** Stylophores and peritremes of adult females of *Mononychellus* spp.: (A) *M. bondari*; (B) *M. caribbeanae*; (C) *M. chemosetosus*; (D) *M. mcgregori*; (E) *M. planki*; (F) *M. tanajoa*.

[314]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.

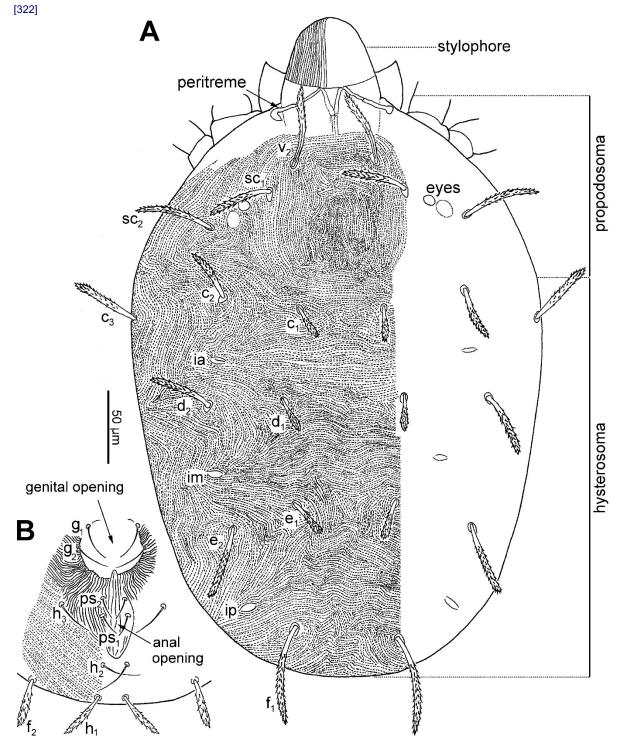
[315]Figures



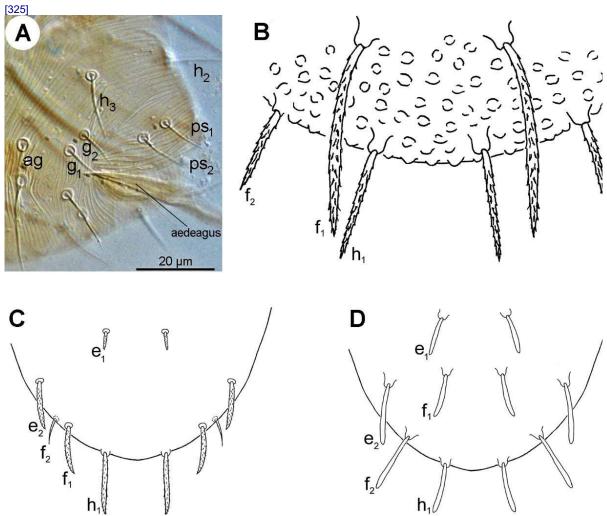
[317]**Figure 1.** Progression of *Mononychellus tanajoa* damage to cassava plant: **(A)** stippled leaves; **(B)** distorted leaves; **(C)** mottled leaves with considerable reduction in leaf size; **(D)** death and fall of upper shoot leaves giving a 'candlestick' appearance.



[320] Figure 2. Mononychellus tanajoa and its principal biocontrol agents: (A) adult and immature stages of M. tanajoa; (B) predatory mite Typhlodromalus aripo; (C) Neozygites tanajaoe infected M. tanajoa.
 [321] Source: (A) J.S. Yaninek, (B & C) G. Goergen, International Institute of Tropical Agriculture

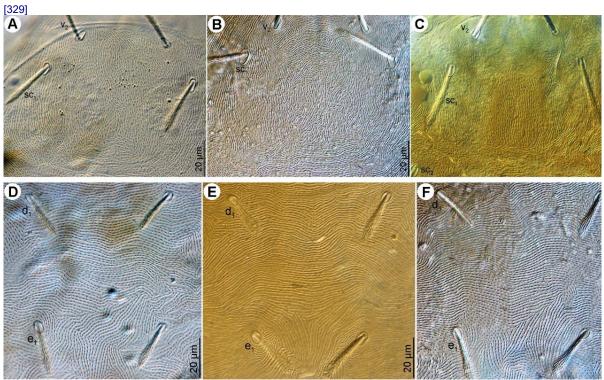


[323]**Figure 3.** Mononychellus tanajoa, adult female: **(A)** dorsal view of idiosoma; **(B)** genital and anal region. [324]Source: Line drawings courtesy of Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand and D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France.

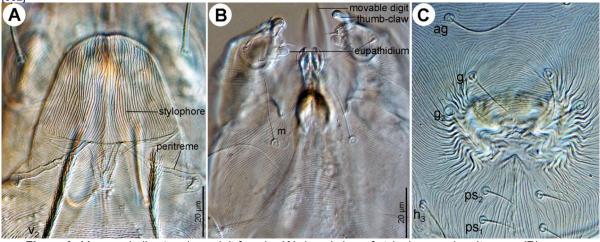


[326]**Figure 4.** Hysterosoma. (**A**) male *Mononychellus*; (**B**) female *Neotetranychus*; (**C**) female *Aponychus*; [327](**D**) female *Eutetranychus*.

[328]Source: Photo and line drawings courtesy of Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand and D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France.



 [330] Figure 5. Mononychellus tanajoa: variation of prodorsal striation in adult female.
 [331] Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.
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[333]**Figure 6.** *Mononychellus tanajoa*, adult female: **(A)** dorsal view of stylophore and peritremes; **(B)** infracapitulum and palp; **(C)** genital and anal region.

[334]Source: Photos courtesy of D. Navia, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, France and Q.-H. Fan, Plant Health & Environment Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, New Zealand.