Universidade de Brasília
Instituto de Ciências Biológicas
Departamento de Fitopatologia
Programa de Pós-Graduação em Fitopatologia

IDENTIFICAÇÃO E VARIABILIDADE GENÉTICA DE *Meloidogyne* spp. EM CANA-DE-AÇÚCAR NO ESTADO DE SÃO PAULO

LEILA LOURENÇO FURTADO

Brasília – DF
2021

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IDENTIFICAÇÃO E VARIABILIDADE GENÉTICA DE *Meloidogyne* spp. EM CANA-DE-AÇÚCAR NO ESTADO DE SÃO PAULO

Dissertação apresentada à Universidade de Brasília como requisito parcial para obtenção do título de Mestre em Fitopatologia pelo Programa de Pós-Graduação em Fitopatologia

Orientadora:
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LEILA LOURENÇO FURTADO

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O Brasil é o maior produtor de cana-de-açúcar do mundo, com destaque para o Estado de São Paulo, que lidera a produção no país. A produção nacional é destinada principalmente à produção de açúcar, álcool, aguardente e alimentação animal. Embora uma alta produtividade seja alcançada por conta das boas condições climáticas para a cultura no país, problemas de ordem bióticas e abióticas ocorrem com frequência e dificultam a condução das lavouras. Dentre os problemas de causa biótica, pode-se destacar as perdas causadas por fitonematoides, que induzem um decréscimo na produtividade na ordem de 20 a 30% já no primeiro corte em cultivares suscetíveis. Fitonematoides de diversos gêneros acometem a cultura, destacando-se o nematoide das galhas (*Meloidogyne* spp.). Espécies de *Meloidogyne* são classificadas como endoparasitas sedentários, com reprodução do tipo partenogênese, seja obrigatória ou facultativa. A identificação de espécies deste gênero consiste em uma abordagem polifásica baseada em características morfológicas, morfométricas, padrão perineal, técnicas bioquímicas e moleculares. Além disso, com os avanços das técnicas moleculares foi possível fazer o uso do DNA genômico para estudos de variabilidade intraespecífica. Ressalta-se que as informações sobre a variabilidade dos fitonematoides são relevantes na adoção de medidas de controle específicas, como recomendação de rotação de cultura e, principalmente, uso de variedades resistentes. Atualmente, já se encontra disponível variedades de cana-de-açúcar resistentes a várias espécies de *Meloidogyne*, entretanto estas são resistentes somente a uma espécie, o que dificulta o controle no campo, onde várias espécies acometem a cultura simultaneamente. Assim, o trabalho teve como objetivo a identificação de espécies de *Meloidogyne* na cultura da cana-de-açúcar no estado de São Paulo e estudar a variabilidade intraespecífica da espécie predominante em cana-de-açúcar. Para isso, identificamos as espécies presentes em 36 canaviais distribuídos em dez municípios do estado de São Paulo por meio do fenótipo de esterase e por marcadores moleculares (SCAR, 28S D2-D3, COI e NADH5). Além disso, foi elucidada a variabilidade genética de populações de *M. javanica* usando os marcadores moleculares RAPD. Foram detectadas as espécies *M. incognita* e *M. javanica* no estado, sendo a última observada em todos os campos de cultivo. Em 33% dos campos amostrados se identificou populações mistas. O estudo de variabilidade de *M. javanica* mostrou que cada isolado é um genótipo com alta variabilidade genética entre os isolados e que a população está estruturada em duas subpopulações, sem qualquer correlação com região geográfica, cultivar, tipo de solo, índice pluviométrico ou bioma. Os resultados deste estudo poderão servir como informação básica para aumentar a eficácia na escolha de métodos de controle em áreas infestadas por *M. incognita* e *M. javanica* em cana-de-açúcar no estado de São Paulo. Este estudo representa uma peça de um grande quebra-cabeça para a melhor compreensão da história evolutiva, variabilidade genética e estruturação das populações de *M. javanica* no mundo.

Palavras-chave: *Meloidogyne incognita*, *Meloidogyne javanica*, *Saccharum hybridum*, SCAR-PCR, esterase, NADH5, RAPD.

Orientadora – Prof. Thaís Ribeiro Santiago – Departamento de Fitopatologia-UnB.

GENERAL ABSTRACT

FURTADO, Leila Lourenço. **Identification and genetic variability of *Meloidogyne* spp. in sugarcane in the state of São Paulo.** 2021. 79p. Dissertation (Master's Degree in Plant Pathology) – Universidade de Brasília, Brasília, DF.

Brazil is the largest sugarcane producer in the world, with emphasis on the state of São Paulo, which leads production in the country. National production is mainly intended to produce sugar, alcohol, beverages, and animal feed. Although high productivity is achieved due to the favorable climatic conditions for the crop in the country, biotic and abiotic problems occur frequently and make it difficult to manage the crop. Among biotic constraints in the crop, we can highlight the losses caused by plant-parasitic nematodes, which can reach a decrease in the productivity in the order of 20 to 30% even in the first cut in susceptible cultivars. Nematodes of several genera of plant-parasitic nematodes affect the culture, especially the root-knot nematode (*Meloidogyne* spp.). *Meloidogyne* species are classified as sedentary endoparasites, with parthenogenesis-type reproduction, either obligatory or facultative. Species identification of this genus consists of a polyphasic approach based on morphological and morphometric characteristics, perineal pattern, biochemical and molecular techniques. Furthermore, with advances in molecular techniques, it was possible to make use of genomic DNA for intraspecific variability studies. It is noteworthy that information on the variability of plant-parasitic nematodes is relevant for the adoption of specific control measures, such as recommendation for crop rotation and, mainly, the use of resistant varieties. Currently, sugarcane varieties resistant to several *Meloidogyne* species are available, however these are only resistant to one species at a time, which makes it difficult to control it in the field, where several species affect the crop simultaneously. Thus, the aim of this study was to identify *Meloidogyne* species in the sugarcane crop in the state of São Paulo and to study the intraspecific variability of the predominant species in sugarcane. For this, it was identified the *Meloidogyne* species present in 36 sugarcane fields distributed in ten municipalities in the state of São Paulo, through the esterase phenotypes and molecular markers (SCAR, 28S D2-D3, COI and NADH5). In addition, it was elucidated the genetic variability of *M. javanica* populations using RAPD molecular markers. *Meloidogyne incognita* and *M. javanica* were detected through sugarcane plantation in the state, the last specie being observed in all fields. In 33% of the sampled fields, mixed populations were detected. The variability study of *M. javanica* showed that each isolate is a genotype with high genetic variation among isolates and that the population is structured in two subpopulations, without any correlation with geographic region, sugarcane cultivar, soil type, rainfall, and biome. The results of this study may serve as basis information to increase the effectiveness in choosing control methods in areas infested by *M. incognita* and *M. javanica* in sugarcane in the state of São Paulo. This study represents one piece of a great puzzle for a better understanding of the evolutionary history, genetic variability, and population structure of *M. javanica* in the world.

Keywords: *Meloidogyne incognita*, *Meloidogyne javanica*, *Saccharum hybridum*, SCAR-PCR, esterase, NADH5, RAPD.

Master's advisor – Prof. Thaís Ribeiro Santiago – Departamento de Fitopatologia.

1. INTRODUÇÃO GERAL

A cana-de-açúcar (*Saccharum hybridum* R.M. Grey) é uma das principais culturas de importância mundial, sendo matéria prima para a fabricação de açúcar, álcool, bebidas e alimentação animal. O Brasil é o maior produtor de cana-de-açúcar do mundo, com geração de 654,8 milhões de toneladas/ano, tendo como destaque o estado de São Paulo, contribuindo com 54% da produção nacional (CONAB, 2021). Entre os diversos fatores que afetam a condução da cultura da cana-de-açúcar, as perdas causadas por fitonematoides tem grande importância, especialmente porque, como patógenos de solo, existem poucas alternativas ecologicamente aceitáveis para o seu controle em solos intensivamente cultivados.

Estima-se que as perdas causadas por fitonematoides podem chegar entre 20 a 40% de redução da produtividade no primeiro corte de cultivares de cana-de-açúcar suscetíveis (Bellé et al., 2017a; Noronha et al., 2017). Entre os nematoides frequentemente associados à cultura destaca-se os do gênero *Meloidogyne*, conhecidos como nematoides das galhas, ocupando o primeiro lugar na lista de nematoides parasitas de plantas devido à sua importância científica e econômica (Jones et al., 2013). Atualmente, sabe-se que as espécies *M. javanica* (Treub, 1885) Chitwood, 1949, *M. incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. enterolobii* (Yang & Eisenback, 1983), *M. ethiopica* Whitehead, 1968, *M. hispanica* Hirschmann, 1986 e *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996, estão associadas à cultura da cana-de-açúcar na região Sul e Nordeste do Brasil. (Severino et al., 2008; Moura et al., 2011; Bellé et al., 2017b; Noronha et al., 2017). Para o estado de São Paulo, não há trabalhos sobre levantamento de espécies disponíveis contendo informações sobre a identificação e distribuição das espécies de *Meloidogyne* nos canaviais.

Diferentes técnicas têm sido utilizadas para a identificação de espécies de *Meloidogyne*, todas com vantagens e desvantagens. A aplicação de métodos clássicos, como o padrão perineal, é difícil devido à subjetividade e alto nível de similaridade entre as características morfológicas que comprometem a precisão da identificação (Moens et al., 2009; Carneiro et al., 2016). Uma alternativa de identificação de caráter taxonômico confiável é o padrão da isoenzima esterase, que é adequado para identificar várias espécies de *Meloidogyne* (Carneiro et al., 2000). Entretanto a necessidade de fêmeas adultas reduz a abrangência do emprego da técnica. O avanço dos estudos empregando o DNA genômico permitiu uma maior possibilidade de técnicas usadas para a identificação do fitonematoide. Dentre esses métodos podemos citar os marcadores de *Sequence Characterized Amplified*

Regions (SCAR) e o estudo das variações das sequências de nucleotídeos ribossomais (18S, ITS - *Internal Transcribed Spacer* e segmento D2/D3 de 28S) e genes mitocondriais (citocromo oxidase I - COI e NADH desidrogenase subunidade 5 - NADH5). Para resolver a limitação de diferentes técnicas e aumentar a precisão na identificação de nematoides, uma abordagem polifásica - combinando técnicas bioquímicas e moleculares - é a metodologia mais utilizada para a identificação das espécies de *Meloidogyne* (Hunt & Handoo, 2009; Mattos et al., 2018).

Outro fator relevante é o estudo da variabilidade genética do gênero *Meloidogyne*, com ênfase em *Random Amplified Polymorphic DNA* (RAPD), *Amplified Fragment Length Polymorphisms* (AFLP), *Simple Sequence Repeats* (ISSR), o gene mitocondrial COI (mtCOI) e, mais recentemente, *Genotyping By Sequencing* (GBS). Os marcadores genéticos são eficientes para diferenciar as espécies de nematoides e estudar a variabilidade genética intraespecífica (Mattos, et al., 2016; Medina et al., 2017; Rashidifard et al., 2018; Santos et al., 2018). No entanto, pouco se sabe sobre a estruturação da população, variabilidade genética e mecanismo evolutivo deste fitopatógeno.

2. OBJETIVOS

Identificar e caracterizar a distribuição das espécies de *Meloidogyne* encontradas nas principais regiões produtoras de cana-de-açúcar do estado de São Paulo empregando técnicas bioquímicas e moleculares, bem como estudar a variabilidade genética intraespecífica de populações de *M. javanica* utilizando os marcadores RAPD.

3. REVISÃO BIBLIOGRÁFICA

A cana-de-açúcar é uma gramínea perene taxonomicamente agrupada na Classe Liliopsida, Ordem Cyperales, Família Poaceae e Gênero *Saccharum* L. Dentro do gênero *Saccharum* encontram-se as espécies cultiváveis *S. barberi* Jeswiet (2n=81-124), *S. edule* (2n=60-80), *S. officinarum* L. (2n=80) e *S. sinense* Roxb. (2n=111-120), e as espécies selvagens *S. spontaneum* L. (2n=40-128) e *S. robustum* Brandes & Jesw. ex Grassl (2n=60-205) (Leme, 2018). A cana-de-açúcar é um material híbrido (*S. hybridum*) advindo do cruzamento de *S. officinarum* (70-75%) e *S. spontaneum* (20-25%), adaptada a regiões tropicais e subtropicais (Butterfield et al., 2001). A espécie *S. officinarum*, originalmente

domesticada na Nova Guiné, tem proporcionado ao híbrido um alto teor de sacarose, enquanto *S. spontaneum* confere resistência a pragas e doenças (Amaral et al., 2015).

Atualmente, a cana-de-açúcar é considerada uma das culturas agrícolas mais cultivadas no mundo (Hoang et al., 2017). A produção mundial soma um total de 1,9 bilhão de toneladas produzidas em aproximadamente 26 milhões de hectares (FAO, 2019). Os primeiros povoados que se dedicaram ao cultivo dessa gramínea estavam na Nova Guiné (Zhang et al., 2018), no entanto, foram os indianos que primeiramente produziram o açúcar a partir do caldo da planta (Daniels et al., 1996). Pequenas e grandes propriedades se dedicam à produção de cana-de-açúcar, não apenas devido ao açúcar, mas a uma versatilidade de produtos, gerando alimento, fibra, combustível e fertilizantes (Berry et al., 2017; Maranhão et al., 2018). As pequenas propriedades se destacam na produção de forragem, alimentação animal e produção de cachaça, rapadura e melado. As grandes propriedades visam fornecer a matéria-prima para as usinas sucroalcooleiras (Berry et al., 2017).

Entre os principais países produtores está o Brasil, com produção mundial que corresponde a 38,6%, seguido pela Índia (20,7%) e China (5,6%) (FAO, 2019). No Brasil, a cana-de-açúcar é cultivada há mais de 500 anos na região Nordeste e, atualmente, seu cultivo se estende por todo território nacional (Maranhão et al., 2008). Embora a região Nordeste tenha sido pioneira no cultivo de cana-de-açúcar, segundo os dados da CONAB de maio de 2021, a região que tem a maior produção do país atualmente é a Sudeste, com produção de 428,5 milhões de toneladas, que corresponde a 65% da produção nacional; destacando-se o estado de São Paulo, responsável por 80% da produção da região. Em seguida, vêm as regiões Centro-Oeste (139,8 milhões de toneladas), Nordeste (48,4 milhões de toneladas), Sul (34,1 milhões de toneladas) e Norte (3,4 milhões de toneladas) (CONAB, 2021). Nos últimos anos, as áreas produtivas no Brasil vêm aumentando com a crescente demanda do setor sucroalcooleiro (Bellé et al., 2017a). Estima-se que a produção no ano de 2020/2021 se mantenha estável com um ligeiro aumento para a produção (Cepea, 2020).

Dentre as dificuldades encontradas pelos produtores para a manutenção e o incremento da produção estão fatores adversos de ordem biótica e abiótica. Dentre os problemas bióticos, destacam-se as perdas causadas por fitonematoides, que podem atingir até 30% da produção (Chaves et al., 2009; Oliveira et al., 2019). Diversas espécies de fitonematoides já foram identificadas em campos de cana-de-açúcar no mundo. Na Província de Guantánamo, 24 espécies pertencentes a 16 gêneros de fitonematoides foram encontradas associadas ao cultivo de cana-de-açúcar, sendo os do gênero *Pratylenchus* o mais encontrado no campo (Peña-Prades et al., 2018). Recentes estudos na África do Sul detectaram a presença

de nematoides dos gêneros *Meloidogyne*, *Pratylenchus*, *Helicotylenchus*, *Xiphinema*, *Paratrichodorus*, *Scutellonema* e *Tylenchorhynchus* (Ramouthar et al., 2018).

No Brasil, considerando os levantamentos nematológicos em diferentes regiões produtoras de cana-de-açúcar, mais de 70% das áreas cultivadas estavam infestadas por uma ou mais espécies. Os fitonematoides mais frequentemente observados em campos foram os dos gêneros: *Meloidogyne*, *Pratylenchus*, *Criconemoides*, *Helicotylenchus*, *Xiphinema*, *Radopholus*, *Trichodorus* e *Paratrichodorus* (Maranhão et al., 2018), sendo o nematoide das lesões radiculares (*P. zeae* Graham, 1951 e *P. brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941) e o nematoide das galhas (*Meloidogyne* spp.) os maiores causadores de perdas para a cultura (Bellé, 2014).

O gênero *Meloidogyne*, anteriormente denominado *Heterodera marioni* (Cornu, 1879) Goodey, 1932, foi descrito pela primeira vez por Miles Joseph Berkeley, na Inglaterra, na cultura do pepino em 1885. Em cana-de-açúcar, o primeiro relato de *Meloidogyne* ocorreu em Java, na Indonésia, em 1885, durante uma investigação envolvendo vários pesquisadores para identificar as doenças da cultura (Winchester, 1969). Após essa descoberta, inúmeros outros relatos atestam a redução da produtividade e aumento populacional do patógeno em decorrência do cultivo sucessivo da cultura em diferentes partes do mundo (Novaretti et al., 1978). No Brasil, os primeiros relatos de *Meloidogyne* em cana-de-açúcar foram feitos por Lordello (1964), em um estudo de identificação das espécies de *Meloidogyne* e levantamento populacional no campo. Atualmente, nematoides deste gênero encontra-se amplamente distribuído em todo território nacional, afetando diferentes culturas e sendo observado até mesmo em áreas do cerrado nunca cultivadas (Souza et al., 1994; Silva et al., 1986; Ye et al., 2015; Mattos et al., 2016; Lopes et al., 2019; Santos et al., 2019; Pimentel et al., 2021).

Membros do gênero *Meloidogyne* são considerados endoparasitas sedentários, pois permanecem no interior da raiz até completar o seu ciclo de vida (Ye et al., 2019). O ciclo de vida de *Meloidogyne* spp. se inicia com a deposição de um aglomerado de ovos, dentro ou na superfície da raiz, que varia de 500 à 2000 ovos, protegidos por uma matriz gelatinosa produzida pela fêmea madura (Calderón-Urrea et al., 2016). Após a embriogênese, o juvenil de primeiro estádio (J1) passa por ecdise tornando-se o juvenil de segundo estádio (J2), que, por sua vez, consegue eclodir do ovo e procurar a planta hospedeira por meio da percepção de compostos atrativos nos exsudados. O J2 penetra nas raízes através de ferimentos causados pelo estilete que perfura a raiz por impulso físico e por meio de moléculas efetoras (celulase, quitinase, proteases e pectinases) secretadas pela glândula dorsal, ocasionando a degradação e modificação da parede celular da planta (Jones et al., 2013). Após a penetração na raiz, o

nematoide no estágio J2 migra intercelularmente dirigindo-se à zona de diferenciação radicular, onde inicia a formação do sítio de alimentação, conhecido como célula gigante (Huang & Maggenti, 1969). Os estádios J3 e J4 ocorrem em uma curta duração de tempo, comparados aos outros estádios, e perdem o estile. Com isso, a fêmea volta a se alimentar no estádio adulto por meio da formação das células gigantes e tornam-se sedentárias com formato piriforme (Ferraz & Monteiro, 1995; Moura, 1996; Ferraz, 2001).

A célula gigante é formada por cinco a sete células multinucleadas que apresentam seu citoplasma bastante denso, podendo conter até 80 núcleos poliplóides e com até oito vezes o número de cromossomos por núcleo (Wiggers, et al., 1990). Consequentemente, ocorre um aumento no tamanho (hipertrofia) e multiplicação (hiperplasia) celular resultando no sintoma típico da doença causada pelos nematoides do gênero *Meloidogyne* - as galhas radiculares. As galhas geralmente desenvolvem-se nas extremidades das raízes; no entanto, muitas vezes, são pequenas e discretas ou até mesmo não são formadas. Portanto, são dificilmente diagnosticadas em plantas lenhosas como cana-de-açúcar (Dias-Arieira et al., 2010).

O tempo do ciclo de vida dos nematoides do gênero *Meloidogyne* varia de acordo com a espécie e as condições ambientais, especialmente a temperatura, podendo durar de três a cinco semanas, fazendo com que seja possível a ocorrência de vários ciclos durante o desenvolvimento da cana-de-açúcar (Ferris & Ferris, 1998; Castagnone-Sereno et al., 2013). Em consequência da alteração no sistema radicular, plantas de cana-de-açúcar infectadas por *Meloidogyne* spp. apresentam sintomas na parte aérea e raiz. Na parte aérea, observa-se alteração no vigor da planta, redução na parte área e amarelecimento das folhas, consequentemente a redução da produção de sacarose. Além disso, raízes da cana-de-açúcar infectadas pelo patógeno tornam-se mal desenvolvidas e pouco eficientes na absorção de água e nutrientes do solo, podendo exibir pequenos engrossamentos que resultam na redução da produção (Dinardo-Miranda, 2005).

A formação de machos na população de *Meloidogyne* é determinada no período pós-embrionário, na transição do estádio J2 para J3, quando ocorre o desenvolvimento do sistema reprodutivo e das gônadas funcionais. No estádio J4 corre uma reversão do formato do nematoide para vermiforme e não existe evidência da sua alimentação, saindo da raiz e movendo livremente no solo (Moens et al., 2009). A presença e proporção de machos na população está diretamente relacionada ao tipo de reprodução. Três tipos de reprodução são observados em espécies de *Meloidogyne*: anfimixia, partenogênese facultativa/meiótica e obrigatória/mitótica (Bird et al., 2009). O macho pode retornar à raiz para o acasalamento com a fêmea quando a reprodução do tipo anfimixia acontece na espécie, ou o macho

permanece no solo até a morte quando a reprodução ocorre por partenogênese (Bird et al., 2009).

Espécies de *Meloidogyne* com reprodução por anfimixia apresentam, usualmente, uma proporção 1:1 de machos e fêmeas, mas esse tipo de reprodução é incomum no gênero e usualmente são espécies com distribuição restrita no mundo, como *M. carolinensis* Eisenback, 1982 e *M. spartinae* Rau & Fassuliotis, 1965 (Moens et al., 2009). Nesse tipo de reprodução, machos e fêmeas recombinam seu material genético aumentando a variabilidade genética a cada geração. Espécies de *Meloidogyne* com reprodução do tipo partenogênese, seja obrigatória ou facultativa, apresentam uma predominância na presença de fêmeas. Nas espécies de *Meloidogyne* amplamente distribuídas no mundo e que causam grandes impactos em culturas de importância econômica predomina a reprodução do tipo partenogênese mitótica (Castagnone-Sereno et al., 2013). Este tipo de reprodução consiste na maturação dos oocistos, a partir de uma simples divisão, com o óvulo se desenvolvendo em um embrião. No entanto, o aparecimento de machos é observado em alta densidade populacional em condições adversas para a planta (Jones et al., 2013). Na tentativa de sobrevivência do nematoide, os primórdios sexuais desenvolvem os testículos, ao invés de ovários, para que ocorra uma menor oviposição e colonização da planta nos próximos ciclos do patógeno. No geral, nesse tipo de reprodução espera-se uma baixa variabilidade dentro da espécie, dependendo de fatores como aneuploidia/polissomia, rearranjos estruturais do cromossomo, fusões cromossômicas, deleções, duplicações e translocações, bem como transferência horizontal de genes adquiridos de outros organismos como fungos e bactérias para aumento do polimorfismo na espécie (Danchin et al., 2010). Outros fatores auxiliam na variabilidade genética, além do acúmulo de mutações pontuais, como os elementos transponíveis, que por sua natureza repetitiva e mobilidade, podem impactar passiva e ativamente a dinâmica do genoma (Kozlowski et al., 2020).

Espécies com reprodução do tipo partenogênese facultativa reproduzem por partenogênese meiótica quando os machos estão ausentes e as fêmeas permanecem não inseminadas. Com isso, primeiramente ocorre uma redução no número de cromossomos do óvulo devido à meiose e, posteriormente, a fusão de um núcleo polar secundário com o pronúcleo do óvulo para o restabelecimento do número de cromossomos somáticos (Van der Beek et al., 1998). Nesse tipo de reprodução, na presença de indivíduos machos, a fecundação cruzada acontece livremente. No entanto, algumas exceções são observadas, como em *M. hapla* Chitwood, 1949 cuja reprodução pode ser partenogenética facultativa e obrigatória (Triantaphyllou, 1985).

As características citogenéticas do gênero *Meloidogyne* estão diretamente relacionadas ao número de cromossomos (Triantaphyllou, 1985). O tipo de reprodução por anfimixia e partenogênese facultativa caracteriza-se geralmente as espécies diplóides e algumas espécies haplóides, enquanto que espécies com reprodução do tipo partenogenética obrigatória demonstram uma ampla variação no número de cromossomos devido à poliploidia (Chitwood & Perry, 2009). Tais características e informações são importantes para a identificação, bem como variabilidade das espécies (Castagnone-Sereno et al., 2013).

Além da característica citogenética, a identificação de espécies de *Meloidogyne* consiste em uma abordagem polifásica baseada em características morfológicas, morfométricas, padrão perineal, técnicas bioquímicas e moleculares (Hunt & Handoo, 2009; Eisenback & Hunt, 2009). Até a década de 1980, a identificação era baseada em caracteres morfológicos, morfométricos e por testes em hospedeiros diferenciadores para determinar as raças fisiológicas (Taylor & Sasser, 1978). A identificação era morosa, subjetiva e muitas vezes sujeita a erro uma vez que a mesma espécie pode apresentar características fenotípicas diferentes dependendo de fatores ambientais, ou, ainda, espécies diferentes apresentam sobreposição no padrão perineal, gerando uma identificação errônea (Willianson et al., 1997; Santos et al., 2019). Com o desenvolvimento e aperfeiçoamento das técnicas bioquímicas, foi possível associar os caracteres morfológicos com fenótipo enzimático espécie-específico. Com isso, foi possível diferenciar mais de 40 espécies baseado principalmente no peso molecular das isoenzimas esterase (EST) e malato-desidrogenase (MDH) (Carneiro et al., 2000). Apesar do grande avanço, a variabilidade da EST e MDH é baixa e não é possível diferenciar todas as espécies usando esse padrão enzimático. Isso se deve ao fato dessas enzimas serem oriundas de genes conservados e com papel essencial na sobrevivência dos nematoides. Além disso, a necessidade da utilização de fêmeas adultas, ausência de bandas definidas e relatos da ocorrência de variantes intra-específicas, como em *M. incognita*, *M. javanica, M. arenaria* e *M. paranaensis*, dificultam o emprego da técnica (Carneiro et al., 2004; Cofcewicz et al., 2004; Carneiro et al., 2008; Muniz et al., 2008;Santos et al., 2012). Apesar da limitação da técnica, o padrão de isoenzimas ainda é bastante empregado e um banco de dados de perfil enzimático de uma gama de espécies de *Meloidogyne* presente no Brasil e no mundo encontra-se disponível (Mattos, 2017).

O avanço das técnicas empregando DNA genômico permitiu a utilização do primeiro método na diferenciação das espécies de fitonematoides a partir das informações do DNA (Curran et al., 1985). RFLP (*Restriction Fragment Length Polymorphisms*) foi a primeira técnica molecular usada para a identificação de espécies de nematoides. A metodologia

consiste no tratamento do DNA com enzimas de restrição, formando um padrão típico de bandas para cada espécie. No entanto, com os avanços da técnica de PCR, foi possível obter uma melhor discriminação entre as espécies de forma mais rápida e utilizando uma menor quantidade de DNA. Dentre os genes capazes de diferenciar os organismos a nível de espécie destacam-se os genes ribossomais (rDNA), regiões intergênicas (ITS) e os genes mitocondriais (mtDNA) (Perry et al., 2007). Essa análise possui alta reprodutibilidade entre laboratórios, é rápida, a obtenção do material genético não está limitada a um estádio de desenvolvimento específico do nematoide e consegue separar espécies com similaridade morfológicas (Zijlstra et al., 2000; Blok & Powers, 2009; Ye et al., 2015).

Marcadores SCAR, originados dos marcadores RAPD, vêm sendo utilizados por muitos autores para a identificação de espécies de *Meloidogyne*. Após a seleção de uma marca única no perfil de RAPD para determinada espécie, esse fragmento é clonado e sequenciado para o desenvolvimento de um primer que amplifica uma região única do DNA genômico de determinada espécie (Zijlstra, 2000; Mattos et al., 2019). Vários marcadores SCAR foram desenvolvidos para diferenciar espécies de *Meloidogyne*, como em espécies predominantes no café (*M. exigua* Goeldi, 1887, *M. incognita* e *M. paranaensis*), no arroz (*M. graminicola* Golden & Birchfield, 1965, *M. oryzae* Maas, Sanders & Dede, 1978 e *M. salasi* Lopez, 1984), espécies quarentenárias (*M. chitwoodi* Golden, O'Bannon, Santo, & Finley, 1980 e *M. fallax* Karssen, 1996), espécies de ampla distribuição geográfica (*M. incognita*, *M. javanica* e *M. arenaria*) e para *M. enterolobii*, *M. ethiopica*, *M. izalcoensis* Carneiro, Almeida, Gomes & Hernandez, 2005 e *M. arabicida* Lopez & Salazar, 1989 (Zijlstra, 2000; Randig et al., 2002; Tigano et al. 2010; Correa et al., 2014; Santos, 2016; Mattos et al., 2019; Ye et al., 2019).

Com a revolução das metodologias de identificação, o gênero *Meloidogyne* passou a ser constituído por mais de 100 espécies (Hunt & Handoo, 2009). A identificação de espécies de *Meloidogyne* em canaviais foi estudada nos estados de Pernambuco, Alagoas, Paraná e Rio Grande do Sul (Severino et al., 2008; Moura et al., 2011; Bellé et al., 2017b; Noronha et al., 2017). As espécies *M. javanica*, *M. arenaria* e *M. incognita* foram as mais frequentemente encontradas no Brasil (Severino et al., 2008; Moura et al., 2011; Bellé et al., 2017b; Noronha et al., 2017).

Na região noroeste do Paraná, um total de 74 isolados de *Meloidogyne* foram coletados em 10 municípios do estado e identificados pelo perfil de esterase. Foi observado a predominância de *M. javanica*, no entanto, *M. incognita* e *M. paranaensis* também foram detectados (Severino et al., 2008). Dinardo-Miranda et al. (2013) observaram que *M. javanica* é a espécie mais comum na região canavieira de Piracicaba, representando cerca de 60% da

população dos nematoides presentes na área. Contudo, diferente da região Sul do Brasil, *M. incognita* é a espécie predominante nos canaviais do estado de Pernambuco e Alagoas. *Meloidogyne hispanica*, *M. enterolobii* e *M. arenaria* também foram identificadas na região utilizando o padrão fenotípico das esterases (Moura et al., 2011; Noronha et al., 2017). As espécies *M. hispanica*, *M. kikuyensis* de Grisse, 1960 e *M. thamesi* Chitwood in Chitwood, Specht & Havis, 1952 foram descritas em cana-de-açúcar fora do Brasil (Cadet & Spaull, 2005; Dinardo-Miranda, 2005).

Como pode-se observar, faltam estudos com abordagens polifásicas na identificação das populações de *Meloidogyne*, uma vez que o padrão enzimático não é capaz de separar todas as espécies. Além disso, até o momento, nenhum estudo emprega um número expressivo de isolados e ferramentas moleculares de alta resolução para identificação desses nematoides no estado de São Paulo, que atualmente é o estado com maior extensão de área plantada de cana-de-açúcar no país. A identificação adequada de *Meloidogyne* spp. tem um papel crucial no estabelecimento das estratégias de controle (Bellé et al., 2017a). Mesmo quando se tem conhecimento sobre a espécie que se encontra na área, a falta de informação sobre a variabilidade dos isolados dificulta a adoção de medidas específicas, como recomendação de rotação de cultura e, principalmente, uso de variedades resistentes (Ye et al., 2019). A escolha errônea de uma variedade em uma determinada área pode levar a grandes perdas para o produtor. Consequentemente, sem o conhecimento da espécie e da variabilidade do patógeno, o produtor fica impossibilitado de fazer uma escolha certeira.

Uma forma de se conhecer a variabilidade local é por meio de ensaios de agressividade; no entanto, a obtenção dos dados do patógeno é uma tarefa árdua, pois exige longos e constantes bioensaios para avaliar com frequência a população do nematoide, uma vez que essas populações podem mudar em resposta à pressão de seleção exercida por culturas resistentes (Netscher, 1977; Janssen et al., 1998). Com isso, a aplicação de marcadores moleculares é uma alternativa para os estudos de variabilidade do patógeno. Além disso, permite conhecer as rotas de introdução, os mecanismos evolutivos e até mesmo predizer o tempo para a suplantação da resistência de determinada população (Silva et al., 2014).

Marcadores moleculares neutros vêm sendo utilizados para estudos da variabilidade intraespecífica do gênero *Meloidogyne*. Entre os marcadores mais utilizados para *Meloidogyne* spp. estão os do tipo RAPD, RFLP e AFLP. RAPD consiste na técnica mais aplicada no estudo de variabilidade de nematoides do gênero, que primeiramente foi usada para diferenciar perfis moleculares de diferentes espécies de nematoides e, posteriormente,

desenvolver marcadores SCAR (Willianson et al., 1997; Donkers-Venne et al., 2000; Zijlstra, 2000; Randig et al., 2002; Santos, 2016; Mattos et al., 2019; Ye et al., 2019). A técnica emprega a utilização de oligonucleotídeos para amplificação do DNA genômico usando a PCR, tendo a vantagem de não necessitar de um genoma de referência da espécie. Entre os fatores limitantes da técnica destacam-se: a demora para obtenção dos resultados; baixa variabilidade por loco, sendo muitas vezes necessárias várias reações de PCR; requer muitos indivíduos para a extração do DNA; e trata-se de um marcador dominante (Ferreira & Grattapaglia, 1998).

No Brasil, o primeiro estudo usando o marcador RAPD para estudar a variabilidade intraespecífica foi realizado por Randig et al. (2002). O trabalho consistiu no estudo das variações polimórficas das espécies *M. arenaria*, *M. exigua* e *M. hapla* na cultura do café (Randig et al., 2002). Após este trabalho, vários outros estudos de variabilidade foram realizados em outras espécies e culturas usando apenas o marcador RAPD ou aliado com o marcador AFLP ou RFLP. Vários estudos têm como foco a caracterização da variabilidade de espécies de *Meloidogyne* no café, como o estudo realizado por Santos et al. (2018) que estudou a variabilidade de *M. paranaensis*, e o de Carneiro et al. (2004) que observou baixa variabilidade intraespecífica em *M. incognita*, *M. exigua* e *M. paranaensis* e alto polimorfismo em *M. arenaria*. No entanto, a variabilidade de *Meloidogyne* também foi investigada em outras culturas, como: soja, batata, goiaba, arroz e algodão. Mattos et al. (2016) estudaram a variabilidade das espécies *M. incognita*, *M. morocciensis* Rammah & Hirschmann, 1990 e *M. javanica* em campos do Cerrado e soja, e Lopes et al. (2019) observaram alta variabilidade de isolados de *M. incognita* coletados principalmente no estado da Bahia. Até o momento, nenhum estudo de variabilidade de *Meloidogyne* spp. foi realizado na cultura da cana-de-açúcar.

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Capítulo 1

IDENTICATION OF ROOT-KNOT NEMATODES FROM SÃO PAULO STATE, THE LEADING SUGARCANE PRODUCING REGION IN BRAZIL

IDENTICATION OF ROOT-KNOT NEMATODES FROM SÃO PAULO STATE, THE LEADING SUGARCANE PRODUCING REGION IN BRAZIL

ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) stand among the most destructive phytopathogens in sugarcane, responsible for yield losses which could reach up to 50% in Brazil. However, there is no updated information about prevalence, distribution, and occurrence of admixture of *Meloidogyne* species affecting the crop for São Paulo State, which is responsible for ~18% of world production of sugarcane. To elucidate the pathogen variability in sugarcane plantations in the state of São Paulo, to contribute to the development of integrated disease control strategies, soil and roots samples were collected in 36 sugarcane fields, in 10 municipalities of the state. Nematode identification was carried out with polyphasic approach using esterase phenotype, Sequence Characterized Amplified Regions (SCAR) markers and study of ribosomal (D2/D3 segment of 28S gene) and mitochondrial genes (cytochrome oxidase I - COI and NADH dehydrogenase subunit 5 - NADH5). With this integrative methodology, *Meloidogyne incognita* and *M. javanica* were identified in the commercial fields surveyed. *Meloidogyne javanica* was observed in all sampled fields and, an admixture of *Meloidogyne* species including *M. incognita* was present in 33% of these areas. Esterase phenotypes, profiles J2 and J3 of *M. javanica* and I1 and I2 of *M. incognita* were detected in the samples, being the first time that the *M. javanica* profile J2 is detected infecting sugarcane. This study will contribute to a rational choice of integrated control measures to the root-knot nematode, especially for the development of resistant cultivars aiming at implementing a sustainable sugarcane production for the state of São Paulo.

KEYWORDS

Meloidogyne incognita, Meloidogyne javanica, Saccharum hybridum, SCAR-PCR, NADH5

INTRODUCTION

Sugarcane (*Saccharum hybridum* R.M. Grey) is one of the main crops in the world destinated especially for the manufacture of sugar, alcohol, spirit beverages, and animal feed. Brazil is the largest sugarcane producing country, yielding 654,8 thousand tons/year, being São Paulo state responsible for 54% and 18% of national and worldwide production,

respectively (CONAB, 2021). The disease constraints caused by plant-parasitic nematodes impose difficulties for sugarcane production to be even higher, especially because, as soilborne pathogens, there are few alternatives for ecologically acceptable disease control under intensively cultivated soils.

It is estimated that the losses caused by plant-parasitic nematodes can reach 20 to 40% yield reduction in the first cut of susceptible sugarcane cultivars (Bellé et al., 2017a; Noronha et al., 2017). Among the nematodes frequently associated with the crop stand out the ones belonging to the genera *Pratylenchus*, *Helicotylenchus* and *Meloidogyne* (Bellé et al., 2014). *Meloidogyne* spp., known as root-knot nematodes, ranks first in the list of plant-parasitic nematodes due to its scientific and economic importance (Jones et al., 2013).

Over 10 species of *Meloidogyne* were described in the literature affecting sugarcane productivity (Cadet & Spaull, 2005; Dinardo-Miranda, 2005; Severino et al., 2008; Moura et al., 2011; Bellé et al., 2017b; Noronha et al., 2017). among them, seven species were identified in Brazilian fields: *M. javanica* (Treub, 1885) Chitwood, 1949, *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. enterolobii* (Yang and Eisenback, 1983), *M. ethiopica* (Whitehead, 1968), *M. hispanica* (Hirschmann, 1986), and *M. paranaensis* (Carneiro, Carneiro, Abrantes, Santos and Almeida 1996). Currently, the largest diversity of *Meloidogyne* species associated with sugarcane is observed in the Northeast region of Brazil, including *M. incognita*, *M. javanica, M. hispanica, M. enterolobii* and *M. arenaria* reported in the region using morphological and biochemical techniques (Moura et al., 2011; Noronha et al., 2017). In the Southern region, *M. incognita, M. paranaensis, M. javanica,* and *M. ethiopica* were detected by biochemical and molecular techniques (Severino et al., 2008; Bellé et al., 2017b). Meanwhile, there is no information available on the identification and distribution of *Meloidogyne* species in sugarcane fields of São Paulo in large scale, the state responsible for the largest share of production in the country.

Identification of *Meloidogyne* species is a complex task, even for experienced nematologists. Different techniques have been used to identify *Meloidogyne* species, all with advantages and limitations. The application of classic methods, such as the perineal pattern, is difficult due to the subjectivity and high level of similarity between morphological characteristics that compromises the accuracy of identification. An example is the identification of *M. enterolobii*, which is constantly confused with *M. incognita* by the absence of morphological markers able to separate these species (Moens et al., 2009). Also, a similar perineal pattern of *M. paranaensis*, *M. incognita*, *M. izalcoensis* (Carneiro, Almeida,

Gomes & Hernandez, 2005), and *M. inornata* (Lordello, 1956) was observed, impairing the correct separation of these species (Carneiro et al., 2016). A reliable taxonomic source of characters is the isoenzyme esterase pattern, which is suitable to identify several *Meloidogyne* species. Moreover, isoenzyme electrophoresis technique allows the species identification in a mix of individuals and the detection of atypical esterase patterns, although there is no typical isoenzyme profile for all *Meloidogyne* species (Carneiro et al., 2016).

With the advent of molecular methods, efficient and high-resolution markers allowed species identification and the exploration of genomic variability. Modern and already popular identification methods of *Meloidogyne* include the use of Sequence Characterized Amplified Regions (SCAR) markers, and the study of ribosomal nucleotide sequences variations (18S, Internal Transcribed Spacer - ITS, D2/D3 segment of 28S) and mitochondrial genes (cytochrome oxidase I - COI and NADH dehydrogenase subunit 5 - NADH5). SCAR markers are efficient for species-level identification, although the variability of sampled individuals used to design the primer is essential for proper marker functioning. Different housekeeping genes have been employed in phylogenetic analysis, such as the ITS; however, due the reduced variation in sequence, it is not reliable to separate some species as *M. incognita*, *M. javanica* and *M. arenaria* in different clades (Blok, 2005). An alternative for phylogenetic analysis is the concatenation of different genomic regions to separate *Meloidogyne* species into distinct clades (Rashidifard et al., 2019). To solve the limitation of different techniques and increase the accuracy of nematode identification, a polyphasic approach - combining biochemical and molecular techniques - is the most used methodology for *Meloidogyne* identification (Hunt & Handoo, 2009; Mattos et al., 2018).

Considering the absence of information on *Meloidogyne* species and the need of robust and accurate methodologies for the identification of root-knot nematodes in sugarcane in São Paulo State, this study was developed to: (i) establish the prevalence and distribution of *Meloidogyne* species in sugarcane fields in the state; (ii) determine whether there are new *Meloidogyne* species in São Paulo sugarcane fields; (iii) study if the pattern of species distribution match with spatial attributes and; (iv) observe the frequency of admixture species in fields.

MATERIALS AND METHODS

Nematode sampling

A total of 36 samples were collected from infected roots and soil of sugarcane in 10 municipalities (Altinópolis, Serrana, Descalvado, São Carlos, Tanabi, Barretos, Guapiaçu, Pirassununga, Brotas and Analândia) of São Paulo state, Brazil. The sampling comprised municipalities, located in the North and Northeast regions of the state. They were chosen due their important contribution to sugarcane production (Rudorff et al., 2010). In each municipality, three commercial sugarcane fields were sampled in March and April/2020 (Figure 1). Except Brotas and Analândia, that a total of six fields were collected in each municipality. A sample consisted of a set of five mixed sub-samples collected in a depth of zero to 15 cm, composed of a central point with the localization georeferenced and other four sites collected in a radius of 10 m around the central point. All samples were collected in stunted, chlorotic plants with roots poor in radicelles or in areas with history of the presence of root-knot nematodes (Supplementary Table 1). Root and soil collected were packed in polyethylene bags, labeled, and stored at 4 °C until nematode extraction. The samples were processed in the Department of Plant Pathology at the University of Brasília, Brasília- DF, Brazil.

Multiplication of *Meloidogyne* spp.

The samples, formed by soil and root, were used for planting tomato plants (*Solanum lycopersicum* L. Santa Cruz Group, cv. Santa Clara Group) for root gall formation. The plants were kept in a greenhouse with a temperature of ~25 ± 5 °C for a period of two months. After this period, galls in roots were removed for extraction of *Meloidogyne* females.

Biochemical identification of *Meloidogyne* spp.

In the first moment, as carried out in routine and research laboratories in Brazil, the biochemical tests were performed to analyze EST phenotypes of isolates according to the profile pattern established to species identified in Brazil (Carneiro et al., 2016). Five adult females were removed from each tomato root system. Four replications were performed per sample. The study was carried out using the vertical electrophoresis system in 7% polyacrylamide gel according to the methodology described by Carneiro and Almeida (2001).

As positive control of the reaction, females of *M. javanica* with a known profile EST J3, MDH N1 were used as positive control and marker.

Molecular and phylogenetic identification of *Meloidogyne* spp.

The nematode eggs were extracted from the galls of tomato roots according to the methodology of Carneiro et al. (2004). The genomic DNA of *Meloidogyne* populations was extracted from 200 to 300 µl of nematode suspension containing eggs by the phenol-chloroform methodology, as described by Randig et al. (2002). The extracted DNA was resuspended in 20 µl of deionized water. In this study, SCAR-PCR primers were used for detection of *Meloidogyne* species *M. arenaria* (Far/Rar), *M. incognita* (Finc/Rinc), *M. javanica* (Fjav/Rjav), *M. paranaensis* (par-C09-F/par-C09-R), *M. enterolobii* (MK7F/MK7R), and *M. ethiopica* (met-F/met-R) in the samples. The PCR reactions were performed using 12.5 µl of DreamTaq Green PCR Master Mix (Thermo Scientific Fermentas Ltd), 1 µL of each primer (10 pmol/µl), 1 µl of genomic DNA (~30 ng) and 9.5 µl of MilliQ water, following the manufacturer's instructions protocol. For each pair of primers, the thermal cycler program used during the PCR followed the description in the base articles (Zijlstra et al., 2000; Randig et al., 2002; Tigano et al., 2010; Correa et al., 2014).

Pure populations of *Meloidogyne* of all samples were, once that more than one species was detected in different samples using SCAR-PCR analysis. Thereunto, roots with egg masses were stained in a Floxin-B solution (0.1 to 0.15 g to 4 liters of water) for 15 min. Subsequently, an egg mass was hand-picked with the aid of tweezers, needle, under a stereoscopic microscope (Leica EZ4). The egg mass of a single female was inoculated in tomato seedling with two pairs of real leaves for nematode´s multiplication. The process to obtain a pure population was repeated five times to each sample. After two months, eggs of pure populations of *Meloidogyne* were used for DNA extraction and phylogenetic analysis of *Meloidogyne* species.

After DNA extraction of pure populations, following the protocol mentioned above, we selected 10 isolates, UnBSP3, UnBSP5, UnBSP8, UnBSP15, UnBSP18, UnBSP21, UnBSP23, UnBSP27, UnBSP29 and UnBSP35, collected from different municipalities of São Paulo State and three genomic regions (28S D2/D3, NADH5, and COI) to confirm the species identification observed in biochemical and SCAR-PCR tests. PCR amplifications were done using the primers RK28SF (CGGATAGAGTCGGCGTATC) and RK28SR (GATGGTTCGATTAGTCTTTCGCC) for 28S D2/D3 (Ye et al., 2015); NADH5F

(TATTTTTTGTTTGAGATATATTAG) and NADH5R (CGTGAATCTTGATTTTCCATTTTT) for NADH (Janssen et al., 2016) and JB3 (TTTTTTGGGCATCCTGAGGTTTAT) and JB4 (TAAAGAAAGAACATAATGAAAATG) for the COI region (Bowles et al., 1992). Reactions were carried out in T100 Thermal Cycler (Bio-Rad®) following the program: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C, 45 °C and 52 °C for 28S D2/D3, NADH5 and COI region, respectively, for 45 sec and elongation at 70 °C for 1 min, concluded with a final extension at 72 °C for 10 min.

The DNA amplification product of each PCR reaction, including SCAR-PCR and genomic regions, were mixed with the loading buffer and GelRed (Biotium®), then loaded on 1% standard TAE buffer agarose gel. The size of amplified fragments was estimated by comparison with a 100 pb molecular marker and photographed using an UV transilluminator (Loccus, L-PIX). The PCR product of 28S D2/D3, NADH5 and COI region were purified using ExoSap-IT® (Affymetrix, Inc., Santa Clara, CA, USA), according to the manufacturer's instruction, and sequenced both strands at Universidade Católica de Brasília (Brasília, DF, Brazil) using the primers previously described.

The quality, corrections at ambiguous positions, contig assembly of all sense and antisense nucleotide sequences were edited in DNA Dragon software (https://www.dna-dragon.com) and aligned using the Muscle algorithm implemented in software Mega v.7.0 (Edgar, 2004; Kumar et al., 2016). Phylogenetic analysis of genes 28S D2/D3, NADH5, and COI regions was constructed using sequence alignment of ten isolates collected in present study and sequences of NCBI's GenBank (https://www.ncbi.nlm.nih.gov/genbank). Two sets of aligned *Meloidogyne* sequences were performed. The first assessment was composed by the phylogenetic analysis of individual genes with their respective sequence alignments and the second set formed by the concatenation of all genetic regions of isolate using the software Mesquite v.3.40 (Maddison & Maddison, 2018).

The appropriate nucleotide substitution model to each genic region was performed using the Mr.ModelTest v.2.3 program (Nylander, 2004), according to the Akaike information criterion (AIC) (Darriba et al., 2012). Bayesian inference (BI) analysis was carried out based on the Monte Carlo Markov chain method (MCMC) and executed in MrBayes v.3.1.1 (Ronquist & Huelsenbeck, 2003) within CIPRES (Miller et al., 2010). The MCMC conducted with two runs, four chains and lasted 10,000,000 generations. A burnin of 25% was applied to calculate the posterior probabilities. The phylogenetic tree was visualized using FigTree v.1.4 (Rambaut, 2018). We used *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle,

Golden, Mamiya & Wergin, 1981) sequences as the outgroup. The *Meloidogyne* species were defined based on the position of clades grouped with other characterized species sequences taken in GenBank.

RESULTS

Esterase phenotypic profile identification

It was possible to identify the species of *M. javanica* and *M. incognita* analyzing the EST phenotype profile of 33 populations (Table 1). *Meloidogyne javanica* was the predominant species in the sugarcane fields and detected in all municipalities sampled. Bands of reference isolate (*M. javanica* EST J3) were present and located in the same level in the polyacrylamide gel indicating the presence of *M. javanica* in 75% of the fields. Phenotypes J2 (Rm:1.00 and 1.23) and J3 (Rm: 1.00, 1.07 and 1.17) from EST were observed for *M. javanica*, being the pattern J2 only found in one population of Altinópolis (Figure 2 and Table1). *Meloidogyne incognita* was detected in four municipalities (Brotas, São Carlos, Analândia, and Guapiaçu) and the profile I1 (Rm 1.00) and I2 (Rm 1.00 and 1.09) observed, each in two municipalities (I1: Brotas and Guapiaçu, and I2: São Carlos and Analândia) (Figure 2 and Table1). No galls were observed on the tomato roots inoculated win root suspension of samples 13, 32, and 33, so it was not possible to perform the EST identification (Table 1).

SCAR-PCR analysis

The SCAR-PCR analyses corroborated the results found by the EST methodology; however, differently from the EST test, SCAR markers allowed the identification of more than one species of *Meloidogyne* in some fields of sugarcane (Table 1). PCR amplification performed using the primer Rjav/Fjav produced a single fragment of ~670 pb in all samples, confirming the presence of *M. javanica* in all areas sampled. Also, it was possible to observe a positive PCR reaction using the specific primers Rinc/Finc in farms localized in eight municipalities, except Altinópolis and Serrana (Table 1). Therefore, a mix of *Meloidogyne* species, composed by *M. javanica* and *M. incognita*, was detected in fields of Brotas (UnBSP7 and 12), Descalvado (UnBSP14), São Carlos (UnBSP16 and 17) Tanabi (UnBSP

20), Barretos (UnBSP 23 and 24), Guapiaçu (UnBSP 25), Pirassununga (UnBSP 29 and 30), and Analândia (UnBSP 35 and 36). PCR reaction was negative for all reactions using the primers *Far*/*Rar*, MK7F/MK7R, met-F/met-R, and par-C09-F/par-C09-R, designed to detect *M. arenaria*, *M. enterolobii*, *M. ethiopica* and *M. paranaensis*, respectively. The sizes of fragments expected to each pair of primers were 420, 520, 350, and 208, respectively. The final distribution of *Meloidogyne* species using SCAR markers in the state of São Paulo is shown in Figure 1.

Phylogenetic analysis

The 28S D2/D3 gene matrix consists of 1077 total characters, including gaps. Of the total character numbers in the matrix, 525 sites are conserved, 239 are variable and 53 are parsimony informative. The evolutionary model selected was GTR+G. The COI gene matrix consists of 629 total characters, including gaps. Of the total character numbers in the matrix, 229 sites are conserved, 221 are variable and 49 are parsimony informative. The evolutionary model selected was GTR. The NADH5 gene matrix consists of 606 total characters, including gaps. Of the total character numbers in the matrix, 368 sites are conserved, 209 are variable and 32 are parsimony informative. The evolutionary model selected was GTR.

The concatenated gene matrix consists of 1,463 total characters, distributed among the COI (380 bp), 28S D2/D3 (595 bp) and NADH5 (488 bp), including gaps. Of the total character numbers in the concatenated matrix, 741 sites are conserved, 512 are variable and 102 are parsimony informative. The evolutionary models selected were: GTR, GTR+G and GTR for, respectively, COI, 28S D2/D3, and NADH5.

The analysis using 28S D2/D3 managed to segregate in separate and well-sustained clades (over 80%) the species of *M. enterolobii*, *M. hispanica*, and *M. ethiopica*, taken from GenBank (Figure S1). However, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. paranaensis*, except for samples of *M. incognita* MF673758 and MF673759, and *M. javanica* UnBSP8, were grouped in the same clade.

Bayesian inference using the COI region managed to separate the species *M. enterolobii*. The other species, *M. hispanica* and *M. ethiopica*, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. paranaensis*, were grouped in the same clade (Figure S2). Although the sequences of the studied populations are in the same clade, each of these species was placed in its subset. The samples studied were close to the species *M. javanica*. Curiously, the *M. javanica* UnBSP15 formed a separate group in the NADH phylogenetic tree that might seem

to be a different species (Figure 3). We believe that the isolate belongs to the species *M. javanica* due the confirmation of species by biochemical and SCAR markers. The other species remained close to each other. A Bayesian analysis with concatenated genes managed to separate only the *M. ethiopica* and *M. enterolobii* with poorly supported clades (Figure S3).

DISCUSSION

The proper identification of nematode species is crucial for the choice of control strategies, including breeding programs for disease resistance. Once the nematode is identified in an area, the use of resistant or tolerant variety is undoubtedly the most economical, efficient, and easy strategy for reducing soilborne pathogen population, especially nematodes in perennial crops (Mazzuchelli et al., 2020). After 57 years of the first report of *Meloidogyne* infecting sugarcane in Brazil by Lordello (1964), few studies describe the occurrence of *Meloidogyne* species in this crop. Moreover, these studies are concentrated in the South and Northeast region of Brazil. Our investigation offers the first survey and regional distribution of the species of *Meloidogyne* present in the leading producing sugarcane area in the world, São Paulo state (Figure 1).

A polyphasic methodology was used to identify the nematode species. Biochemical and SCAR-PCR analysis demonstrated a high similarity of the results observed, although only in molecular analysis it was possible to distinguish two *Meloidogyne* species in the same farm/sample. Genomic markers frequently demonstrate more sensitivity for routine laboratory testing compared to the EST profile test (Mattos et al., 2019). The EST phenotype technique has the advantage of being faster, simpler, and less laborious for some crops; however, it is necessary to keep tomato plants for a long time in contaminated soil with nematodes, due to the absence of visible galls in sugarcane roots. Thus, the identification of *Meloidogyne* in sugarcane using specific primers would be faster due the direct DNA extraction of eggs present in the sugarcane root and would allow the identification of more than one species in the same sample.

Conversely, SCAR markers and EST profile had a different result of phylogenetic analysis of mitochondrial and ribosomal genes. The phylogenetic relations, inferred by Bayesian analysis, among root-knot nematode isolates of the present study showed the discrimination among two species in São Paulo sugarcane fields in NADH5 analysis, separating the isolates in two independent clusters, while 28S D2/D3, COI, and concatenated

regions grouped all sequences in only one cluster (Supplementary Figure S1, S2 and S3). It has already been shown that the phylogenetic analysis of NADH5 region can separate the common tropical species of *Meloidogyne* (*M. enterolobii, M. incognita, M. javanica,* and *M. arenaria*) into well-sustained clades and individual subsets (Janssen et al., 2016; Rashidifard et al., 2019). However, the same authors found that the ribosomal genes are inefficient for distinguishing between *M. incognita* and *M. javanica*, confirming our results. This inconsistency is due to the high conservation of 28S D2/D3 region, with a nucleotide similarity between 97-100%, of *M. incognita, M. javanica* and *M. arenaria* (Ye et al., 2015; Ye et al., 2019). The COI region also presented low genetic variability, despite the relatively high rate of mutation observed in mitochondrial DNA genes (Blok and Powers, 2009).

All the results were combined, and a more reliable species identification was obtained. We found that *M. javanica* is predominant and present in all sugarcane fields sampled in São Paulo, although the mixture of root-knot species, also containing *M. incognita*, was observed. So far, seven species have been reported infecting sugarcane in Brazil. A greater number of *Meloidogyne* species was detected in the state of Pernambuco (*M. arenaria, M. incognita, M. javanica, M. hispanica* and *M. enterolobii*) followed by Alagoas (*M. arenaria, M. incognita* and *M. javanica*) and Paraná (*M. incognita, M. paranaensis* and *M. javanica*) (Severino et al., 2008; Moura et al., 2011; Noronha et al., 2017). Therefore, São Paulo is the state with the lowest number of reported species. Recently, *M. ethiopica* was described as infecting sugarcane in the state of Rio Grande do Sul (Bellé et al., 2017b). Apparently, the wide distribution of *M. incognita* and *M. javanica*, phytopathogens with low efficiency of dispersion, in several Brazilian States and different hosts, indicate that these nematodes are endemic in Brazil. This is reinforced by the presence of these species in native areas of the Cerrado (Mattos et al., 2016). We speculate that the high diversity of species in other states compared to São Paulo can be attributed to the introduction of emerging species (e.g., *M. hispanica* and *M. ethiopica*) by propagative material infected coming from other countries and/or the frequent crop rotation in those areas. Severino et al. (2008) described the presence of *M. paranaensis*, species frequently associated to coffee, in an area of Paraná that has been cultivated with sugarcane for over 25 years; however, coffee was previously planted in the same area, which may explain the presence of this species in the area. It is speculated that *M. ethiopica* was introduced in the state of Rio Grande do Sul by means of commercial kiwi orchards coming from Chile (Carneiro et al., 2003). After the report, *M. ethiopica* has been spread by infected planting material and introduced in different areas of the state (Bellé et al., 2017c; Márquez et al., 2019), as well as other emerging *Meloidogyne* species, which could

reach the largest producing region in Brazil. Unfortunately, there are still few studies that allow the understanding of the epidemiological dynamics of the dispersion of these pathogens in the sugarcane crop.

It has already been shown that EST variants showed aggressiveness differences to different cultivars of the same crop. In coffee, the EST variants P2 and P2a of *M. paranaensis* were more aggressive to susceptible cultivar than P1 (Santos et al., 2018). Moreover, the EST phenotypic variability gives indication of both interspecific and intraspecific genetic variation exist in the *Meloidogyne* population (Carneiro et al., 2008; Muniz et al., 2008; Santos et al., 2012; Santos et al., 2018). In this work, the EST profile J2 and J3, variants characteristic of *M. javanica*, and I1 and I2, of *M. incognita*, were found. A similar scenario was observed in Paraná state with predominance of *M. javanica* profile EST J3, followed by *M. incognita* profile EST I1 prevalence of the variant in 46% and 22% of fields, respectively (Severino et al., 2008). The phenotypes EST J2 and I2 were not identified in Paraná. The esterase variant J2 was found in other important economic crops such as potato (Lima-Medina et al., 2013) and banana (Cofcewicz et al., 2004) in Brazil. To our knowledge, this is the first official report of the occurrence of the phenotype EST J2 in sugarcane. The EST profile I1 and I2 were also observed in Pernambuco sugarcane fields (Moura et al., 2011). In Brazil, both subpopulations of *M. incognita* (I1 and I2) are widely dispersed in different hosts and states. Future studies must be carried out to assess the genetic difference and aggressiveness of different EST phenotypes in sugarcane cultivars, as performed to other cultures.

Unlike São Paulo and Paraná, *M. incognita* and *M. arenaria* are the species more commonly found in the Northeast region of Brazil. Probably, the greater similarity between the results of the Southest and South regions is due to the smaller geographic distance, climatic conditions, or competition during long term coexistence with native plant-parasitic nematodes. Hamza et al. (2017) suggested that *M. incognita* could not reproduce in olive orchards due the competition with *M. javanica* or unfit local habitats. Alternatively, the predominance of *M. javanica* could be explained by the adoption of resistant cultivars. Indeed, the more frequently cultivar in 2019-2020, RB966928, is susceptible to *M. javanica* (Bellé et al., 2017a). Unfortunately, the occurrence of admixture species of *Meloidogyne* is frequently observed in sugarcane fields, and information on eventual interactions between the two species is not available for sugarcane.

Overall, the frequency of fields with two *Meloidogyne* species in São Paulo were similar to other states (Noronha et al., 2017). This result directly impacts the control of the pathogen in the area. Resistant varieties are available on the market for the main *Meloidogyne*

species in sugarcane. The variety RB-071001 and SP70-1143 are recognized for resistance to *M. javanica* and RB-863129, RB-867515 and RB-92579 to *M. incognita* (Silva et al., 2012, 2013). However, varieties resistant to more than one pathogen or nematode species are rarely seen in the market. The use of cultivars resistant to a single species of the pathogen in a field with mixed populations allows the gradual increase of the unaffected species, making the control of the pathogen inefficient.

In conclusion, this study demonstrates that *M. javanica* is the key *Meloidogyne* species of culture with the coexistence of *M. incognita* in the same fields in São Paulo state. Constant monitoring and sanitary measures need to be carried out to prevent the entry of additional species of *Meloidogyne*, already present in Brazil, in the state. The results obtained will allow to choose more specific cultivars to control or reduce the nematode population in the area.

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TABLES AND FIGURES

Table 1: *Meloidogyne* species identified by SCAR and Esterase methods in samples collected in different municipalities of São Paulo State. Esterase profiles are listed in the last column.

Samples	Origin	SCAR identification	Esterase identification	Esterase profile
UnBSP1	Altinópolis	*M. javanica*	*M. javanica*	J3
UnBSP2	Altinópolis	*M. javanica*	*M. javanica*	J2
UnBSP3	Altinópolis	*M. javanica*	*M. javanica*	J3
UnBSP4	Serrana	*M. javanica*	*M. javanica*	J3
UnBSP5	Serrana	*M. javanica*	*M. javanica*	J3
UnBSP6	Serrana	*M. javanica*	*M. javanica*	J3
UnBSP7	Brotas	*M. javanica/M. incognita*	*M. incognita*	I1
UnBSP8	Brotas	*M. javanica*	*M. javanica*	J3
UnBSP9	Brotas	*M. javanica*	*M. javanica*	J3
UnBSP10	Brotas	*M. javanica*	*M. javanica*	J3
UnBSP11	Brotas	*M. javanica*	*M. incognita*	I1
UnBSP12	Brotas	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP13	Descalvado	*M. javanica*	-	-
UnBSP14	Descalvado	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP15	Descalvado	*M. javanica*	*M. javanica*	J3
UnBSP16	São Carlos	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP17	São Carlos	*M. javanica/M. incognita*	*M. incognita*	I2
UnBSP18	São Carlos	*M. javanica*	*M. javanica*	J3
UnBSP19	Tanabi	*M. javanica*	*M. javanica*	J3
UnBSP20	Tanabi	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP21	Tanabi	*M. javanica*	*M. javanica*	J3
UnBSP22	Barretos	*M. javanica*	*M. javanica*	J3
UnBSP23	Barretos	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP24	Barretos	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP25	Guapiaçu	*M. javanica/M. incognita*	*M. incognita*	I1

UnBSP26	Guapiaçu	*M. javanica*	*M. javanica*	J3
UnBSP27	Guapiaçu	*M. javanica*	*M. javanica*	J3
UnBSP28	Pirassununga	*M. javanica*	*M. javanica*	J3
UnBSP29	Pirassununga	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP30	Pirassununga	*M. javanica/M. incognita*	*M. javanica*	J3
UnBSP31	Analândia	*M. javanica*	*M. javanica*	J3
UnBSP32	Analândia	*M. javanica*	-	-
UnBSP33	Analândia	*M. javanica*	-	-
UnBSP34	Analândia	*M. javanica*	*M. javanica*	J3
UnBSP35	Analândia	*M. javanica/M. incognita*	*M. incognita*	I2
UnBSP36	Analândia	*M. javanica/M. incognita*	*M. javanica*	J3

Table S1: List of *Meloidogyne* species used in the phylogenetic study with their respective identification numbers (ID), and NCBI accession numbers for NADH5, 28S, and COI genes. The list includes the *Bursaphelenchus xylophilus* species that was used as an outgroup.

Specie	ID	NADH5	28S	COI
M. enterolobii	P1	MG920325	KY033176	KY203706
M. enterolobii	P2	MG920329	KY033177	KY203677
M. javanica	P14	MG948220	MF673746	MF673782
M. javanica	P15	MG948221	MF673747	MF673781
M. javanica	P17	MG948223	MF673750	MF673780
M. javanica	P27	MG948226	MF673753	MF673777
M. incognita	P32	MG948249	MF673758	MF673773
M. incognita	P33	MG948230	MF673759	MF673772
M. incognita	P34	MG948231	MF673762	MF673771
M. arenaria	MeloSA22		KC287192	
M. arenaria	6304.1		KP901082	
M. arenaria	US1			JX683704
M. arenaria	LHS62			KM887153
M. arenaria	T332	KU372354		
M. arenaria	Y19	KU372355		
M. paranaensis	PAR5		KY911105.1	
M. paranaensis	189293		AF435800.1	
M. ethiopica	325748		KF482373	
M. ethiopica	325748.2		KX579055.1	
M. ethiopica	T612	KU372360.1		
M. hispanica	520120		KT359553.1	
M. hispanica	520120.2		EU443608.1	
M. hispanica	HN2			JX683713
M. hispanica	HN1			JX683712.1
B. xylophilus	6326	JN596461.1	EU295504.1	KU531399.1

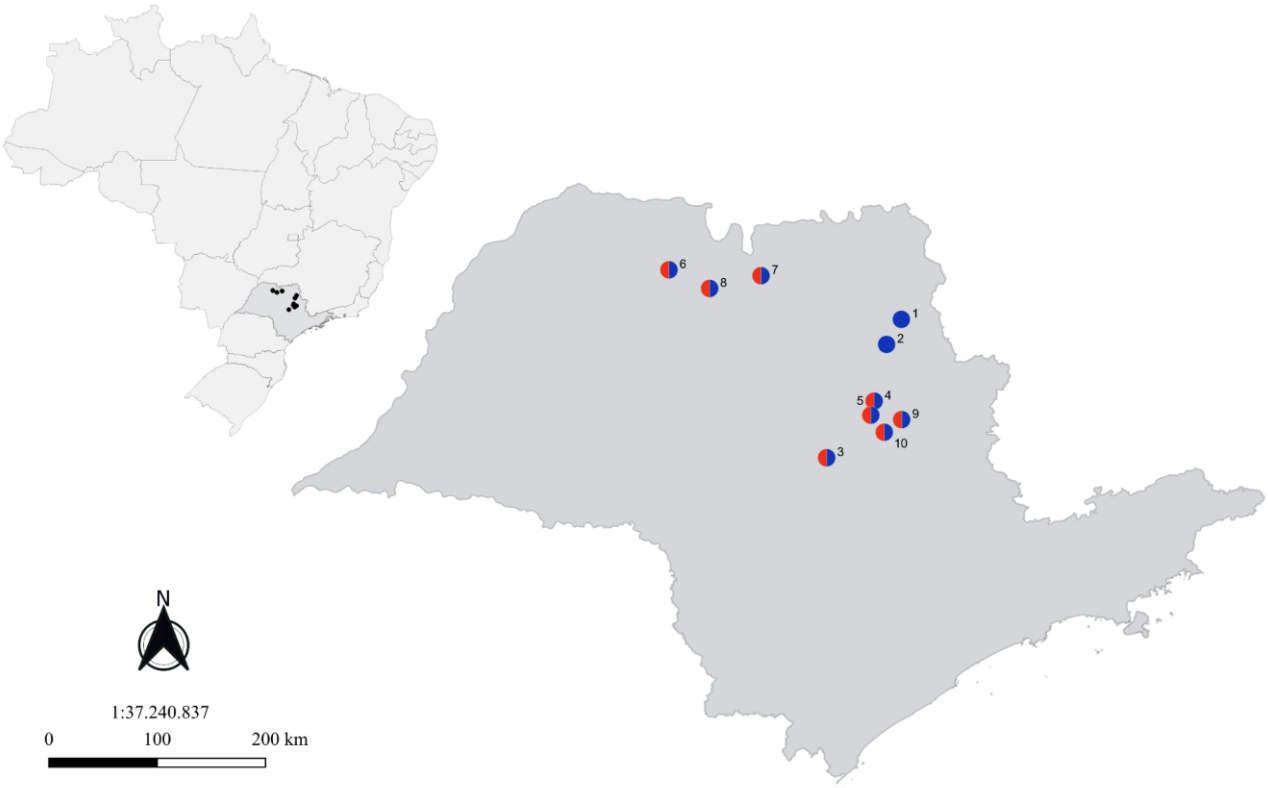

Figure 1: Distribution of *Meloidogyne incognita* (red) and *Meloidogyne javanica* (blue) sampled in municipalities in São Paulo state: (1) Altinópolis, (2) Serrana, (3) Brotas, (4) Descalvado, (5) São Carlos, (6) Tanabi, (7) Barretos, (8) Guapiaçu, (9) Pirassununga, and (10) Analândia. The species were identified by SCAR-PCR method.

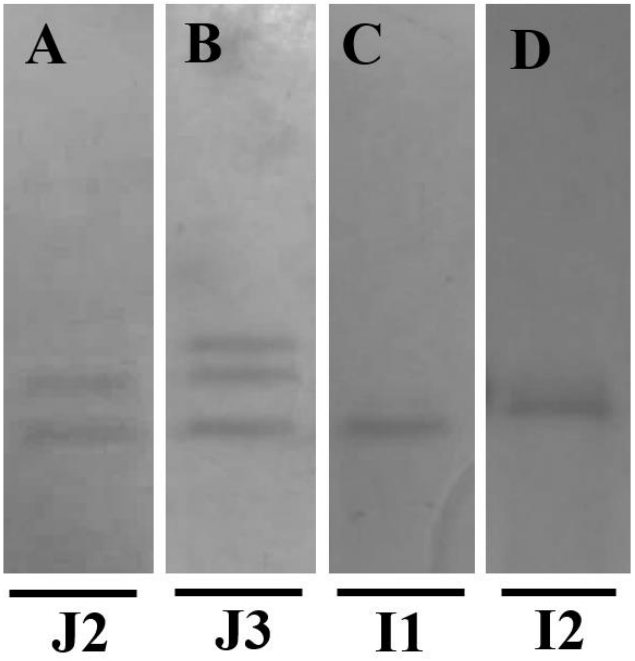

Figure 2: Esterase profile of *Meloidogyne javanica* (A and B) and *Meloidogyne incognita* (C and D) identified in samples from São Paulo State using the methodology described by (Carneiro and Almeida, 2001).

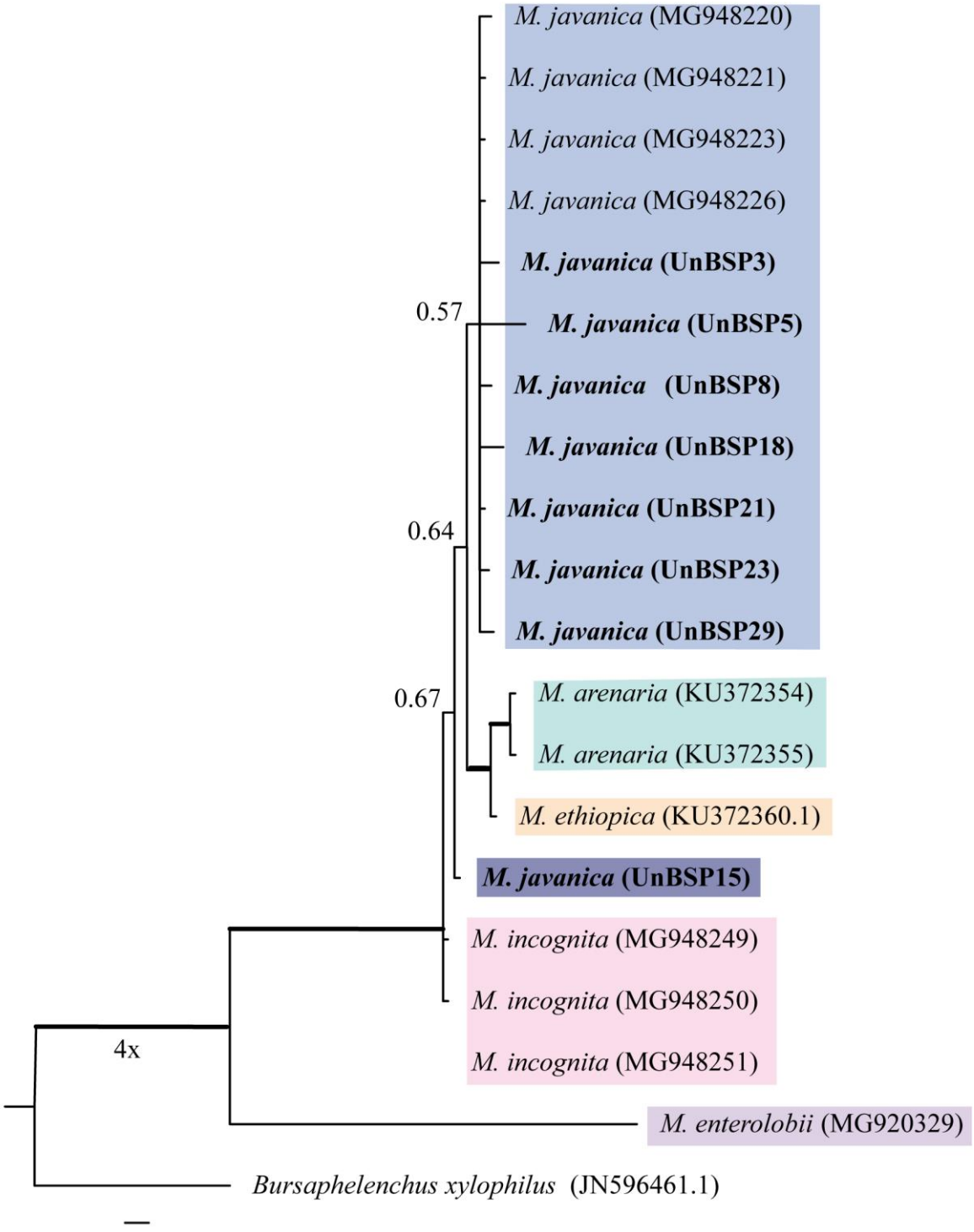

Figure 3: Phylogenetic tree of the NADH5 gene obtained by Bayesian Inference from samples of *Meloidogyne* spp. Collected in São Paulo state. Higher probability values are indicated to the left of the nodes, with thick lines indicating PP ≥ 0.99. The tree was rooted with *Bursaphelenchus xylophilus*. The specimens of this study are highlighted in bold; the other species were obtained from Genbank and their accession numbers are written along with the species name. Scale bar: 0.01.

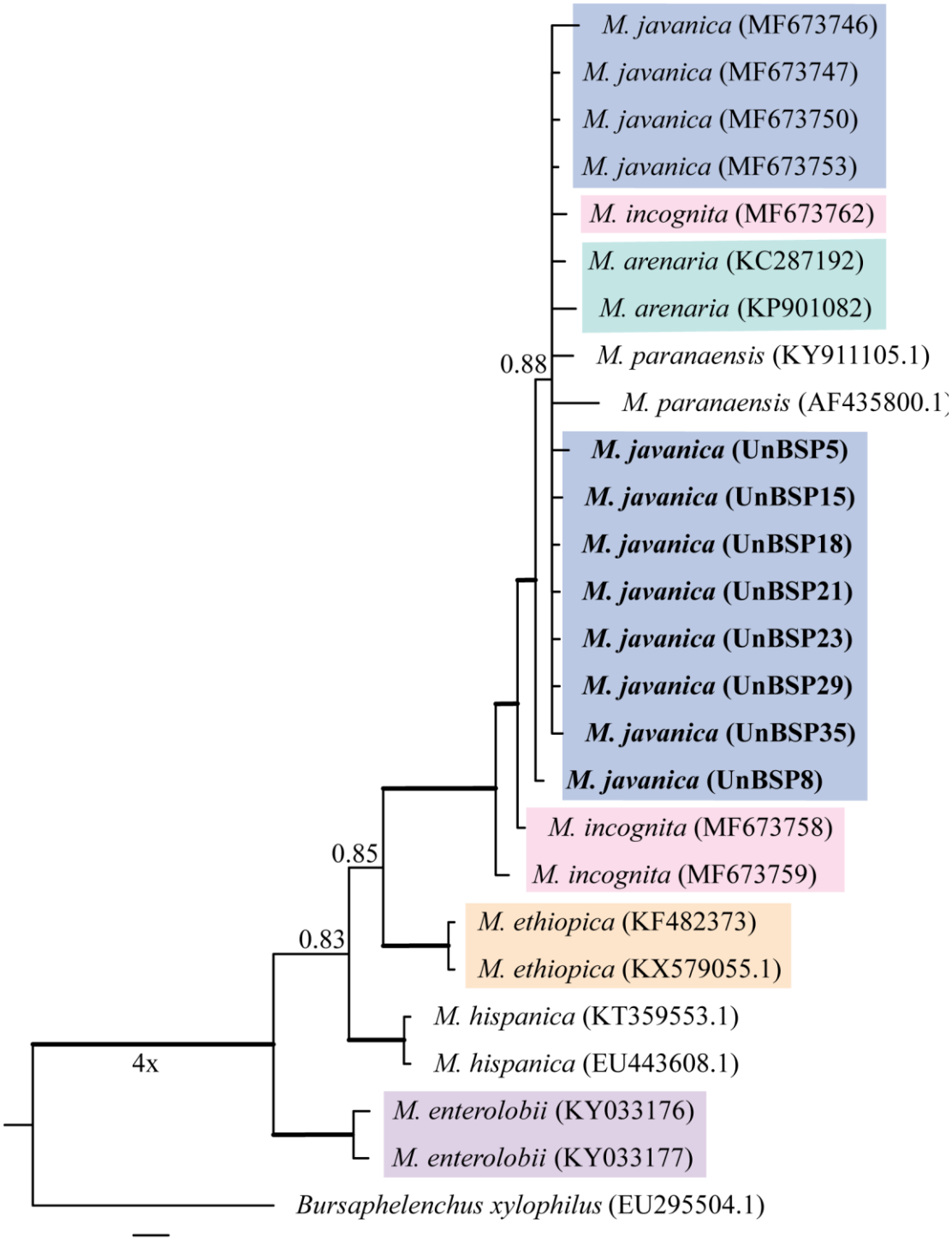

Figure S1: Phylogenetic tree of the 28S D2/D3 gene obtained by Bayesian Inference from samples of *Meloidogyne* spp. Collected in São Paulo state. Higher probability values are indicated to the left of the nodes, with thick lines indicating PP ≥ 0.99. The tree was rooted with *Bursaphelenchus xylophilus*. The specimens of this study are highlighted in bold; the other species were taken from Genbank and their accession numbers are written along with the species name. Scale bar: 0.01.

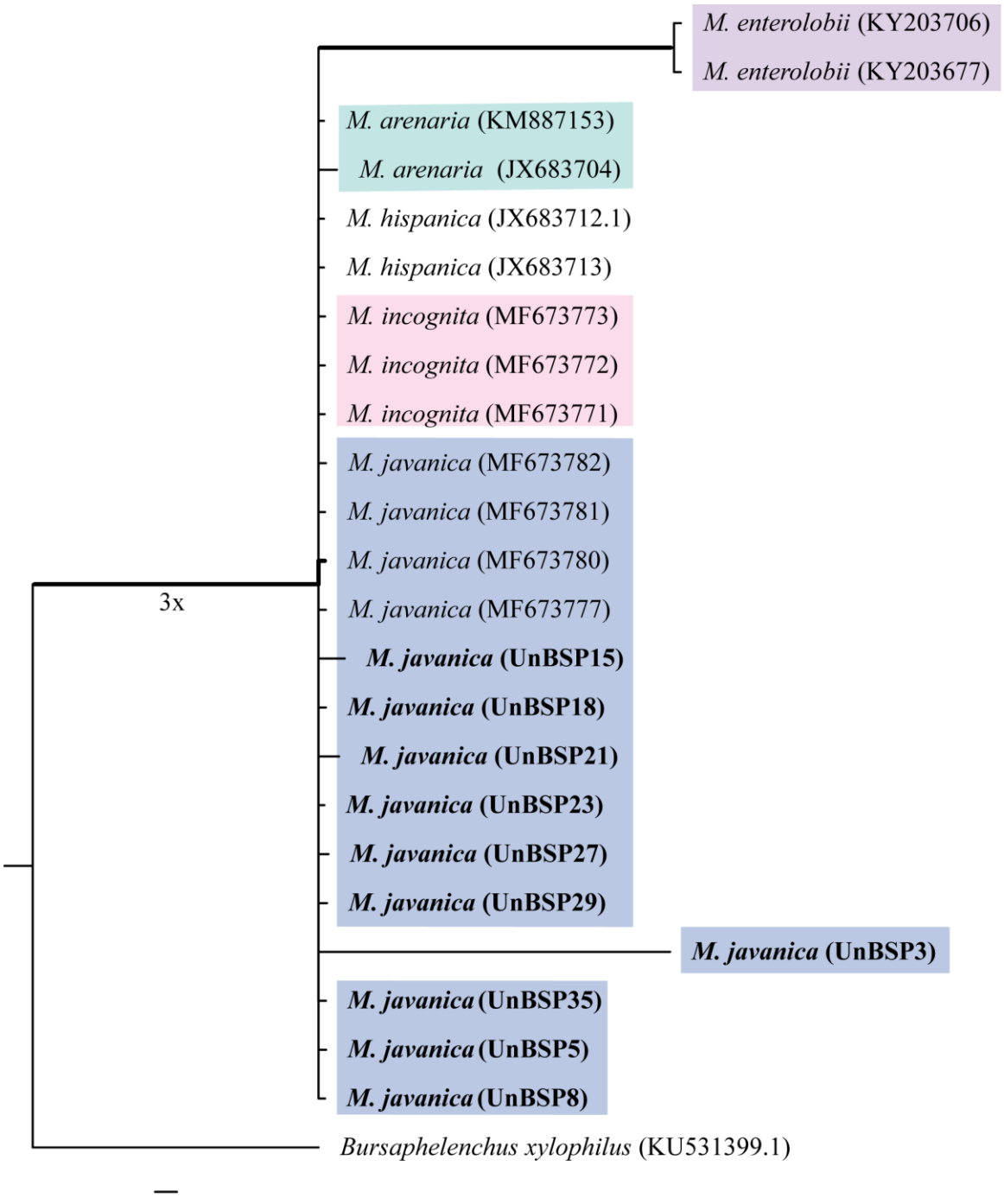

Figure S2: Phylogenetic tree of the COI gene obtained by Bayesian Inference from samples of *Meloidogyne* spp. Collected in São Paulo state. Higher probability values are indicated to the left of the nodes, with thick lines indicating PP ≥ 0.99. The tree was rooted with *Bursaphelenchus xylophilus*. The specimens of this study are highlighted in bold; the other species were taken from Genbank and their accession numbers are written along with the species name. Scale bar: 0.01.

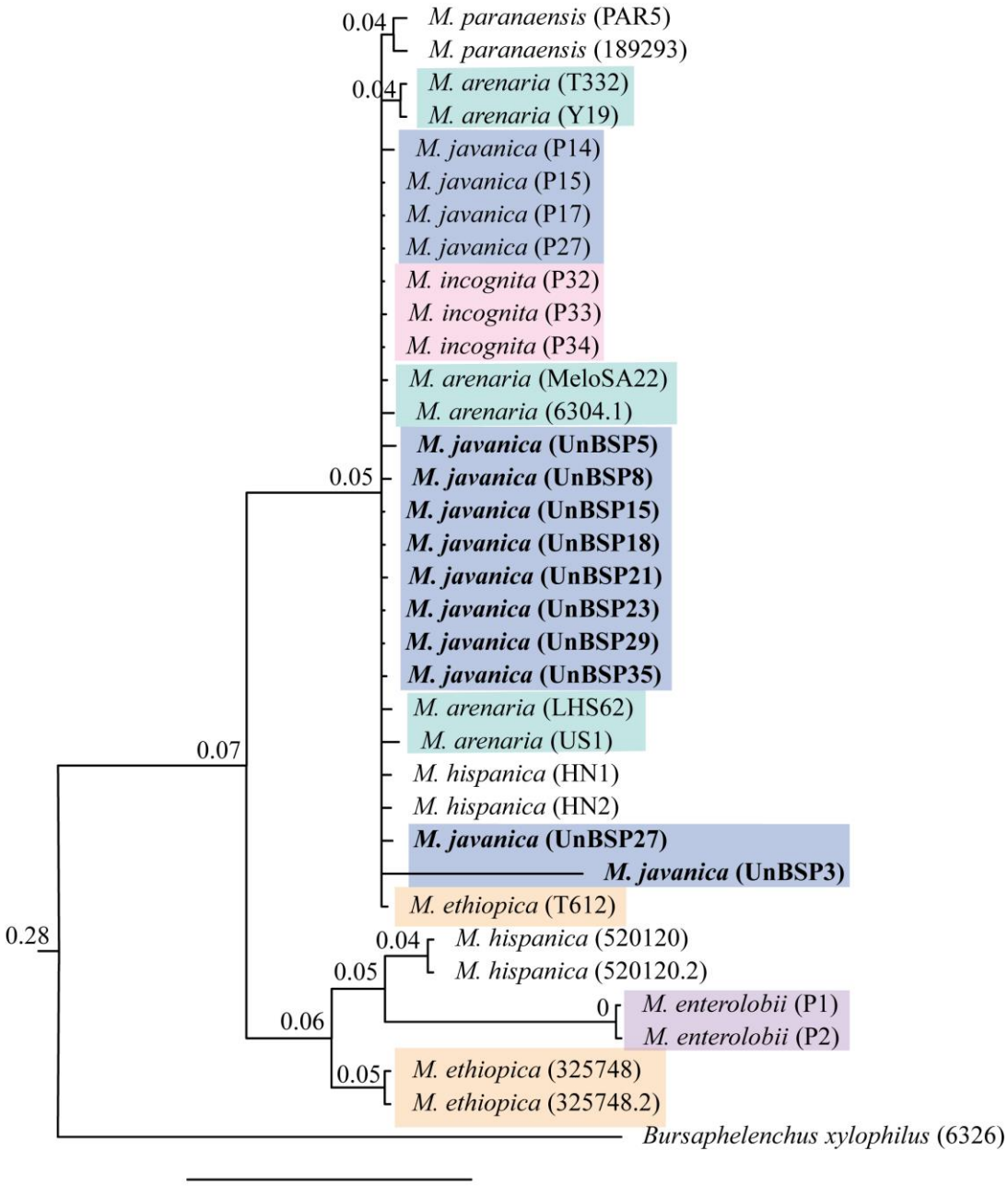

Figure S3: Phylogenetic tree concatenated with genes 28S, COI, and NADH5 obtained by Bayesian Inference from samples of *Meloidogyne* spp. Collected in São Paulo state. The tree was rooted with *Bursaphelenchus xylophilus*. The specimens of this study are highlighted in bold; the other species were taken from Genbank and their accession numbers are described in Table S1. Scale bar: 0.01.

Capítulo 2

GENETIC VARIABILITY OF *Meloidogyne javanica* IN COMMERCIAL SUGARCANE FIELDS IN SÃO PAULO, BRAZIL

GENETIC VARIABILITY OF *Meloidogyne javanica* IN COMMERCIAL SUGARCANE FIELDS IN SÃO PAULO, BRAZIL

ABSTRACT

Meloidogyne javanica is one of the most harmful plant parasitic nematodes in tropical and subtropical agricultural areas of the world. It is a serious problem in sugarcane (*Saccharum hybridum*) in Brazil, including the State of São Paulo, responsible for 54% of national production. For the control of this plant-parasitic nematode, the most efficient method is the use of resistant varieties; however, for the species *M. javanica* and *M. incognita*, such varieties are not fully available. Furthermore, the intraspecific variation observed in *Meloidogyne* isolates allows some of them to overcome resistance. Therefore, the evaluation of the genetic variability and population structure of *M. javanica* is essential for the success of the sugarcane breeding programs. In this context, it was assessed the genetic variability of 23 isolates of *M. javanica* on a microscale using the RAPD markers. Sampling was carried out in ten municipalities located in two important agricultural poles for sugarcane production of São Paulo state. The variability study of *M. javanica* showed that each isolate is a genotype with high genetic variation among isolates and that the population is structured in two subpopulations, without any correlation with geographic region, sugarcane cultivar, soil type, rainfall, and biome. The movement of infected propagation material might have influenced the weak genetic structure of *M. javanica* in a short distance.

***Keywords*:** Root-knot nematode; RAPD markers; *Saccharum hybridum*; molecular epidemiology

INTRODUCTION

Meloidogyne javanica (Treub, 1885) Chitwood, 1949 is one of the most damaging plant-parasitic nematodes in tropical and subtropical agricultural areas in the world (Moens et al., 2009). With the advent of climate changes, the species is seen as a serious threat to temperate regions (Bebber et al., 2014). The apomictic species stands out by high adaptive capacity, wide host range, and has a sophisticated mechanism of survival (Trudgill and Blok, 2001; Oka, 2019). Thousands of plant species, encompassing dicots and monocots, are described as hosts of *M. javanica* (Trudgill and Blok, 2001). Moreover, it has been frequently

observed the occurrence of nematode reproduction in host species yet not described (Phan et al., 2021).

Sugarcane (*Saccharum hybridum* R.M. Grey) is one of the crops seriously affected by *M. javanica* with yield losses estimated in 20 to 35% (Dinardo-Miranda et al., 2008). Brazil is the largest producer of sugarcane in the world and São Paulo State is responsible for 54% of national production (CONAB, 2021). In the north and northeast regions of São Paulo concentrate the major commercial production fields of sugarcane (IBGE, 2017). These regions are characterized by warm temperatures with significant rainfall throughout the year. Thus, the intrinsic characteristics of the pathogen, coupled with ideal environmental conditions for the pathogen development and the monoculture of susceptible sugarcane cultivars for a long time, favor the increase of *Meloidogyne* populations and, consequently, the occurrence of serious epidemics (Dinardo-Miranda, 2006).

The employment of resistant varieties is an economical, practical, and efficient measure to reduce the nematode population and epidemic risk in the area. The genetic basis of modern sugarcane is derived basically from the crosses between *Saccharum officinarum* L. and *S. spontaneum* L. accessions (Arceneaux, 1967; Roach, 1989). The limited number of commercial sugarcane genotypes available are derived from backcrosses with *S. officinarum* used to increase resistance to pests and diseases in the new cultivars (Yu et al., 2018). Furthermore, *S. officinarum* contains additional desirable genetic traits for vigor, drought tolerance, including nematode resistance (Bonnett and Henry, 2011; Bhuiyan et al., 2019). As a result, commercial sugarcane varieties resistant to *M. javanica* and *M. incognita*, are rare. Additionally, the intraspecific variation observed in isolates of *Meloidogyne* allows some of them to overcome crop resistance, what makes necessary the assessment of the genetic variability and structure of nematode populations in a given area (Williamson et al., 2009).

Different molecular markers have been used to study genetic variability for nematodes of the genus *Meloidogyne*, with emphasis on Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP), Simple Sequence Repeats (ISSR), the mitochondrial COI gene (mtCOI) and, more recently, Genotyping By Sequencing (GBS). The genetic markers are efficient to differentiate the common tropical root-knot nematode species (*M. incognita*, *M. arenaria*, and *M. javanica*), and, also, the intraspecific genetic variability of *M. javanica* (Mattos, et al., 2016; Medina et al., 2017; Rashidifard et al., 2018; Santos et al., 2018). However, limited information is available on the origin, genetic variation, and evolutive mechanism that influence *M. javanica*, especially due to the existent studies were conducted using a restricted number of isolates. To current knowledge, only five studies

focused on understanding the genetic variability of *M. javanica* (Mattos, et al., 2016; Medina et al., 2017; Szitenberg et al., 2017; Rashidifard et al., 2018; Santos et al., 2018). In a general scenario, considerable polymorphism is related; however, most studies indicate a low genetic variability in populations of *M. javanica* at the continental or regional levels using RAPD markers. Santos et al. (2018) characterized the variability of this species on samples collected from diverse vegetables in sub-Saharan Africa and Latin America (Brazil and El Salvador). The Latin American populations were compared with isolates of African origin and low polymorphism was observed among populations from the two continents. The low variability among isolates of the African and American continents can be explained by the possible origin of the genus *Meloidogyne* while the Earth was a supercontinent (Poinar et al., 2008). At the regional level, 37 populations of *M. javanica* were collected also on vegetables in seven distinct sites in the southern region of Iran (Ghaderi et al., 2020). A total of four clades was observed without correlation with geographical region and host plants. Despite the importance of *M. javanica* to Brazilian agriculture, only two investigations were performed, in soybean and in potato (Mattos, et al., 2016; Medina et al., 2017). Similar to previous works, Medina et al. (2017) reported 14% of polymorphism in 17 populations of nematodes affecting potatoes collected in Rio Grande do Sul, Santa Catarina, and Paraná states. In another study conducted in soybean plants, even comparing the variability of *M. javanica* collected in native areas of Cerrado and cultivated areas, a small variability was observed. (Mattos et al., 2016)

Robust studies employing a larger number of isolates collected in different regions and hosts need to be carried out to obtain a better understanding of the real variability of the pathogen, the evolutionary mechanisms that influence the breakdown crop resistance and the genetic structure of the population. In this context, it was assessed the genetic variability of *M. javanica* on a microscale of ~ 250 km using the RAPD marker on isolates sampled in ten municipalities localized in two important agricultural poles of sugarcane production of - the north and northeast regions of São Paulo state.

MATERIALS AND METHODS

Populations and DNA extraction

A total of 23 pure populations belonging to the nematode collection of the University of Brasília were analyzed in the study. The isolates were obtained from root and soil collected in sugarcane fields with history of root-knot disease caused by *M. javanica* (Furtado et al.,

unpublished). The isolates were collected in 10 municipalities located in the north (N) and northeast (NE) regions of São Paulo state. Isolates from the N region were collected in Tanabi (UnBMj20), Barretos (UnBMj22 and UnBMj23), and Guapiaçu (UnBMj26 and UnBMj27). Samples of NE region were obtained in Altinópolis (UnBMj1, UnBMj2 and UnBMj3), Serrana (UnBMj4, UnBMj5 and UnBMj6), Brotas (UnBMj7, UnBMj8, UnBMj10 and UnBMj11), Descalvado (UnBMj13), São Carlos (UnBMj16 and UnBMj18), Pirassununga (UnBMj28, UnBMj29 and UnBMj30), and Analândia (UnBMj34 and UnBMj36) (Table 1).

These municipalities are responsible for 5% of the state's total sugarcane production (IBGE, 2017). The pure populations were kept in tomato (*Solanum lycopersicum* L. cv. Santa Clara) due to the high nematode multiplication in this susceptible host. After two months, eggs were extracted from tomato roots following the methodology described by Carneiro et al. (2004). Genomic DNA of pure populations was obtained from 200 to 300 µl of nematode eggs extracted by the phenol-chloroform methodology (Randig et al., 2002). The genomic DNA was quantified and stored at -20 °C.

The SCAR primer species-specific Fjav (GGTGCGCGATTGAACTGAGC) and Rjav (CAGGCCCTTCAGTGGAACTATAC) of *M. javanica* were used to confirm the identification of species (Zijlstra et al., 2000). The PCR reactions were performed in the PTC-100 Thermal Cycler, MJ Research with a final volume of 25 µl using 12.5 µl of DreamTaq Green PCR Master Mix (Thermo Scientific Fermentas Ltd), 1 µl of each primer (10 pmol/µl), 1 µl of genomic DNA (~30 ng) and 9.5 µl of water MilliQ. The amplifications included a negative control without template DNA. The amplifications were conducted using cycling conditions of 94 °C for 2 min, followed by 45 cycles at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 60 s, with a final cycle of 72 °C for 7 min. The amplification was confirmed by electrophoresis in a 1.0% (w/v) standard TAE buffer agarose gel, stained with GelRed (Biotium®) and visualized under UV light. Amplification products generated were compared with a 100 bp ladder.

Genomic fingerprint

RAPD analysis was performed using a set of 30 random 10 mer oligonucleotide primers (Table S1), similar to other studies of genetic variability of *Meloidogyne* species (Tigano et al., 2010; Santos et al., 2012). The RAPD reaction was conducted according to the methodology described by Randig et al. (2002). Briefly, the PCR reaction was performed in a final volume of 18.7 µl containing 9.3 µl of DreamTaq, 6.9 µl of water MilliQ, 0.9 µl of each

primer (10 mM), and 1.5 µl of genomic DNA. PCR amplification was carried out at an initial denaturation at 94 °C for 5 minutes, 35 cycles containing the step of denaturation (94 °C for 30 s), annealing (36 °C for 45 s) and elongation (72 °C for 2 min), and a final extension of 72 °C for 10 min. The PCR reaction was performed in a PTC-100 thermocycler, MJ Research. PCR reactions were performed for each primer separately and repeated twice to demonstrate the reproducibility of the bands. The amplified bands were separated by electrophoresis on 2% agarose gel at 100 V for 2.5 h, stained with ethidium bromide and photographed under UV light. The bands were manually recorded as present (1) or absent (0) for each primer on a concatenated data matrix.

Population structure and differentiation

Initially, discriminant analysis of principal components (DAPC) and Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) trees generated based on Euclidean distances with 1,000 bootstraps were used to examine the clustering of genetically similar individuals. The analyses were constructed in R package *adegenet* v. 2.0.1 and *ade4* v. 1.7-5, respectively (Excoffier et al., 1992; Dray and Dufour, 2007; Jombart, 2008; Jombart et al., 2010). The genetic differentiation between municipalities and subpopulations previously separated by DAPC and UPGMA was computed using the pairwise fixation indices (F''_{ST}) in the GENODIVE (Meirmans and Hedrick, 2011). Analysis of molecular variance (AMOVA) was performed based on Euclidean distance in R package *poppr*. AMOVA estimated the variance of individual samples within the population or among populations.

The Mantel test was used to evaluate the relationship between genetic and geographic distance (km). The genetic distance between population pairs was compared using the F''_{ST} parameter. The geographic distance between sampling locations was measured using the R package *geosphere* (Karney, 2013). If there is no transit of infected material between areas, we would expect a significant pattern of isolation by distance. Conversely, no relationship between genetic and geographic distance would be expected if the populations were translocated. Beside, the movement rate between geographic regions and cities was estimated using the equation $F_{ST} = 1/(1+4Nm)$.

Genotyping analysis and random-mating

The general descriptors of genetic variability were analyzed by municipality and subpopulations. The clonality of multilocus genotypes was calculated by the formula: 1-[(number of different genotypes)/(total number of isolates)] (Zhan et al., 2003). The genotype richness (N0), exponential of Shannon's entropy (N1), and the inverse of the Simpson's concentration indices (N2) were assessed by Hill's numbers in R package *iNEXT* (Chao et al., 2014; Hsieh et al., 2016). A confidence interval of 95% was employed to compare the variability of different populations. The analysis of Hill's numbers allowed the comparison of populations with different sizes due to the rarefaction/extrapolation of sampled numbers (Colwell et al., 2012). Shannon-Wiener diversity (H) and Gene diversity and evenness index (E5) was calculated for each population using the R package *poppr* (Kamvar et al., 2014). With a similar R package, linkage disequilibrium was calculated to test the nonrandom association of alleles at different loci through the *rD* index.

RESULTS

Meloidogyne javanica isolates and RAPD reproducibility

We confirmed that all the 23 isolates belong to *M. javanica* species with the amplification of a PCR product of 670 pb using the SCAR species-specific primer Fjav/Rjav (Figure S1). The PCR reaction without template DNA was not amplified. The RAPD-PCR products of 30 random oligonucleotide primers produced a total of 216 bands with a size between 300 to 6,500 bp (Table S1). A total of 44.4% of polymorphic bands were detected. The banding pattern of RAPD remained consistent in both rounds of PCR.

Population structure of *M. javanica* in São Paulo State

The individuals collected in sugarcane fields clustered in two well-defined groups in DAPC and UPGMA (Figure 1A and S2). The genotypes clustered similarly in both analyzes with a total of 12 and 11 isolates allocated to subpopulation 1 and 2, respectively. Isolates collected in the same municipalities were detected in both groups. The first group was composed of isolates collected in six municipalities of NE (Altinópolis, Serrana, Brotas, São Carlos, and Pirassununga) and two of N region (Barretos and Guapiaçu) (Figure 1B and S2). The second group included isolates collected in all municipalities sampled, except Analândia (NE). The isolates collected in cultivar RB96-6928 were grouped into Subpopulation 1

(UnBMj1, UnBMj2, UnBMj7, UnBMj10, and UnBMj11) and Subpopulation 2 (UnBMj3 and UnBMj8). The same fact occurred with individuals sampled in cv. RB867515 (UnBMj16 and UnBMj18) (Figure S2). We did not get information on the cultivars planted in the other fields, making it difficult to compare the results. Therefore, no signal of population structure by municipality, or cultivar was observed in both grouping analyses.

No genetic differentiation was observed among municipalities. The value of the index F''_{ST} ranges from 0 to 1, being that the grade 0 means no genetic difference between the populations and the value 1 to populations that are fully distinct. The F''_{ST} value varied from 0.08 comparing the genetic difference between Serrana and Brotas with Altinópolis to 0.2 between Pirassununga and Altinópolis (Table 2). A moderate genetic difference was observed in Pirassununga compared with Altinópolis. Conversely, no genetic structure was observed in the other comparations (Table 2 and Figure 2). The nematode genetic structure of Analândia, Tanabi, Descalvado, São Carlos, Barretos, and Guapiaçu was not analyzed due to the reduced number of isolates collected in these municipalities. Regarding subpopulations, also, a moderate genetic structure was observed between subpopulation 1 and 2 (F''_{ST} = 0.23, P <0.05). The AMOVA analysis supports the evidence of the absence of *M. javanica* population structuration by municipalities (7.17%) and the relevance of subpopulations (21.6%) (Table 3). However, the higher molecular variance of species occurs within species (78.39-92.82%). No structure population was observed between N and NE regions (F''_{ST} = 0.007).

Although the occurrence of the same haplotype was not observed in different municipalities, immigrants was detected in between Serrana and Altinópolis (2.88), Brotas and Altinópolis (3.32), Pirassununga and Altinópolis (1), Pirassununga and Serrana (2.88) and Pirassununga and Brotas (1.53) (Table 2). Therefore, isolates with minor genetic differences are not necessarily geographically close to each other.

Genotyping and random mating

Of a total of 23 isolates of *M. javanica*, the same number of haplotypes were identified. In this way, the clonal fraction (1) and evenness (E5 = 1) were similar to all municipalities and subpopulations (Table S2). Similar to the F''_{ST}, the E5 index ranges from 0 to 1, being the value 0 when there is the predominance of same genotypes and low frequency of other genotypes, and 1 to the same frequency of all genotypes. The general descriptor was analyzed only for populations with a minimum number of three isolates. The Shannon-Wiener index showed intermediate diversity in Pirassununga, Serrana and Altinópolis (1.09) and

Brotas (1.38) and high diversity in subpopulation 1 and subpopulation 2 (2.48 and 2.40) (Table S2). A higher gene diversity (Hexp) was obtained in Pirassununga (0.26) followed by Brotas (0.21), Serrana (0.17), and Altinópolis (0.14) (Table S2). The gene diversity was higher in subpopulation 2 (0.23) than in subpopulation 1 (0.18). Similar genotypic diversity and richness were detected in municipalities and subpopulations (Figure 3 and S3). For six of the municipalities, the diversity and genotypic richness cannot be inferred due to the low number of samples. There is no evidence of linkage disequilibrium in the subpopulations 1 and 2 ($P < 0.05$). The gene diversity ranged from 0.08 to 0.37. Markers AB03, AB04, and AQ12 had the greatest diversity (0.37) followed by AS08 and E15 (0.35), D13 (0.34), and N07, N10, and P05 (0.33) and E18 (0.30). Markers with median values were B11 (0.25), A7 (0.23), A10 (0.24) and G05 and G06 (0.21). The other markers had low values ranging from 0.08 to 0.18 indicating little diversity (Table S1).

DISCUSSION

Understanding the distribution, genetic variability, and factors that may delimit subpopulations of a given species is fundamental in several aspects of plant parasitic nematode disease management. Studies on variability of *M. javanica* are very scarce, none of them in sugarcane. Here, we sought to clarify the genetic variability of *M. javanica* in the state of São Paulo, the largest sugarcane producer globally. We found that each individual is a genotype and the population present high genetic variability. Moreover, we identified that the population is structured in two subpopulations, with similar variability, dispersed along the study area, without any apparent spatial or biological correlations, *i.e.*, with no indication of population structure by geographic region, cultivar, biome, rainfall and soil type.

In previous studies, a reduced number of polymorphic bands was found in isolates of *M. javanica* collected in Brazil and Iran, the percentage of polymorphic bands was between 24.6% (Mattos et al., 2016) and 26.7% (Ghaderi et al., 2020). Moreover, a comparative genomic analysis also showed that *M. javanica* displays low genetic diversity, even though using only five isolates they were collected in distinct continents (Morocco and USA) (Szitenberg et al., 2017). In contrast, we observed an elevated number of clones and a higher percentage of polymorphic bands (44.4%). Direct comparison of variability should be limited because we investigated six times more isolates than the soybean Brazilian study and analyzed three times more markers than the Iranian population (Mattos et al., 2016; Ghaderi et al., 2020). Such a high genotypic diversity led to speculate the occurrence of sexual meiotic

recombination of population or influence of transposon elements (TE). Actually, apomict nematodes could occasionally produce male individuals; it was observed the mating and spermatozoa into spermatothecae of females, but the sperm nucleus was always degraded (Triantaphyllou, 1981). Crucially, it was not observed any male (data not shown) and the data rejected the occurrence of recombination (Table S2). On the other hand, Kozlowski et al. (2020) documented the influence of TE as the primary source of genomic variation and adaptability of *M. incognita*, a closely related root-knot species. *Meloidogyne javanica* genome is rich in transposon and their movement can induce genome rearrangement and lead to an increase in gene copy number, as well as the creation or reversion of genome sequences (Blanc-Mathieu et al., 2017).

For pathogens that have limited dispersal, genetic similarity is expected to decrease with increasing geographic distance, however this was not observed in isolates of *M. javanica* collected in São Paulo state. The absence of genetic and spatial correlation and wide distribution of lineages can be explained by the dispersion of nematodes alone or in infected propagative material. Although this nematode is a soil inhabitant, *M. javanica* can easily be dispersed, in short, or long distances, by fresh and seawater, strong winds, infested soils, propagative material, agricultural machinery and implements, movements of animals and people in the area (Martin, 1969). Other studies have also demonstrated the movement of *Meloidogyne* isolates between regions. Shao et al. (2020) observed that the same haplotype of *M. enterolobii* was sampled in Guangdong, Guangxi, and Hunan Provinces in China. In sugarcane, probably, the introduction of new *M. javanica* variants into new areas occurred through stalks or pre-sprouted seedling or the introduction of sugarcane in a new area already contaminated with *Meloidogyne*. The dispersion of *Meloidogyne* spp. by propagative material is widely known. Hamza et al. (2017) demonstrated that the presence of *M. javanica* in olive orchards is directly related to contaminated substrates of nurseries in Morocco. Short distances, as analyzed in this work, allow more intense gene flow, consequently preventing regional genetic differentiation. Moreover, Ghaderi et al. (2020) also found *M. javanica* isolates subgroups without correlation with host plant or distance. In contrast, Asamizu et al. (2020) stated that the host can be a determining factor in *M. incognita* genomic diversity.

Two models explain the existence of organisms with parthenogenetic reproduction: the general-purpose genotype (GPG) and frozen niche variation (FNV). The first model suggests that the success of organisms is due to the presence of generalist lineages with good

fitness in different and variable environments (Vrijenhoek and Parker, 2009). The FNV model assumes the existence of clonal lineages with high specialization in specific sub-niches. Based on the data, the wide spatial distribution, subpopulation variability, and the lack of correlation with hosts, one can speculate that these two lineages were both adapted to different environments studied in São Paulo state. Unfortunately, the little knowledge on the lineage's origin, genomic structure of *M. javanica* regionally and at a global level prevents a complete understanding of the origin and interaction of these subpopulations.

Overall, the discovery of two lineages in São Paulo State is the first step to the breeding programs to evaluate the resistance of existing and new cultivars and to investigate differences in aggressiveness within these two lineages. Further studies must be carried out to determine whether these lineages are restricted to the state of São Paulo or widely distributed in Brazil and the world.

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TABLES AND FIGURES

Table1: Identification of *Meloidogyne javanica* isolates with their respective municipalities and geographic locations, as well as the cultivars planted in the sampled fields.

Isolates	Municipalities	Geographic locations		Cultivar
UnBMj1	Altinópolis	-21 01.93960'	-47 30.24308'	RB96-6928
UnBMj2	Altinópolis	-21 01.32313'	-47 29.95428'	RB96-6928
UnBMj3	Altinópolis	-21 01.72483'	-47 30.86490'	RB96-6928
UnBMj4	Serrana	-21 15.79481'	-47 38.40285'	*
UnBMj5	Serrana	-21 15.53034'	-47 38.53383'	*
UnBMj6	Serrana	-21 15.54855'	-47 38.79892'	*
UnBMj7	Brotas	-22 18.46100'	-48 11.48695'	RB96-6928
UnBMj8	Brotas	-22 18.09523'	-48 11.62475'	RB96-6928
UnBMj10	Brotas	-22 20.77967'	-48 01.99461'	RB96-6928
UnBMj11	Brotas	-22 20.41283'	-48 02.25690'	RB96-6928
UnBMj13	Descalvado	-21 51.20416'	-47 45.72910'	CTC96-1007
UnBMj16	São Carlos	-21 54.92862'	-47 47.09963'	RB867515
UnBMj18	São Carlos	-21 55.18749'	-47 47.14427'	RB867515
UnBMj20	Tanabi	-20 34.26207'	-49 38.46668'	*
UnBMj22	Barretos	-20 37.79759'	-48 47.68708'	*
UnBMj23	Barretos	-20 37.65800'	-48 47.53345'	*
UnBMj26	Guapiaçu	-20 44.77778'	-49 16.07483'	*
UnBMj27	Guapiaçu	-20 44.83121'	-49 16.20460'	*
UnBMj28	Pirassununga	-21 57.37465'	-47 30.07169'	*
UnBMj29	Pirassununga	-21 56.98409'	-47 30.13675'	*
UnBMj30	Pirassununga	-21 56.43809'	-47 30.23588'	*
UnBMj34	Analândia	-22 04.17966'	-47 39.94014'	*
UnBMj36	Analândia	-22 04.07880'	-47 39.87660'	*

*Unknown information

Table 2: Genetic differentiation (F''_{ST}), lower diagonal, and number of immigrants (Nm) estimated by $F''_{ST} = 1/(1+4Nm)$, upper diagonal, among isolates of *Meloidogyne javanica* collected in four Municipalities of São Paulo state estimated in Genodive.

Populations	Altinópolis	Serrana	Brotas	Pirassununga
Altinópolis	-	2.88	3.32	1
Serrana	0.08	-	0	2.88
Brotas	0.08	0	-	1.53
Pirassununga	0.20	0.08	0.15	-

Table 3: Analysis of molecular variance (AMOVA) of 23 isolates of *Meloidogyne javanica* sampled in São Paulo conducted to test grouping according to subpopulations and municipalities Altinópolis, Serrana, Brotas, São Carlos, Tanabi, Barretos, Guapiaçu, Pirassununga, and Analândia.

Source of variation	d.f.	Sum of squared deviations	Variance components	Proportion of variance components (%)
Subpopulation				
Variations between Subpopulation	1	49	3.24	21.6
Variation within samples	21	247.16	11.76	78.39
Total	22	296.16	15.01	100
Municipalities				
Variations between Municipalities	9	132.84	0.97	7.17
Variation within samples	13	163.33	12.56	92.82
Total	22	296.17	13.53	100

Table S1: RAPD primers used in this study with their nucleotide sequence, the number of bands generated, the size of the fragments, gene diversity and linkage disequilibrium.

Primer	Sequence 5'-3' (DNA)	Total number of locus observed	Band size range	Gene diversity	rD
A7	GAAACGGGTG	21	6000-350	0.23	-
A10	GTGATCGCAG	11	4000-650	0.24	0.23
A14	TCTGTGCTGG	3	1600-1000	0.09	-
AB02	GGAAACCCCT	14	3800-500	0.09	-
AB03	TGGCGCACAC	12	2800-550	0.37	0.07
AB04	GGCACGCGTT	10	3000-650	0.37	0.07
AQ12	CAGCTCCTGT	9	3000-850	0.37	0.07
AS08	GGCTGCCAGT	15	4000-650	0.35	-
B05	TGCGCCCTTC	7	4000-1000	0.13	-0.6
B11	GTAGACCCGT	8	3000-830	0.25	-0.05
C07	GTCCCGACGA	5	3000-1500	0.08	-0.04
C09	CTCACCGTCC	6	2500-500	0.16	0.45
D13	GGGGTGACGA	9	3000-575	0.34	0.17
E07	AGATGCAGCC	12	4000-500	0.15	0.12
E15	ACGCACAACC	9	3000-400	0.35	-
E18	GGACTGCAGA	9	5000-850	0.30	0.06
F06	GGGAATTCGG	6	2500-1300	0.09	1
G05	CTGAGACGGA	12	4000-750	0.21	0.2
G06	GTGCCTAACC	7	5000-500	0.21	0.2
G13	CTCTCCGCCA	11	3050-950	0.18	0.08
H01	GGTCGGAGAA	6	4500-400	0.18	0.08
J20	AAGCGGCCTC	11	3500-450	0.18	0.08
K07	AGCGAGCAAG	7	4800-500	0.18	0.08
K19	CACAGGCGGA	8	4800-550	0.18	0.08
M10	TCTGGCGCAC	10	4000-800	0.18	0.08
M20	AGGTCTTGGG	4	4200-800	0.18	0.08

N07	CAGCCCAGAG	4	5000-750	0.33	-
N10	ACAACTGGGG	5	3000-300	0.33	-
P05	CCCCGGTAAC	8	6000-1000	0.33	-
R07	ACTGGCCTGA	11	6500-550	0.14	0.46

rD; association index and multilocus estimate of linkage disequilibrium and *significant at P<0,05.

Table S2: General descriptors of variability of *Meloidogyne javanica* populations (genetic diversity indices, gene diversity and linkage disequilibrium) from four municipalities (Altinópolis, Serrana, Brotas and Pirassununga) and two subpopulations collected in São Paulo state.

	N	**Genotypes**	**R**	**E5**	**H**	**He**	**rD**
Altinópolis	3	3	0	1	1.09	0.14	-
Serrana	3	3	0	1	1.09	0.17	-
Brotas	4	4	0	1	1.38	0.22	-
Pirassununga	3	3	0	1	1.09	0.26	-
Total	**13**	**13**	**0**	**1**	**3.13**	**0.24**	**0.03***
Subpopulation 1	12	12	0	1	2.48	0.18	-
Subpopulation 2	11	11	0	1	2.40	0.23	-
Total	**23**	**23**	**0**	**1**	**3.14**	**0.23**	0.03*

N: Number of isolates; Genotypes: Number of genotypes; R: Clonal fraction; E.5: Evenness index; H: Shannon-Wiener Index of MLG diversity; G: Stoddart and Taylor's Index of MLG diversity; He: Gene diversity (Nei 1978), averaged over all loci and rD; association index and multilocus estimate of linkage disequilibrium and *significant at P<0,05.

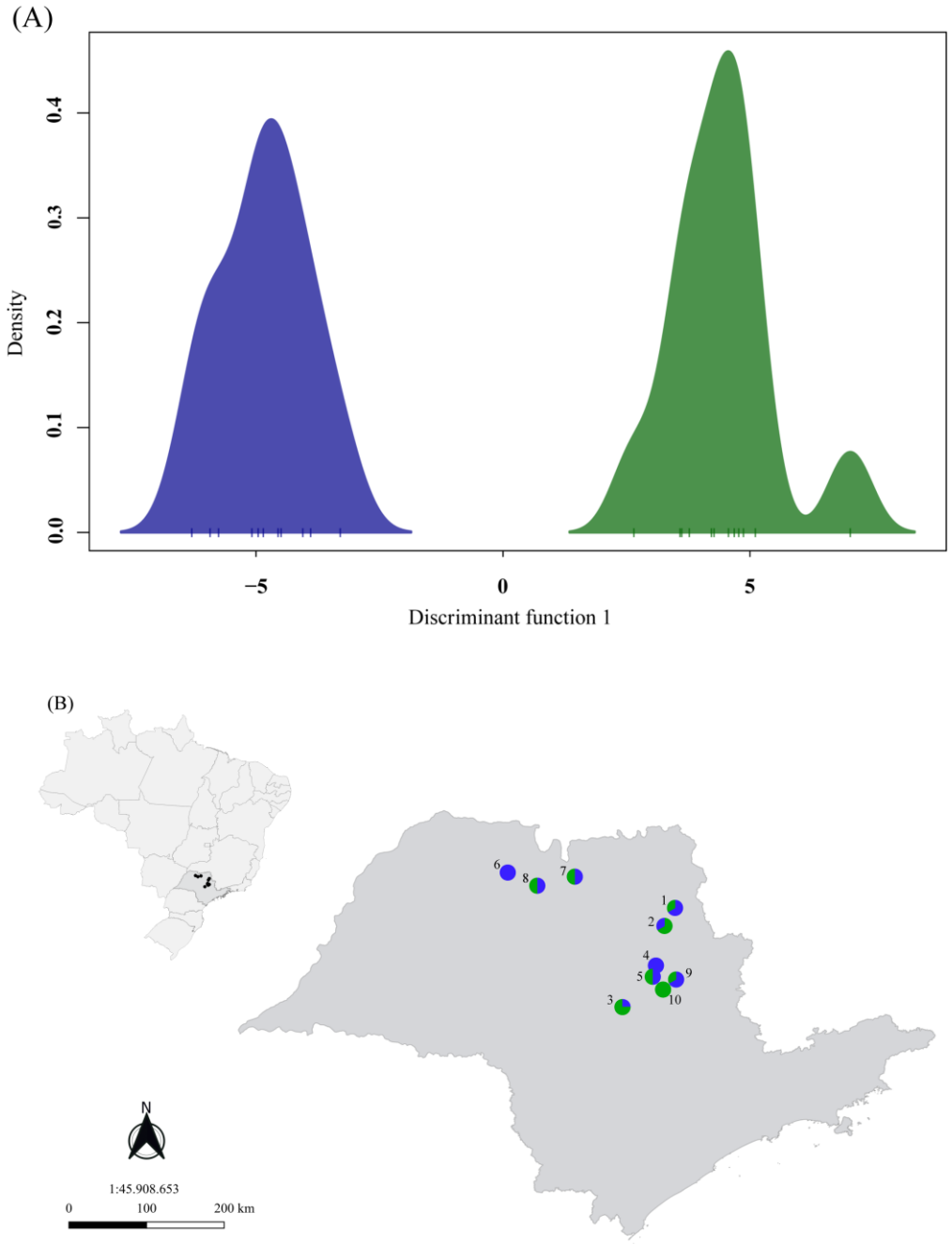

Figure 1: (A) Discriminant Analysis Principal Component (DAPC) of samples collected in the state of São Paulo. The scatter plot showed clustering into two subpopulation: subpopulation 1 (green) and subpopulation 2 (blue). **(B)** Geographic distribution of isolates clustered into two subpopulations. Isolates clustered in subpopulation 1 were found in (1) Altinópolis, (2) Serrana, (3) Brotas, (4) Descalvado, (5) São Carlos, (6) Tanabi, (7) Barretos, (8) Guapiaçu, and (9) Pirassununga. Isolates of subopulation 2 were found in (1) Altinópolis,

(2) Serrana, (3) Brotas, (5) São Carlos, (6) Tanabi, (7) Barretos, (8) Guapiaçu, (9) Pirassununga, and (10) Analândia.

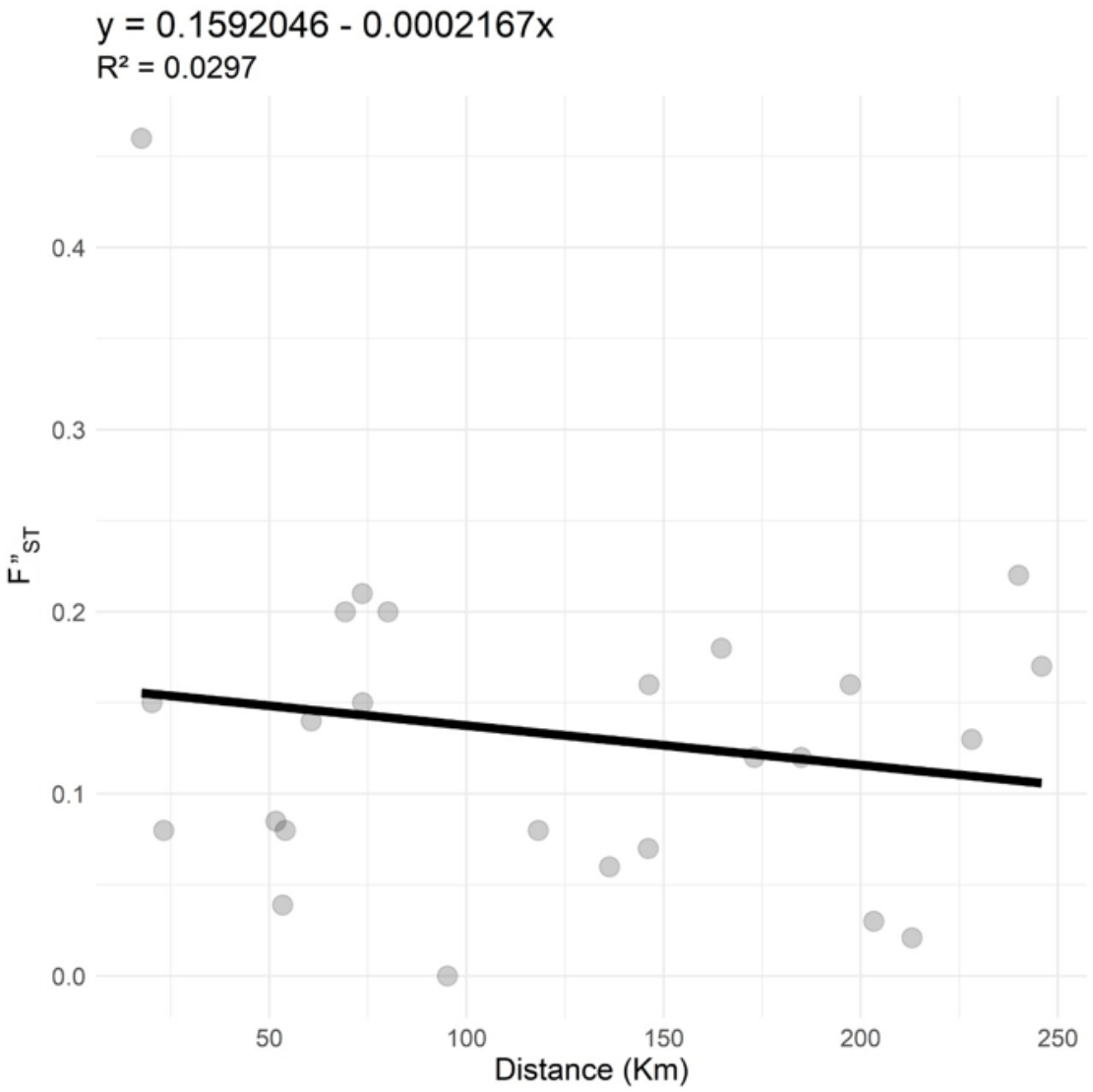

Figure 2: Mantel test for matrix correlation between genetic distance (F''_{ST}) and geographic distance of *Meloidogyne javanica* isolates collected in Altinópolis, Serrana, Brotas and Pirassununga, São Paulo.

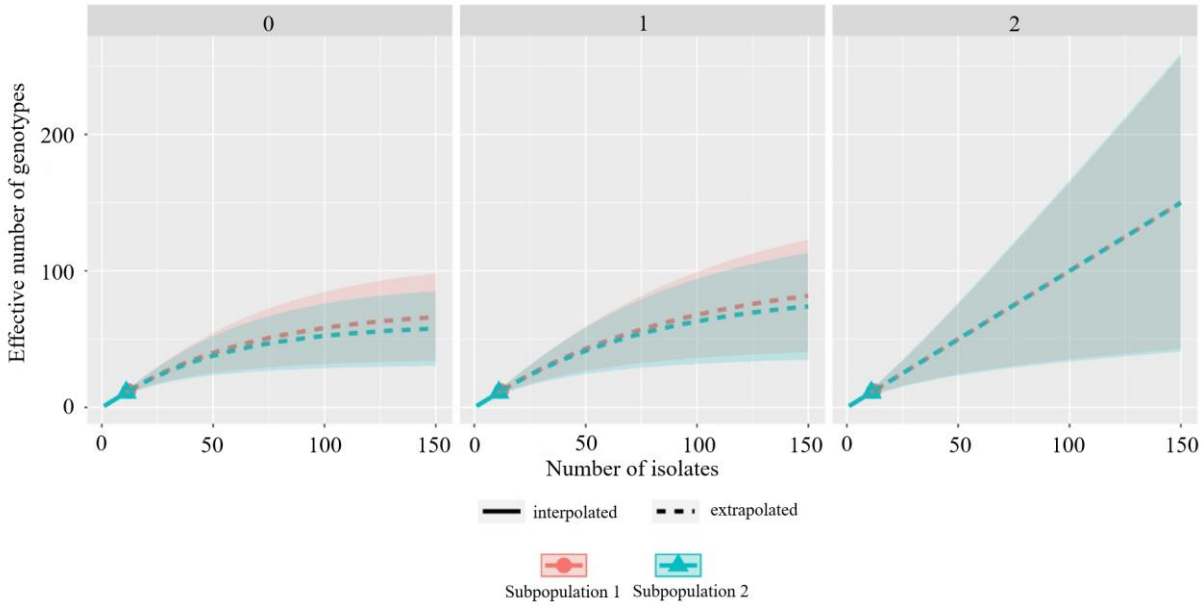

Figure 3: Diversity accumulation curves of Hill's number and effective number of genotypes order of 0 (N0), 1 (N1) and 2 (N2) of two subpopulation of *Meloidogyne javanica* collected in São Paulo state. The confidence interval is represented by shaded area.

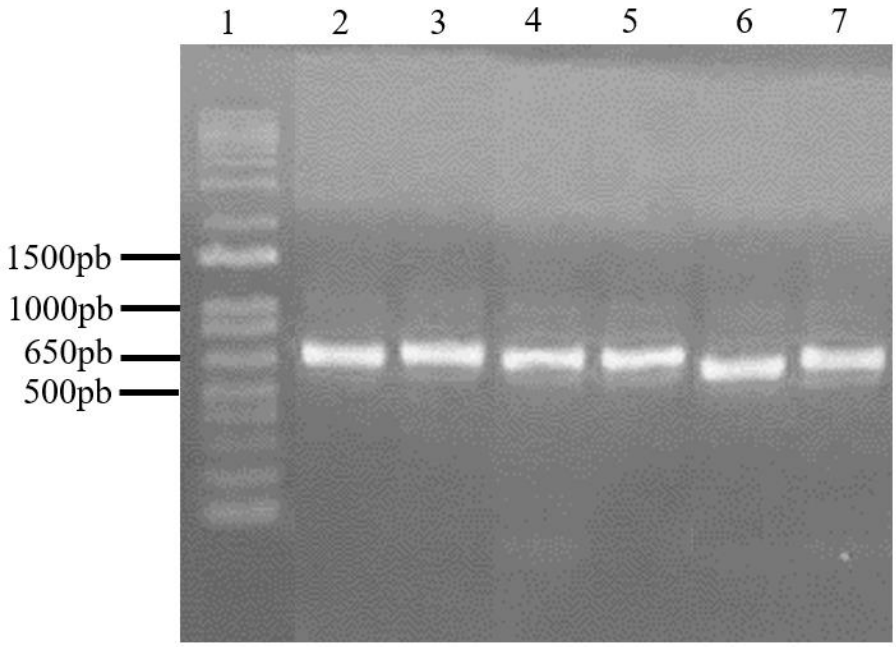

Figure S1: PCR amplification of genomic DNA of *Meloidogyne javanica* using Fjav and Rjav primers. 1=1kb DNA ladder; 2= isolate UnBMj1; 3= isolate UnBMj3; 4= isolate UnBMj4; 5= isolate UnBMj5; 6= isolate UnBMj6 and 7= isolate UnBMj7.

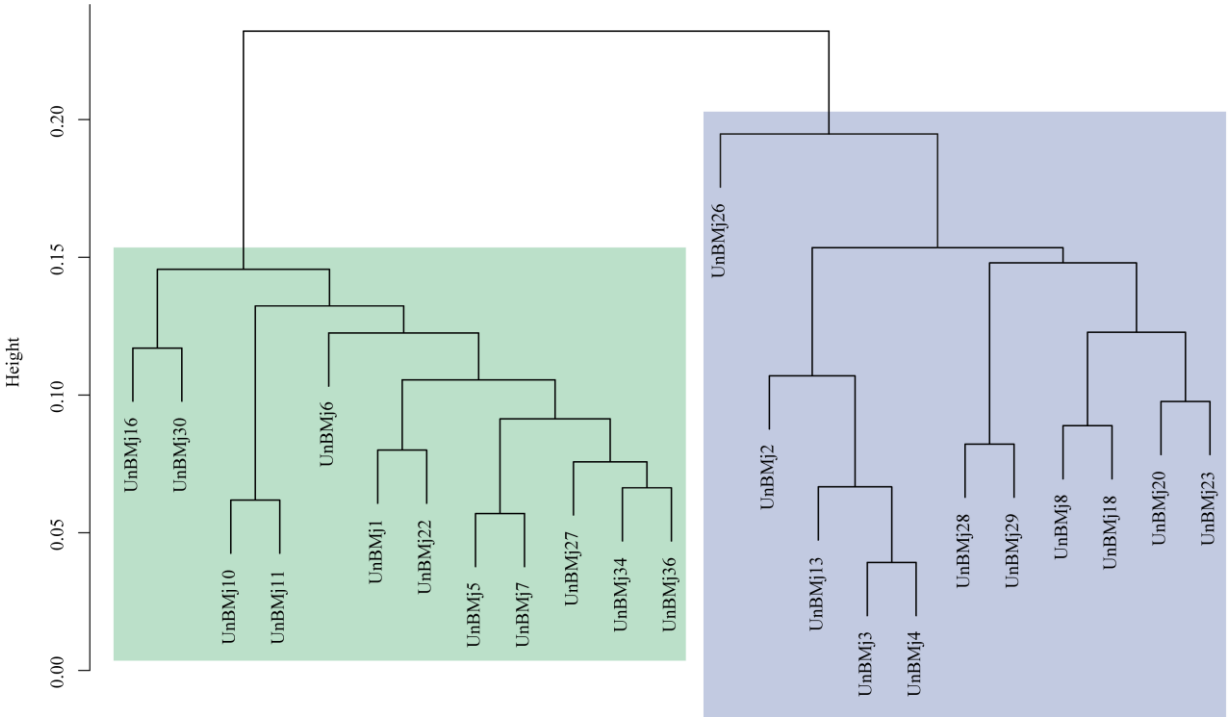

Figure S2: UPGMA dendrogram of Euclidean distance showing the genetic relationship among 23 isolates of *Meloidogyne javanica* collected in São Paulo state and the formation of two groups.

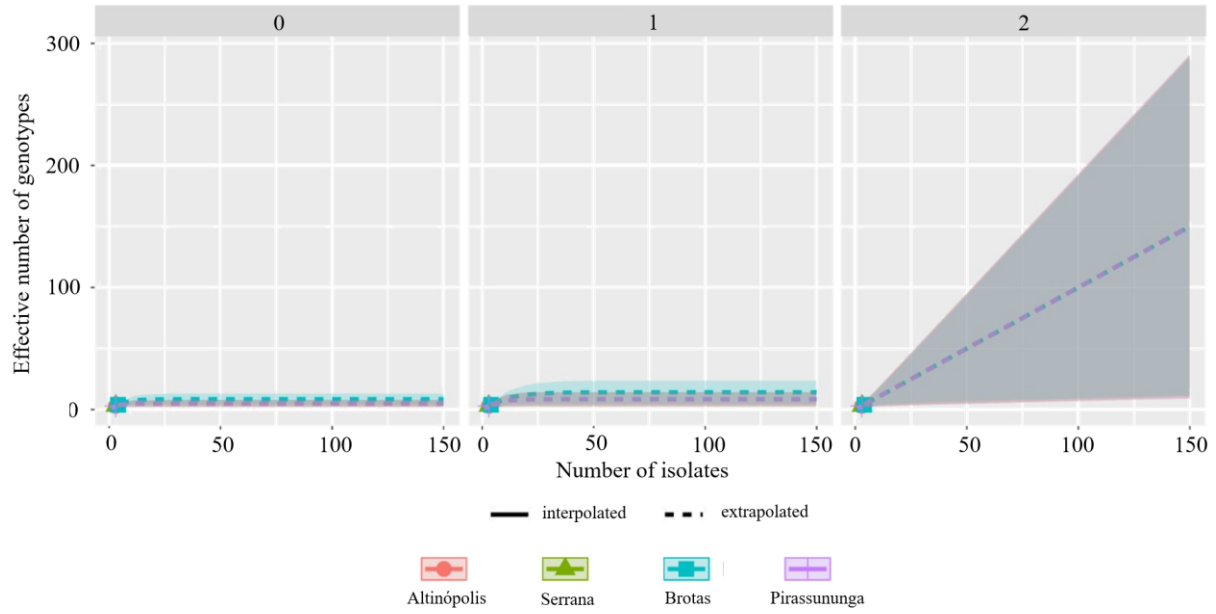

Figure S3: Diversity accumulation curves of Hill's number and effective number of genotypes order of 0 (N0), 1 (N1) and 2 (N2) of *Meloidogyne javanica* collected in four municipalities of São Paulo state. The confidence interval is represented by shaded area.