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Characterization of *Meloidogyne chitwoodi* and *M. fallax* populations from Switzerland

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Abstract

Meloidogyne chitwoodi and *M. fallax* are temperate-adapted root-knot nematodes (RKN) with exceptionally wide host ranges, including potato, tomato and other high-value crops, to which they inflict severe yield and quality loss. Both species are regulated as quarantine organisms in Switzerland. Recent detections of these nematodes have raised concerns about their introduction, establishment, and spread within Swiss agriculture. This study provides the first integrated characterization of one *M. chitwoodi* and four *M. fallax* populations from Switzerland. A newly discovered *M. chitwoodi* population was classified as Race 1, Roza pathotype through differential host assays and molecular marker analysis. Genetic relationships were further assessed with a multilocus barcoding approach targeting nuclear rRNA (18 *S*, 28 *S*, *ITS*), mitochondrial genes (*COI*, *COII*), and the 5–18 *S* intergenic spacer. RKN population sequences were analyzed together with global reference datasets to infer phylogenetic placement and putative origins. Phylogenetic analyses confirmed the identity of all Swiss populations. Intraspecific resolution was consistently low across all barcoding loci, indicating that standard multilocus barcodes have limited power to distinguish among populations of *M. chitwoodi* and *M. fallax*. Despite this limitation, the results contribute to a better understanding of the genetic diversity of *M. chitwoodi* and *M. fallax* in Switzerland and their relationship to global populations, supporting future surveillance and management efforts. These findings establish a genetic baseline for Swiss RKN surveillance, facilitate tracing of future incursions, and support the design of more effective phytosanitary and management strategies aimed at containing these economically important pests.

Keywords Quarantine pest, Plant-parasitic nematodes, Nematode race characterization, Pathotype roza, Molecular phylogenetics, Nematode diagnostics

1 Background

Root-knot nematodes (RKN; *Meloidogyne* spp.) are the largest and most economically damaging group of plant-parasitic nematodes (PPN), collectively parasitizing almost every species of higher plant and causing severe reductions in global food production [1–3]. RKN infestations can cause substantial yield losses in both field and greenhouse-grown plants, while aesthetic root defects decrease marketability and economic return of below-ground crops, even when overall yield losses are relatively modest [2,

4]. Within this genus, the temperate species *M. chitwoodi* and *M. fallax* have become particularly problematic in cool-climate production zones where potatoes, carrots and other root or tuber crops constitute major revenue streams [5, 6]. After hatching, infective second-stage juveniles (J2) enter host roots, migrate to the vascular cylinder and induce the formation of multinucleate giant cells that serve as permanent feeding sites [7]. Subsequently, the sedentary nematodes advance through the third and fourth developmental stages before reaching maturity as either sessile egg-laying females or mobile males. The nematode feeding behavior causes visible root galling, which can significantly impact plant physiology, leading to stunted growth, yield losses, reduced crop market quality and consequent rejection of the crop [7, 8].

The Columbia RKN, *M. chitwoodi* Golden, O'Bannon, Santo and Finley, 1980, was first described on potato (*Solanum tuberosum* cv. Russet Burbank) in the north-western United States [9]. While potatoes are among the most economically important hosts, *M. chitwoodi* is very polyphagous. As of 2025, the European and Mediterranean Plant Protection Organization (EPPO) lists 116 host species, including tomato, wheat, maize, onion, rape, beet, chili pepper, carrot, and barley [10].

The closely related *M. fallax* Karssen, 1996, also known as the false Columbia RKN, was first described in The Netherlands in 1992 on black salsify (*Scorzonera hispanica* L.), after initially being misidentified as *M. chitwoodi* [11]. Its host range largely overlaps that of *M. chitwoodi*, encompassing the same major crops [12]. Due to their broad host ranges and their capacity to infect economically important crops, both species cause severe economic losses. Consequently, they are listed as A2 quarantine pests by the EPPO [13] and classified in Switzerland [14] and several other countries as quarantine organisms. Within Europe, the presence of *M. chitwoodi* (*Mc*) and *M. fallax* (*Mf*) has been confirmed in Denmark (*Mc*), Sweden (*Mc*, *Mf*), The Netherlands (*Mc*, *Mf*), Belgium (*Mc*, *Mf*), Germany (*Mc*, *Mf*), France (*Mc*, *Mf*), Switzerland (*Mc*, *Mf*), Spain (*Mc*), Portugal (*Mc*), the UK (*Mf*) and, as a trans-continental state, Turkey (*Mc*). Outside Europe, *M. chitwoodi* and *M. fallax* occur in the United States, with first reports in 1980 and 2013, respectively [9, 15]. Ongoing range expansion is fueled by international trade and lapses in phytosanitary enforcement.

Variation in virulence and reproductive potential among *M. chitwoodi* and *M. fallax* populations directly influences sustainable management programs [16–18]. Consequently, race identification, pathotype testing and phylogenetic studies have been used to delineate races and pathotypes [19, 20]. For example, historical evidence suggests that *M. chitwoodi* may have been present in Europe and America much earlier than originally reported [9, 21], making its origin uncertain [22, 23]. Two distinct races of *M. chitwoodi* are currently recognized based on host differential tests: Race 1 (reproducing on *Daucus carota* (carrot) cv. Red Cored Chantenay) and Race 2 (on *Medicago sativa* (alfalfa) cvs. Sanditi and Thor) [2, 24, 25]. While both races are present in the USA [26], only Race 1 populations have been reported in Europe to date [27, 28]. *Meloidogyne chitwoodi* Race 1 can be further differentiated into the Roza pathotype, which commonly occurs in Europe and can propagate on *Solanum bulbocastanum* SB22, a wild potato species known to harbor resistance to *M. chitwoodi* and late blight [29, 30].

In Switzerland, *M. fallax* was first morphologically described in 2002, followed by *M. chitwoodi* in 2003, both from greenhouse soil samples collected in the canton of Valais, south of the Alps [31]. Since then, *M. fallax* has been detected, north of the Alps, in

greenhouses in Bern (2019) and Aargau (2023), as well as two new sites in Valais in 2023. The first *M. chitwoodi* detection north of the Alps occurred in 2021 in an agricultural field in Bern [32]. Despite stricter regulations after these detections, little is known about the genetic structure, origin(s) and potential diversity of Swiss populations. Such knowledge is essential for tracing invasion pathways and tailoring containment measures.

Therefore, this study aimed to characterize the currently known Swiss populations, including one *M. chitwoodi* population and four *M. fallax* populations. Race identification for *M. chitwoodi* was conducted using host differential testing, and both species were analyzed through multilocus phylogenetic approaches based on nuclear and mitochondrial markers. The resulting dataset provides the first genetic baseline for *M. chitwoodi* and *M. fallax* in Switzerland, facilitates comparison with global collections, and supports ongoing surveillance and quarantine programs.

2 Results and discussion

2.1 Morphological and molecular characterization

Morphological analysis of J2 of *M. chitwoodi* and *M. fallax* was performed as a preliminary assessment to support subsequent molecular identification, with emphasis on tail shape and hyaline tail region. Both species exhibited a clearly visible hyaline terminus, a critical feature for identification. *Meloidogyne fallax* J2 exhibited a typical conical hyaline tail, whereas *M. chitwoodi* displayed a distinctly segregated hyaline tail tip (Fig. 1), supporting their differentiation based on tail morphology.

In accordance with EPPO protocols [34], which describes the hyaline tail of J2 as 12–16 µm long for *M. fallax* and 8–12.5 µm long for *M. chitwoodi*, the hyaline tail length was measured for the Swiss populations of both species. Although several morphological characters can be used for species identification, only these traits were selected for quantitative assessment in this study, followed by molecular analysis. The average hyaline tail length was 13.8 µm for *M. fallax* and 10.7 µm for *M. chitwoodi* ($n = 8$). These values fall within the respective diagnostic ranges and are consistent with species-specific morphological descriptions. A Student's t-Test confirmed the statistical significance of the observed difference in hyaline tail length between the two species ($p = 6.55 \times 10^{-4}$), supporting their morphological separation, as seen in Supplementary (Fig. S1). These morphological observations were finally validated by molecular diagnostics targeting the intergenic spacer region between the *5 S* and *18 S* genes. PCR amplification yielded distinct amplification products: *M. chitwoodi* at 540 bp, *M. fallax* at 670 bp, and the *M. hapla* control at 440 bp (Fig. 2). These results are consistent with Wishart et al. (2002) [35] and confirm the identification of one *M. chitwoodi* and four *M. fallax* populations in Switzerland.

3 Race and pathotype identification of the Swiss Meloidogyne chitwoodi population

Host-differential assays were conducted to determine the race status of the Swiss *M. chitwoodi* population. *Meloidogyne chitwoodi* Race 1 is known to occur on carrot cv. Red Cored Chantenay but not on alfalfa cvs. Sanditi or Thor, whereas Race 2 shows the reciprocal phenotype [2, 24, 25]. In controlled infection assays, the Swiss population reproduced successfully on carrot but not on alfalfa, thereby identifying it as Race 1. This is

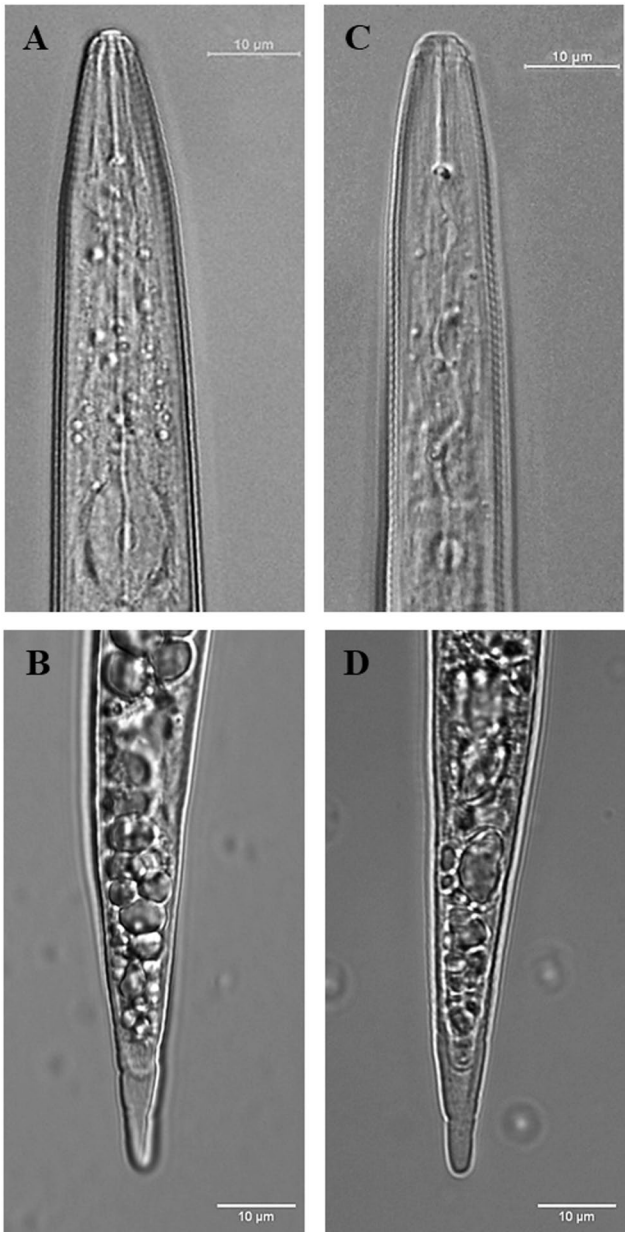


Fig. 1 General morphology of *Meloidogyne chitwoodi* and *Meloidogyne fallax* juveniles from newly sequenced Swiss populations. **A** Anterior end of *M. chitwoodi* J2; **B** Tail region of *M. chitwoodi* J2; **C** Anterior end of *M. fallax* J2 from the MFV population; **D** Tail region of *M. fallax* J2 from the MFV population. Light microscopy, 100× magnification. Scale bars at 10 µm. Pictures were prepared for publication with Fiji (ImageJ; [33])

consistent with field observations from 2021, where severe damage occurred on carrot crops. Interestingly, in the initial 7-week Falcon tube experiment, a marked difference in egg development was observed between carrots and tomato control. Egg development of *M. chitwoodi* was significantly delayed on carrot roots compared to tomato, suggesting differential host suitability or temporal variation in nematode development (Fig. S2). Based on these observations, the pot experiment was extended over 8 weeks (corresponding to 840 degree-days). In alfalfa, no reproduction was observed, while measurable reproduction occurred in both carrot (Rf = 1.3) and tomato (Rf = 3.1), with Rf significantly higher in tomato than in carrot (Student's t-Test, $p = 0.006$), confirming the

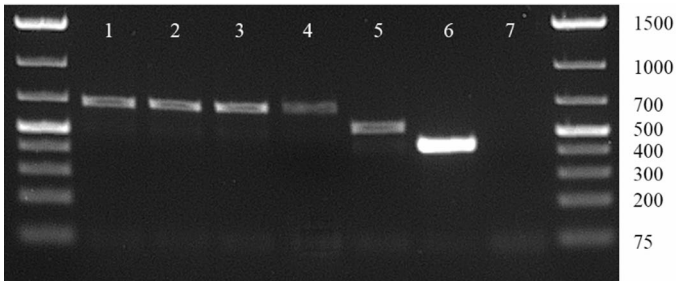


Fig. 2 PCR amplification of the intergenic spacer between 5 S and 18 S rRNA in *Meloidogyne* species. Amplified products include *M. fallax* (670 bp) from populations: Bern (lane 1), Valais-1 (lane 2), Valais-2 (lane 3), and Aargau (lane 4); *M. chitwoodi* (540 bp) from population Bern (lane 5); *M. hapla* control (440 bp, lane 6). Water was used as negative control (lane 7). PCR was performed using primers JMV1, JMV2, and JMVhapla. A 1 kb Plus DNA ladder was used as a molecular size

Table 1 Race identification of the Swiss *Meloidogyne chitwoodi* population using differential host cultivars

Meloidogyne chitwoodi	Daucus carota cv. Red Cored Chantenay	Medicago sativa cv. Sanditi	Solanum lycopersicum cv. moneymaker
Egg masses	35.13 ± 14.89	0	36.20 ± 9.68
Eggs and J2	6392.17 ± 1800.11	0	15658.21 ± 2006.21
Eggs and J2/ Egg mass	181.98	–	432.55
Reproduction factor (Rf)	1.27	–	3.13*

Values are based on eight replicates per host species ($n = 8$)

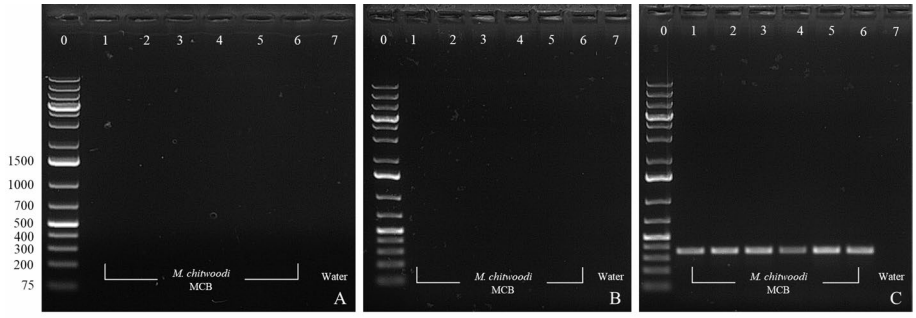
Differences in reproduction factors (Rf) between carrot and tomato were tested using a Student's t-Test ($\alpha = 0.05$)

*Rf differs significantly between carrot and tomato ($p = 0.006$).

Host plants include *Daucus carota* cv. Red Cored Chantenay, *Medicago sativa* cv. Sanditi, and the positive control *Solanum lycopersicum* cv. Moneymaker

inability of the Swiss *M. chitwoodi* population to propagate on alfalfa and supporting its classification as Race 1 (Table 1). Although the number of egg masses per root system was comparable between carrot (35.13 ± 14.89) and tomato (36.20 ± 9.68), the total recovery of eggs and J2 was significantly higher in tomato (15,638 ± 2006) than in carrot (6392 ± 1800). Consequently, the average number of J2 and eggs per egg mass was significantly greater in tomato (432.55) than in carrot (181.98) (Table 1).

This difference may reflect variation in host suitability, generation time, or nutrient availability in tomato versus carrot roots; however, this interpretation remains hypothetical and would require targeted experiments to confirm. Interestingly previous studies have demonstrated that the number of eggs per egg mass in *M. chitwoodi* decreases with increasing host plant age [36]. Although plant age was not assessed in this study, it may have influenced the results, and its potential impact on nematode reproductive output cannot be excluded. Nonetheless, the absence of reproduction on alfalfa and successful reproduction on carrot confirm that the Swiss *M. chitwoodi* population belongs to Race 1. The use of ROZMR primers (Fig. 3) produced a 360 bp amplification product for all the tested samples confirming the classification of the Swiss *M. chitwoodi* population as Race 1 and, more specifically, as pathotype Roza. This indicates its capacity to overcome the SB22-derived resistance gene in commercial potato cultivars [37]. The detection of the Roza pathotype has direct practical impact, as resistance sources based on *Solanum bulbocastanum* SB22 may not remain effective where this pathotype occurs. This highlights the need to verify the performance of SB22-derived resistant cultivars under



Each gel is left to right: Ladder (0), juveniles hatched from young tomato plants from resistance test (1) (2) and (3), juveniles hatched from older tomato plants from propagation moneymakers (4) (5) and (6), water negative control (7), n=2.
A has primers that amplify Race 1 *M. chitwoodi*, B has primers that amplify Race 2 *M. chitwoodi* and C has primers that amplify Race 1 pathotype Roza ($Race1_{Roza}$).
Primers for *M. chitwoodi* $Race1_{Roza}$ show an amplification product at 360 bp, consistent with Hu *et al.* 2023

Fig. 3 Molecular race identification of *Meloidogyne chitwoodi* using SCAR markers and agarose gel electrophoresis. Three PCRs were run using specific primer pairs targeting Race 1 (A: MC1MR), Race 2 (B: MC2MR) and Race 1 pathotype Roza (C: ROZMR) [28]. DNA replicates were performed for each reaction ($n = 6$). All tested nematode samples produced a 360 bp band in the ROZMR PCR (C), indicating the presence of the Roza pathotype. A 1 kb Plus DNA ladder was used as a molecular size marker for the agarose gels. Water was included as a negative amplification control

local conditions and to include pathotype-specific diagnostics in routine surveillance. Targeted monitoring for resistance-breaking populations should therefore be integrated into Swiss RKN management programs when *M. chitwoodi* is detected.

To further validate this identification, the PCR product amplified with the ROZMR primers was sequenced and used as a query in a BLAST search on the NCBI database. While the core nucleotide database yielded no matches, a search of the whole-genome shotgun contigs database revealed 100% query coverage and 100% identity match with the *M. chitwoodi* Race 1 pathotype Roza (JACZZN010000019.1, [38]), thus confirming the molecular identification of the Swiss population.

These findings emphasize the importance of integrating non-host crops, such as alfalfa, into crop rotations to mitigate nematode spread. Interestingly, all *M. chitwoodi* populations described in Europe to date, including the Swiss population, have been identified as Race 1 [39].

4 Phylogenetic reconstruction

To identify potential genetic variation between geographically distinct populations, a phylogenetic analysis was conducted on Swiss populations of *M. chitwoodi* and *M. fallax*. Six barcoding regions were sequenced and submitted to the NCBI database: the nuclear 18 *S*, and 28 *S* ribosomal RNA genes, *ITS*, the mitochondrial *COI* and *COII*, and the intergenic spacer region between 5 *S* and 18 *S* rRNA genes, referred to as JMV in this work (Table S1). These sequences were then compared against global *Meloidogyne* populations for a comprehensive analysis.

A multigene phylogenetic tree was constructed using concatenated sequences from 18 S, 28 S, ITS, and COI regions (Fig. 4). Reference sequences for *Meloidogyne* species were retrieved from NCBI, following the methodology outlined by Álvarez-Ortega et al. [3]. *Pratylenchus vulnus* and *P. penetrans* were used as outgroups to root the tree. The resulting phylogenetic tree revealed three main clusters, consistent with previous phylogenetic studies of the genus *Meloidogyne* [3, 40, 41].

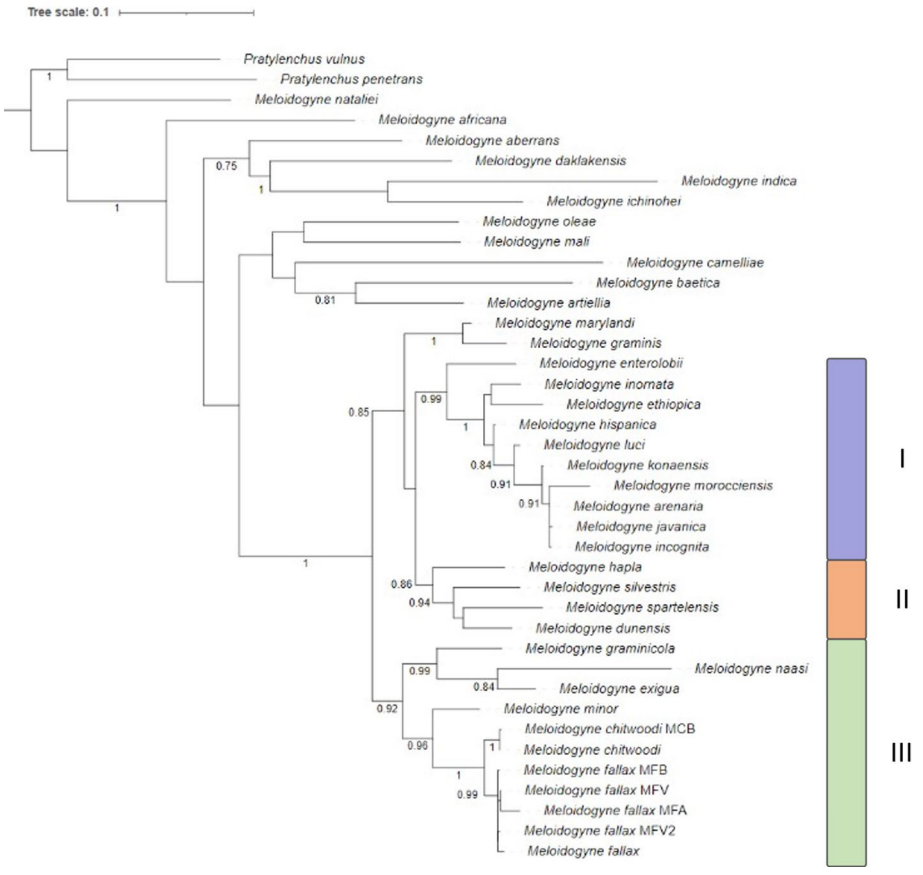


Fig. 4 Multigene phylogenetic analysis of *Meloidogyne* spp. A Maximum Likelihood consensus tree was inferred from concatenated sequences of 18 S rRNA, 28 S rRNA, ITS and COI using the GTR + I + G nucleotide substitution model in MEGA X (v. 10.2.6). Bootstrap support values (1000 replicates) are shown for nodes with support > 0.75. *Pratylenchus* spp. sequences were used as an outgroup to root the tree. Clusters I, II and III are highlighted according to Álvarez-Ortega et al. [3]

Cluster I comprised tropical species such as *M. incognita*, *M. arenaria*, and *M. enterolobii*. Cluster II included species such as *M. hapla*, and Cluster III contained two subclades: one of cereal-parasitic nematodes (*M. graminicola*, *M. naasi*, and *M. exigua*) and another comprising temperate RKN, *M. chitwoodi*, *M. fallax*, and *M. minor*.

This phylogenetic analysis was used to further confirm the species identification of the newly sequenced *M. chitwoodi* and *M. fallax* populations. The Swiss *M. chitwoodi* and *M. fallax* populations grouped within their respective species clades, clustering closely with corresponding reference sequences from NCBI.

This result provides strong molecular confirmation of species identity. Furthermore, within Cluster III, *M. minor* was the closest common ancestor to both *M. chitwoodi* and *M. fallax* and was therefore selected as the outgroup for subsequent phylogenetic analyses in this study.

Previous studies have investigated the use of commonly used barcode regions for phylogenetic analyses within *Meloidogyne* spp. Among these, the 18 *S* ribosomal DNA gene is considered to be highly conserved within RKN, often showing limited interspecific variability. Powers et al. and Kiewnick et al. [25, 40] reported no significant sequence variation between closely related species such as *M. fallax* and *M. chitwoodi*, to the point

of preventing species differentiation. Our 18 S phylogenetic analysis confirmed this, as *M. chitwoodi* and *M. fallax* clustered closely together in a single clade (Fig. S3). No clear sub-clustering based on geographic origin was observed, except for a weakly supported group of four *M. chitwoodi* sequences from The Netherlands (bootstrap support (BS) = 0.62). Consequently, the 18 S marker was unsuitable for resolving intraspecific variation or inferring phylogeographic structure in our dataset.

The 28 *S* rDNA gene, although highly conserved among RKN [42], has previously demonstrated sufficient resolution for distinguishing nematodes at the species level, except for some closely related species within the tropical RKN complex, such as *M. incognita*, *M. arenaria*, and *M. javanica* [40]. In our analysis, the 28 *S* rRNA gene provided better resolution than the 18 *S* rRNA gene, clearly separating *M. chitwoodi* and *M. fallax* into distinct groups (Fig. S4). However, this marker has limitations in providing detailed information at population level. No distinct subclades were observed within species, and the newly sequenced Swiss populations grouped within their respective species-defined groups without further differentiation. The clustering of *M. chitwoodi* sequences as a sister group to *M. fallax* was strongly supported, corroborating previous findings regarding their close phylogenetic relationship.

The phylogenetic analysis using the ITS region and the mitochondrial loci COI and COII showed higher resolution than the ribosomal subunits, corroborating former phylogenetic studies on RKN [25, 40, 42]. While previous studies have suggested that the ITS region may be too polymorphic for species-level phylogenetic reconstruction in tropical *Meloidogyne* species [3, 43, 44], it has shown good resolution for temperate species such as *M. chitwoodi* and *M. fallax*.

In our analysis, ITS sequences clustered *M. chitwoodi* and *M. fallax* into two distinct, species-specific sister groups with BS values of 0.86 and 0.87, respectively (Fig. 5). Within *M. fallax*, a subgroup comprising the Swiss populations MFV, MFA and MFB was observed (BS = 0.65). This result is notable since the populations MFV and MFV2 are geographically close, both originating from the Canton of Valais, but are phylogenetic separated by 3 single nucleotide polymorphisms (SNPs). Specifically, populations MFB, MFA and MFV share one SNP (A [adenine] instead of G [guanine] at position 234), while MFB also displays 2 additional SNPs (T [thymine] instead of C [cytosine] at position 134, and an A instead of G at position 270). Although the relatively modest BS suggests the need for further investigation with additional replicates and extended amplicon sequencing, this grouping supports the potential utility of ITS for intra-specific population studies in *M. fallax*.

The phylogenetic tree constructed from *COI* sequences showed that *M. chitwoodi* sequences clustered as a subgroup to *M. fallax*, with strong BS value of 0.93 (Fig. S5). No distinct subgroups were observed within *M. fallax*, except for two sequences from the UK, which formed a separate cluster with moderate support (BS = 0.62), based on a shared SNP.

In the *COII* phylogenetic analysis, *M. fallax* sequences formed a subcluster within *M. chitwoodi* sequences, supported by a BS value of 0.87 (Fig. 6). Like the ITS results, only minor subgroups with geographic proximity were observed. A low-support cluster (BS = 0.46) was observed for the *M. fallax* populations MFV, MFV2 and MFA, with MFV2 and MFA forming a subcluster supported by a BS value of 0.65.

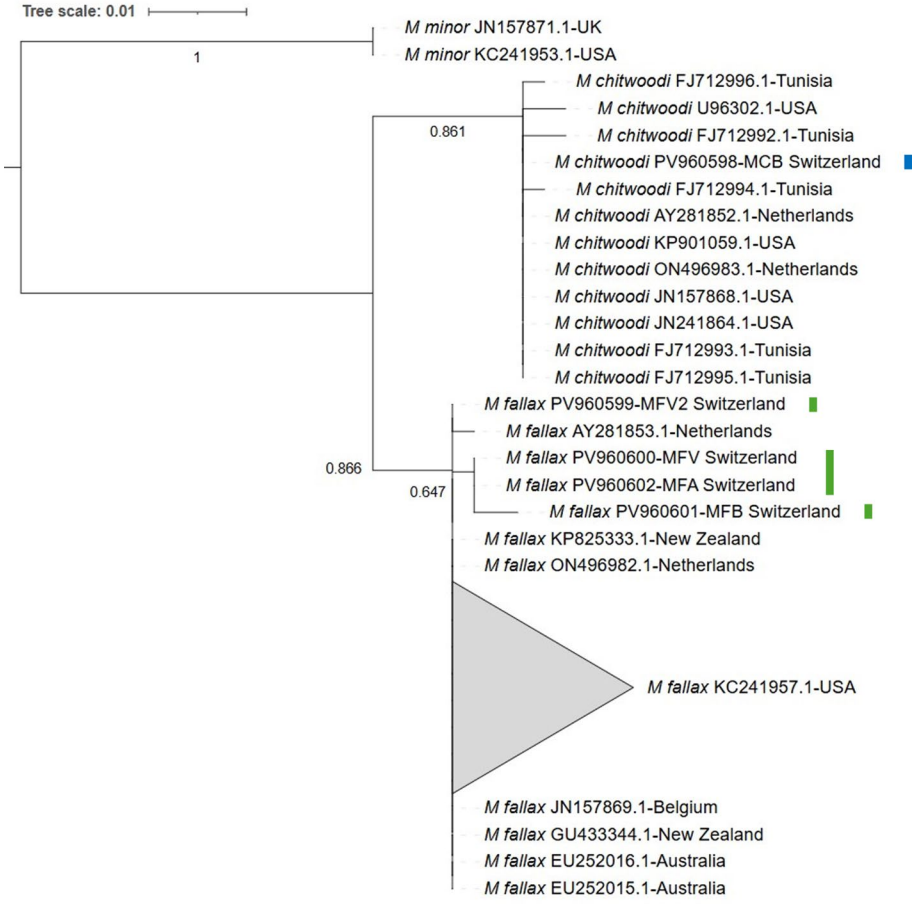


Fig. 5 Phylogenetic tree based on the ITS region of *Meloidogyne chitwoodi* and *M. fallax*. The tree was generated using the Tamura-Nei nucleotide substitution model and the Maximum Likelihood method with 1000 bootstrap replicates in MEGA X (v. 10.2.6). Bootstrap support values greater than 0.60 are shown at the nodes

Within this cluster, MFA and MFV shared a SNP at position 71 (G instead of T), while MFA and MFV2 shared two SNPs at positions 290 and 421 (both A instead of G). As in the ITS-based analysis, these results suggest that *COII* region may be useful for detecting population-level differences. However, the relatively low bootstrap support highlights the need for further validation using additional replicates or complementary markers.

The sequences obtained from the intergenic region between 5 S and 18 S rRNA (referred to as JMV) proved valuable for the molecular identification and phylogenetic analysis of the two *Meloidogyne* species. The phylogenetic tree based on JMV sequences showed clear species differentiation and provided the highest resolution among all barcoding regions analyzed. These findings were expected, as JMV barcodes are primarily designed for species-level identification (Fig. 7). Consequently, sequences of *M. chitwoodi* and *M. fallax* clustered distinctly, with strong separation between the two species. No intraspecific subgroups were observed in the JMV-based phylogenetic tree, suggesting that this barcode region may be too conserved within each species to resolve population-level variation. Similar findings were reported by Aslan et al. [20], who used JMV sequences to compare *Meloidogyne* populations from Turkey with those from Switzerland, Canada, the United Kingdom, and the USA. However, after excluding the JMV primer-binding regions and standardizing the sequence lengths of all database-derived

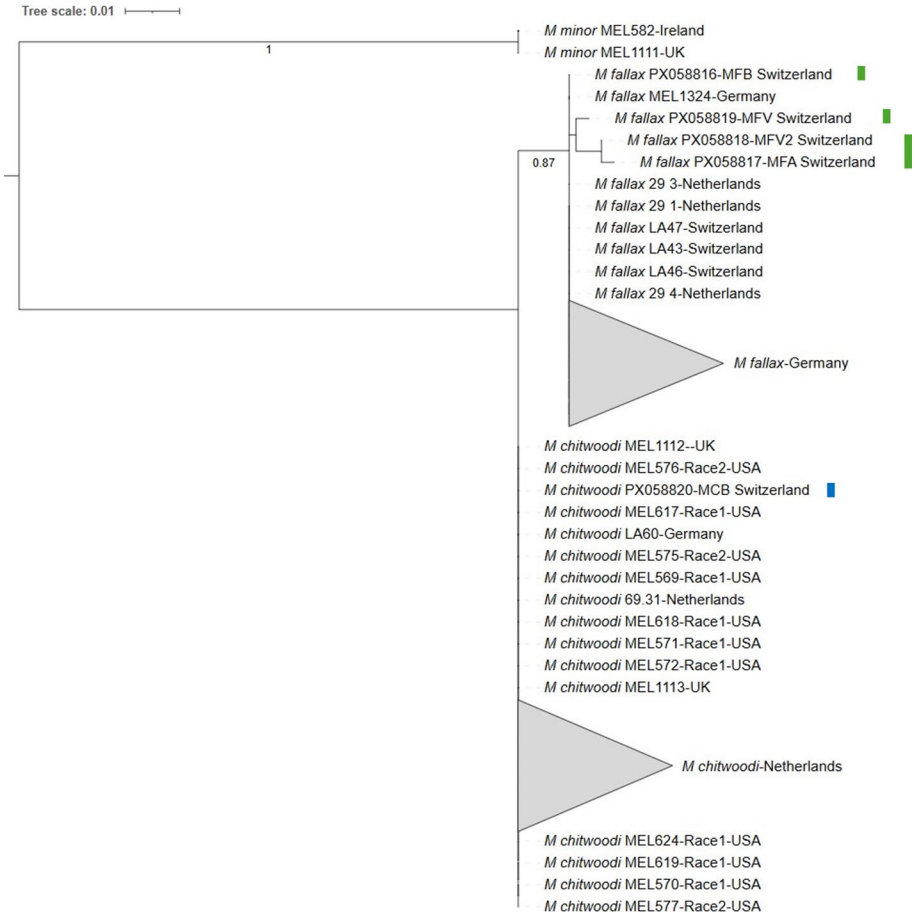


Fig. 6 Phylogenetic tree based on the mitochondrial *COII* gene of *Meloidogyne chitwoodi* and *M. fallax*. The tree was constructed using the Tamura-Nei nucleotide substitution model and the Maximum Likelihood method with 1000 bootstrap replicates in MEGA X (v. 10.2.6). Bootstrap support values greater than 0.60 are shown at the nodes

sequences to match our dataset, our phylogenetic analysis did not reproduce the same tree topology as reported by Aslan et al. [20].

Overall, our results indicate that many of the commonly used genes for *Meloidogyne* phylogenetic analyses are too conserved to reliably detect population-level differences between *M. chitwoodi* and *M. fallax*.

This low sequence variability across standard barcoding loci indicates that these temperate *Meloidogyne* species are genetically highly conserved at the selected diagnostic markers. This pattern may reflect relatively recent geographic expansion, limited evolutionary divergence among introduced populations, and/or predominantly clonal reproduction. At the same time, our findings show that standard barcode regions reach their resolution limits when applied to population-level analysis in *M. chitwoodi* and *M. fallax*. In this context, our study not only confirms species identity but also shows that the multilocus barcodes used here have limited power to distinguish among Swiss and foreign populations.

Nevertheless, phylogenetic analyses based on ITS and COII sequences showed some population-level differentiation, although with relatively low BS. These findings suggest that ITS and COII may be suitable markers for population studies, particularly if longer fragments are amplified or more replicates are included in the analysis. However,

Fig. 7 Phylogenetic tree of *Meloidogyne chitwoodi* and *M. fallax* species based on JMV sequences. The tree was generated using the Tamura-Nei nucleotide substitution model and the Maximum Likelihood method with 1000 bootstrap replicates in MEGA X (v. 10.2.6), with *M. minor* sequences used as the outgroup. Bootstrap support values greater than 0.60 are shown at the nodes

achieving higher phylogenetic and epidemiological resolution will likely require moving beyond common standard barcodes toward more variable genomic datasets, such as complete mitochondrial genomes, or whole-genome sequencing approaches. These approaches would enable more precise differentiation among populations and help clarify their origins and spread pathways.

5 Conclusion

This study provides the first molecular characterization of *M. chitwoodi* and *M. fallax* in Switzerland, complementing earlier morphological records. Differential-host assays and species-specific markers showed that the Swiss *M. chitwoodi* population (MCB) belongs to Race 1, pathotype Roza: it reproduced on carrot cv. 'Red Cored Chantenay' but not on alfalfa cvs. 'Sanditi', 'Thor'. The presence of Roza can also overcome the *S. bulbocastanum*

SB22 resistance gene. Consequently, its presence poses a direct threat to breeding programs that rely on this resistance source for potato improvement.

Furthermore, phylogenetic analyses based on the commonly used barcoding loci 18 S, 28 S, ITS, COI, and COII, together with the diagnostic JMV marker, confirmed species identity but offered little resolution at population level. These results therefore not only provide a genetic baseline for Swiss populations but also define a practical performance benchmark for standard barcoding markers in *M. chitwoodi* and *M. fallax*. Future studies should focus on higher-resolution genomic approaches to better trace outbreaks, identify sources, and support risk assessment.

To better understand the distribution and potential spread of *M. chitwoodi* and *M. fallax*, identifying more variable informative genetic markers is critical and could enhance phylogenetic resolution and support monitoring efforts. Incorporating such tools into regulatory diagnostics would enable the collection of informative sequences globally, aiding in the mapping of nematode spread and improving control strategies at national and international levels.

Race and pathotype identifications should also be incorporated as a dual-purpose diagnostic tool, supporting both treatment recommendations for infested fields and at the same time contributing to broader population genetic studies. In the long term, whole-genome sequencing or full mitochondrial genome analyses could uncover signatures of adaptation, host specificity, or resistance-breaking traits in nematode populations, useful for risk assessments and quarantine policies.

Finally, durable management of RKN will continue to depend on resistant cultivars deployed within integrated nematode-control programs. Future efforts should prioritize collaborative surveillance and data-sharing platforms to strengthen early warning systems and accelerate responses to emerging virulent populations, regardless of their regulatory status.

6 Materials and methods

6.1 Nematode isolation and culture

One *M. chitwoodi* and four *M. fallax* nematode cultures were developed on tomato plants (*Solanum lycopersicum* cv. Moneymaker) from single second-stage juveniles (J2) extracted from soil samples collected from commercial farms in Switzerland, either from open fields (*M. chitwoodi*) or from greenhouse tunnels (*M. fallax*), using the Oostenbrink dish technique, as described by Hallmann and Subbotin [45], or from *M. fallax* egg masses collected from infected tomato roots grown in the respective greenhouse tunnels (*M. fallax*). Egg masses were carefully removed with tweezers and a needle under a stereomicroscope (model SZX12, Olympus Optical Co Ltd., Japan) and subsequently incubated in water within 6-well plates at 21.5 °C until J2 hatching. Species identification was performed through morphological and molecular analyses following the EPPO standard [34]. These analyses were performed on pure nematode cultures initiated either by inoculation of a single J2 onto tomato plants or from single hatched J2 obtained from a single egg mass containing approximately 200 eggs or J2.

6.2 Nematode fixation and morphological observation

Morphological observations were performed on heat-killed J2 (n = 8) mounted on temporary slides and examined under a Zeiss AXIO Imager Z1 light microscope (Zeiss,

Oberkochen, Germany) equipped with a Zeiss AxioCam MRm camera. Zeiss Immersion oil 518 C (ISO 8036/1) was used at 100X magnification. Image acquisition, analysis and editing were performed using ZEN software (ZEISS Efficient Navigation), version 2.6 (blue edition), and the software Fiji [33], version 2.16.0.

6.3 DNA extraction, PCR amplification

A single J2 from each *Meloidogyne* population was transferred to cavity slides and bisected using sterile disposable scalpels (Surgical Scalpel Blade No.11, Swann-Morton, UK) to facilitate DNA extraction. DNA was extracted using Kawasaki's modified proteinase-K KAWA buffer as described by Holterman et al. [46] and stored at -20 °C until further use.

PCR amplification was carried out using the HotStartTaq Master Mix Kit (Qiagen, Hilden, Germany) on SensoQuest Labcycler thermal cycler (SensoQuest, Göttingen, Germany). Species differentiation among *M. chitwoodi*, *M. fallax*, and *M. hapla was* performed using the species-specific primers JMV1, JMV2, and JMV hapla, following the protocol of Wishart et al. [35]. PCR conditions were as follows: initial denaturation at 95 °C for 3 min, 45 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; followed by a final extension at 72 °C for 15 min. Amplification products were visualized by electrophoresis on a 1% TAE agarose gel and subsequently sent to Microsynth AG (Balgach, Switzerland) for Sanger sequencing. Refer to Fig. S6 for the original gel picture.

7 Phylogenetic analysis

Barcoding regions of the nuclear and mitochondrial genomes, including *18S* rRNA, *28S* rRNA, internal transcribed spacer (*ITS*), *COI* and *COII*, were amplified as described by Kiewnick et al. and Álvarez-Ortega et al. [3, 40], using the following primer pairs: *18S* rRNA: G18SU (5'-GCT TGT CTC AAA GAT TAA GCC-3') and R18Tyl1 (5'-GGT CCA AGA ATT TCA CCT CTC-3'), *28 S* rRNA: D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3'), *ITS* region: TW81 (5'-GTT TCC GTA GGT GAA CCT GC-3') and AB28 (5'-ATA TGC TTA AGT TCA GCG GGT-3'), *COI*: JB3 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and COI2R10 (5'-CAG GAA ACA GCT ATG ACA TAT TAA TTA AAG AGA AAG AAA ACA AAT C-3'), *COII*: COIIF1 (5'-TAR ATT KNT TTC ATR RTT TTA ATT GT-3') and COIIR1 (5'-CAC AAA TTT CTG AAC ATT GMC C-3').

The intergenic spacer region between *5 S* and *18 S* rRNA, referred to as JMV in this work, was sequenced using the species-specific primers JMV1, JMV2, and JMV hapla, according to Wishart et al. [35]. Obtained sequences were aligned using the online software MultAlin [47] and trimmed to generate consensus sequences from sequence overlaps between forward and reverse reads. Consensus reads were submitted to the NCBI (National Center for Biotechnology Information) GenBank database (Table S1).

For the multigene phylogenetic tree, sequences of barcodes 18 S, 28 S, ITS and COI from several *Meloidogyne* species were retrieved from the NCBI and EPPO-Q-bank databases (Table S2). Sequences for each locus were aligned separately using MUSCLE as implemented in MEGA X (version 10.2.6). The resulting alignments were visually inspected and trimmed to remove obvious peripheral misalignments and overhanging ends. The individual locus alignments were then combined in MEGA X into a multilocus dataset in the following order: 18 S, 28 S, ITS, COI. The final constructed alignment

had a total length of 2605 bp (18 S: 485 bp; 28 S: 706 bp; ITS: 976 bp; COI: 438 bp). The best-fit nucleotide substitution model for the combined dataset was determined using the model selection function in MEGA X based on the Bayesian Information Criterion (BIC). The GTR + I+G model showed the best fit and was therefore used for tree construction.

For the single-locus phylogenetic trees, sequences of barcodes 18 S, 28 S, ITS, COI, COII and JMV of *M. chitwoodi* and *M. fallax* were retrieved from the NCBI and EPPO-Q-bank databases (Tables S3–S8). Sequences for each locus were aligned using MUSCLE in MEGA X and resulting alignments were inspected and trimmed to remove clear misalignments and overhangs at both ends. The model selection function in MEGA X was used to check the best-fit nucleotide substitution models of each barcode, and all trees were run with the T93 (Tamura-nei) substitution model.

The combined multigene and all single-locus phylogenetic trees were run with the Maximum Likelihood statistical method and 1000 bootstrap replicates in MEGA X.The resulting trees were visually enhanced using the Interactive Tree of Life (iTOL) online tool [48].

8 Meloidogyne chitwoodi race identification

For inoculum production, previously established *Meloidogyne chitwoodi* cultures were reared on *S. lycopersicum* cv. Moneymaker (HILD samen, Kirchenweinbergstraße 115, Marbach am Neckar, Germany) in the greenhouse under constant climatic conditions (20 °C ± 1 °C, 60% relative humidity, 16/8 h light/dark photoperiod). To increase the nematode population and obtain sufficient inoculum for the race identification assays, six tomato plants were inoculated with 15,000 J2 per plant and harvested 7 weeks after inoculation for nematode propagation.

Eggs were extracted from heavily galled roots by rinsing of the root-attached soil: silver sand mixture (1:3, v/v), followed by cutting the roots into 1 cm pieces and shaking them twice for 4 min in a 1.5% chlorine solution. *Meloidogyne chitwoodi* eggs were separated from roots and chlorinated solution using a cascade of mesh screens (pore sizes of 500, 100, 75, 50, and 25 µm), followed by thorough rinsing with water. Eggs were incubated in water at 20 °C for 5 days to allow J2 to hatch. Freshly hatched J2 were separated from the eggs using the Oostenbrink dish method [45].

A host differential test was conducted to determine the race of the Swiss *M. chitwoodi* population using *Daucus carota* cv. Red Cored Chantenay (Select Samen AG, Bergstrasse 14, 3612 Steffisburg, Switzerland), *Medicago sativa* cv. Sanditi (UFA-Samen, In der Euelwies 34, Winterthur, Switzerland) and *Solanum lycopersicum* cv. Moneymaker. Plants were grown for three weeks before inoculation. A preliminary experiment was performed using 50 mL Falcon tubes ($n = 8$ replicate plants per host) filled with steamed soil: silver sand mixture (1:3, v/v), 150 J2 inoculated per plant. The main experiment was performed in 13 cm plastic pots ($n = 8$ replicate plants per host species), also filled with the same substrate, with each pot containing a 3-week-old plant inoculated with 5000 J2. Plants were maintained for 7 weeks (Falcon tubes) or 9 weeks (pots), after which they were uprooted and washed free of soil with water.

Nematode egg masses were stained using 1% Ponceau red (Ponceau 4R - CI 16255 [49]), and counted under a stereomicroscope. Eggs were extracted collectively from all egg masses obtained from each single plant, as described above, and counted under

an inverted microscope (model CKX53, Olympus Optical Co Ltd., Japan). The mean number of eggs per egg mass was subsequently calculated from the total egg count and the total number of egg masses per plant. Soil populations of J2 (from the pot experiment using 100 mL of soil) were extracted using the Oostenbrink dish method [45] and counted under the inverted microscope. The Reproduction factor (RF = FP/IP, or final population/ initial population) was determined for each host. Differences in Reproduction factor (Rf) between host species were tested using Student's t-Test (n = 8 per host), using RStudio v2024.04 (Posit, Boston, USA). Significance was assessed at.

$p < 0.05$.

For molecular race confirmation, DNA was extracted as previously detailed. PCR amplification was performed on extracted DNA using the three primer pairs: MC1MR (Forward: 5'-TTT TGT GCG ATT GGC AAG GA-3', Reverse: 5'-ATA CAT TCG CTC GGT TCC CG-3'), MC2MR (Forward: 5'-TCG ACT CAT GTC AGC TCG TT-3', Reverse: 5'-ACG CTA GTC GAA GAC GCT TT-3'), and ROZMR (Forward: 5'-CAC GGA ACGT TGG AAG GGT A-3', Reverse: 5'-CCT CGT GAT CAG CGC AGT AA-3'), as outlined in Hu et al. [28]. PCR was performed using the HotStartTaq Master Mix Kit (Qiagen, Hilden, Germany) on SensoQuest Labcyclers, under conditions detailed in Hu et al. [28]. PCR products were analyzed by gel electrophoresis as described above. Refer to Fig. S7 and Fig. S8 for the original gel pictures.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44372-026-00643-8.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions
Conceptualization: PD.; Methodology: PD, AC.; Investigation: AC, TS, JE.; Data curation: AC.; Formal analysis: AC.; Resources: PD, AC, TS, JE.; Writing – original draft: AC.; Writing, review & editing: PD, AC, SR.; Supervision: PD, SR.; Project administration: PD.; All authors read and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article and its supplementary information files. Sequence data supporting the findings of this study have been deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under the primary accession codes: PV954789, PV954787, PV954786, PV954785, PV955396, PV955395, PV955397, PV955398, PV955399, PV960607, PV960606, PV960609, PV960608, PV960610, PX058817, PX058816, PX058819, PX058818, PX058820, PV960602, PV960601, PV960600, PV960599, PV960598, PX058821, PX058824, PX058822, PX058823, and PX058825.

Declarations

Ethics approval and consent fo participate
All plant materials used in this study (*Solanum lycopersicum* cv. Moneymaker, *Daucus carota* cv. Red Cored Chantenay, and *Medicago sativa* cv. Sanditi) were obtained from commercial suppliers and cultivated under controlled greenhouse conditions. No wild plant species were collected. All procedures involving plant material were conducted in accordance with relevant institutional, national, and international guidelines and legislation. No specific permits or licenses were required for the described research.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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