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RESEARCH ARTICLE

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Integrating quantitative morphological and qualitative molecular methods to analyse soil nematode community responses to plant range expansion

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Abstract

1. Below-ground nematodes are important for soil functioning, as they are ubiquitous and operate at various trophic levels in the soil food web. However, morphological nematode community analysis is time consuming and requires ample training. qPCR-based nematode identification techniques are well available, but high-throughput sequencing (HTS) might be more suitable for non-targeted nematode community analyses.
2. We compared effectiveness of qPCR- and HTS-based approaches with morphological nematode identification while examining how climate warming-induced plant range expansion may influence below-ground nematode assemblages. We extracted nematodes from soil of *Centaurea stoebe* and *C. jacea* populations in Slovenia, where both plant species are native, and Germany, where *C. stoebe* is a range expander and *C. jacea* is native. Half of each nematode sample was identified morphologically and the other half was analysed using targeted qPCR and a novel HTS approach.
3. HTS produced the highest taxonomic resolution of the nematode community. Nematode taxa abundances correlated between the methods. Therefore, especially relative HTS and relative morphological data revealed nearly identical ecological patterns. All methods showed lower numbers of plant-feeding nematodes in rhizosphere soils of *C. stoebe* compared to *C. jacea*. However, a profound difference was observed between absolute and relative abundance data; both sampling origin and plant species affected relative abundances of bacterivorous nematodes, whereas there was no effect on absolute abundances.
4. Taken together, as HTS correlates with relative analyses of soil nematode communities, while providing highest taxonomic resolution and sample throughput, we propose a combination of HTS with microscopic counting to supplement important quantitative data on soil nematode communities. This provides the most cost-effective, in-depth methodology to study soil nematode community

responses to changes in the environment. This methodology will also be applicable to nematode analyses in aquatic systems.

KEYWORDS
biodiversity, high-throughput sequencing, microscopy, molecular approaches, nematodes, qPCR, soils

1 | INTRODUCTION

Nematode communities are highly diverse and include the most abundant animals in any environment (Ettema, 1998). Soil nematodes are functionally versatile and are active at various positions in the soil food web, and include bacterivores, fungivores and animal and plant parasites (Bongers & Bongers, 1998). Well-developed extraction methods enable collection of nematodes from soil, plant roots and shoots, routinely followed by morphological identification to feeding guilds, based on their distinct mouthparts (Yeates, Bongers, De Goede, Freckman, & Georgieva, 1993). However, a major obstacle in nematode community analysis is the choice of applied methodology along with the required expertise.

Nematode identification approaches vary from detecting single plant parasites in soil or plant materials for export purposes (Vervoort et al., 2012) to entire community composition-oriented studies for monitoring purposes (Bongers, 1990; Bongers & Ferris, 1999; Chen, Daniell, Neilson, O'Flaherty, & Griffiths, 2010; Neher, 2001; Ritz & Trudgill, 1999). Nematode communities might be among the best, most widely accepted and easiest to use indicators (Griffiths et al., 2016; Ritz, Black, Campbell, Harris, & Wood, 2009), but costs of whole-community nematode analyses constrain large-scale application. Currently, most studies that require nematode identification apply microscopic identification after their extraction from soil and roots. Most commonly, nematode identification resolves taxa into coarse functional units, as higher resolved taxonomic rank identification requires profound identification skills (Yeates & Bongers, 1999). Even then, some groups, especially smaller and younger individuals (juveniles), are hardly identifiable beyond family level (Floyd, Abebe, Papert, & Blaxter, 2002; Powers, 2004). For convenience, a fraction of 100–150 individuals within individual samples are identified to determine the nematode community structure, which usually takes skilled morphological identification experts more than 1 hr for completion (Griffiths, de Groot, Laros, Stone, & Geisen, 2018). Therefore, morphological analyses of environmental nematode samples are constrained by taxonomic resolution, time and, consequently, money (Griffiths et al., 2018).

Although molecular methods represent the standard to study microbial bacteria, fungi and protists (Geisen & Bonkowski, 2017; Prosser, 2015), they are much less frequently applied to study soil nematode communities. Still, an array of molecular approaches are available to study soil nematodes. Abundances of specific nematode groups can be determined using qPCR, and several specific primer pairs have been developed to study different nematode taxa in soils (Green, Wang, Lilley, Urwin, & Atkinson, 2012; Quist et al., 2016; Vervoort et al., 2012; Wiesel, Daniell, King, & Neilson, 2015). However, these targeted quantitative approaches are not effective at providing diversity information on multi-species nematode communities, as only overall abundances of targeted nematode taxa are obtained; in addition, information on taxon representation within a studied group remains unknown. In order to determine entire nematode community compositions, molecular diversity analyses are applied; these include DGGE (e.g. Foucher, Bongers, Noble, & Wilson, 2004), T-RFLP (e.g. Donn, Neilson, Griffiths, & Daniell, 2012) and now predominantly high-throughput sequencing (HTS) (Darby, Todd, & Herman, 2013; Kerfahi et al., 2016; Porazinska et al., 2009; Porazinska, Fujisaki, Purcell, & Giblin-Davis, 2014; Porazinska, Giblin-Davis et al., 2010; Porazinska, Morgan et al., 2014; Porazinska, Sung, Giblin-Davis, & Thomas, 2010; Sapkota & Nicolaisen, 2015).

While molecular methods circumvent labour-intensive, expert-dependent identification, they introduce other biases. PCR amplification of the target gene can artificially change the true community composition due to differences in copy numbers and primer biases (e.g. Porazinska et al., 2009; Behnke et al., 2011; Darby et al., 2013; Geisen, Laros, Vizcaíno, Bonkowski, & de Groot, 2015). Furthermore, current molecular diversity analyses only can target a short (≤550 bp) barcode region of the nematode DNA that has to be chosen carefully to allow high-resolution nematode community analyses. Arguably most importantly, diversity analyses such as HTS provide only relative abundance data and do not provide information on absolute (nematode) numbers (Vandeputte et al., 2017).

Regardless of the applied methodology to study soil nematode communities, soil nematodes are functionally well characterized. The role of pests of important crop plants has given soil nematodes a notorious reputation, especially in agricultural and horticultural systems, and the vast majority of work on soil nematodes focuses on individual species or even genotypes within the plant parasites, also named plant or root-feeders or herbivores (Neher, 2010). There is increasing interest in the role of plant-feeding nematodes in controlling natural plant populations (Brinkman, Duyts, Karssen, van der Stoel, & van der Putten, 2015; Cortois et al., 2017). For instance, plant-feeding nematodes are studied in the context of climate warming-induced plant range expansion (Morriën, Duyts, & van der Putten, 2012; Viketoft & van der Putten, 2015; Wilschut, Geisen, ten Hooven, & van der Putten, 2016). As climate warming is enabling the poleward spread of plant species (Parmesan & Yohe, 2003; van der Putten, 2012; Walther et al., 2009), those plants can escape natural

enemies, contributing to invasiveness (van der Putten, 2012), as shown for introduced exotic plant species (Keane & Crawley, 2002). Indeed, range-expanding plant species on average have reduced exposure to plant-feeding nematodes when grown in soil from the new range (Morriën et al., 2012). However, it remains unknown how plant exposure to nematode communities in the new range compares with that of the original habitat of the range-expanding plant species. This information is essential in order to determine if escape from plant-feeding nematodes may contribute to enemy release as a result of plant range expansion.

Because of their roles in various trophic levels of the soil food webs (Bongers & Bongers, 1998; Hunt et al., 1987), freshwater (Pusch et al., 1998; Traunspurger, 2000) and marine systems (Middelburg et al., 2000; Moens & Vincx, 2009), free-living, non-plant–feeding nematodes are also receiving increasing interest. For example, in soil food webs, free-living nematodes feed on bacteria and fungi or act as omnivores and predators. These nematodes as well as protists stimulate nutrient mineralization as well as microbial and faunal turnover thereby affecting other soil organisms and plants (Bonkowski, 2004). Therefore, non-plant–feeding nematodes are of high importance for ecosystem functioning; however, functionally different from their plant-feeding "relatives" (Ferris et al., 2012; Yeates et al., 1993).

The aim of our study was to compare state-of-the-art methods for qualitative and quantitative nematode community analyses. As a study system, we used nematodes in the root zone soil of the range-expanding plant species *Centaurea stoebe* in its native range (Slovenia) and its expanded range (Germany). We compared nematode community composition of this range expanding plant species to the congener *C. jacea*, which is native in both the original and novel range of *C. stoebe*. We morphologically identified half of the extracted nematodes to family or (mostly) genus level by microscopy. Furthermore, we extracted DNA from the other half of each sample, which was subsequently subjected to targeted quantification of seven common nematode taxa using qPCR (Vervoort et al., 2012) as well as non-targeted nematode community analyses using a newly developed HTS approach. We tested the hypotheses that (1) ecological patterns are recovered independent of applied methodology and (2) relative and absolute abundances of plant-feeding nematodes in the root zone of *C. stoebe* are lower than those of *C. jacea*, especially in the expanded range of *C. stoebe*.

2 | MATERIALS AND METHODS

2.1 | Soil sampling and nematode extraction

Nematode community identifications were done on the range expanding *Centaurea stoebe* L., a neophyte originating in south-eastern Europe that arrived in north-western Europe during the last 100 years (Welss, Reger, & Nezadal, 2008) and the related *C. jacea* L., which is native in both the original and expanded range of *C. stoebe*. Both plant species commonly co-occur in riverine habitats and were sampled from nearby locations in similar soil conditions.

Soils (*c.*1 kg) underneath each of nine plants (three populations with each three plants) of both congeners were sampled in both Slovenia (native to *C. stoebe* and *C. jacea*) and Germany (Frankfurt region, native to *C. jacea* only) (Supplementary Table S1). Individual soil samples were kept separately to form real biological replicates and were stored in plastic containers at 4°C for up to 2 weeks after sampling until nematodes were extracted from 100 g of gently homogenized soils, using an Oostenbrink elutriator (Oostenbrink, 1960). The nematodes were concentrated in glass jars with 20 ml of tap water, thoroughly homogenized and subdivided into two subsamples, one for morphological identification and one for molecular work. Both subsamples were allowed to settle for 24 hr at 4°C and then concentrated to 2 ml by carefully removing the supernatant using a Pasteur pipette attached to a Venturi pump. Another subsample of 10 g of soil was dried at 105°C for 3 days in order to determine soil moisture percentage for subsequent calculation of nematode numbers per unit of soil dry weight.

2.2 | Nematode quantification and community analyses

2.2.1 | Morphological determination

One half of the nematode solution was fixed by diluting the suspension with 4 ml hot formalin (90°C) instantly followed by 4 ml cold formalin (4°C). These subsamples were stored at RT until morphological determination using an inverse light microscope (Olympus CK40, 400× and 1,000× magnification). Between 90 and 170, nematodes were morphological identified up to genus or family level from the entire or a defined subset of the samples. Subsequently, all nematode groups were categorized into feeding guilds according to Yeates et al. (1993) and Bongers and Bongers (1998) (Supplementary Table S2). Nematode numbers were calculated and standardized to numbers per 100 g of dry soil.

2.2.2 | Molecular determination of abundances and community structure of nematodes

DNA from the other subsample was extracted using the Clear Detections Nematode DNA extraction and purification kit™ (Clear Detections, Wageningen, the Netherlands). DNA isolates were stored for further use at −20°C.

Quantitative determination of nematode groups using qPCR

After evaluating the performance of 13 nematode taxon-specific primer pairs for nematode quantitative analyses (see supplementary methods for details), we focused our final approach on nematodes of the families Aphelenchidae, Aphelenchoididae, Cephalobidae, Monhysteridae and Plectidae and the genera *Helicotylenchus* and *Rotylenchus*. These are representatives of the most abundant functional groups of soil nematodes including plant

feeders (*Helicotylenchus*, *Rotylenchus*), bacterivores (Cephalobidae, Monhysteridae, Plectidae) and fungivores (Aphelenchidae, Aphelenchoididae) (Yeates et al., 1993). The targeted qPCRs of genera and families were performed at NIOO-KNAW with the ClearDetection kit (http://www.cleardetections.com/) according to manufacturer's instructions.

Determination of the nematode community composition using DNA metabarcoding

A novel high-throughput sequencing approach was designed to investigate the community structure of nematodes using DNA metabarcoding. Instead of using nematode-specific primers that can miss several nematode taxa, we applied universal eukaryotic primers targeting the most variable V4 region of the 18S rDNA, a barcoding region most suitable for almost all eukaryotes (Pawlowski et al., 2012). We used the universal eukaryotic primers 3NDf (Cavalier-Smith, Lewis, Chao, Oates, & Bass, 2009) in combination with 1132rmod (5'–TCCGTCAATTYCTTTAAGT–3'), modified from 1132r (Hugerth et al., 2014) to amplify a *c*.570 bp long fragment. For all primers, we used pre-tagged primers, containing Illumina adapters, a 12 bp long barcode to allow demultiplexing of the reads after sequencing, a primer linker and the sequencing primers.

PCRs were conducted in duplicates, visually quality verified on agarose gel and duplicates pooled before PCR cleanup with Agencourt AMPure XP magnetic beads (Beckman Coulter). Samples were pooled in equimolar ratios after determining concentrations with a fragment analyser (Advanced Analytical) before sending for sequencing to BGI, China (see supplementary methods for further details).

2.3 | Bioinformatics

To obtain an annotated OTU table for nematodes, we used the following pipeline: the raw MiSeq-reads were merged with a minimum overlap of 10 bp and at least a PHRED score of 25 using the RDP extension to PANDASeq (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012) named Assembler (Cole et al., 2014). Flexbar v2 (Dodt, Roehr, Ahmed, & Dieterich, 2012) was used to remove the primer sequences from the FASTQ files, after which the sequences were converted to FASTA format and concatenated into a single file. VSEARCH (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) was used for sequence clustering into OTUs based on 97% sequence similarity, using the UPARSE strategy of de-replication, sorting by abundance (with at least two sequences) and clustering using the UCLUST smallmem algorithm (Edgar, 2010). Chimeric sequences were detected and removed using the UCHIME algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011) implemented in VSEARCH. All steps were implemented in a workflow made with Snakemake (Köster & Rahmann, 2012) as available at: (de Hollander, 2016).

Nematode OTUs were taxonomically assigned to genus level against the quality curated PR2 database (Guillou et al., 2013), while sub-genus level classification was treated as OTUs. All sequences assigned as Nematoda with similarity of less than 90% were manually blasted against NCBI GenBank to ensure that the best matches of these OTUs in fact resembled nematodes, which was confirmed.

2.4 | Statistical analyses

All statistical analyses were performed in R 3.4.1 (R Core Team, 2017). We calculated relative abundances of all genera and families that were identified by MorphoID and qPCR to be able to compare it with HTS method. The effect of sampling origin and plant species and their interaction on nematode absolute and relative abundances were analysed using ANOVA. To meet the requirements of normality and homoscedasticity of errors, the total number of nematodes, the plant feeders and omnivores+predators absolute abundances identified by MorphoID were square-root transformed and the bacterivores and fungivores absolute abundances identified by MorphoID were log transformed; all data obtained by qPCR and HTS were square-root transformed. To examine the differences between absolute nematode abundance identified by morphological and qPCR methods, a two-sided pairwise *t* test was used. For the pairwise comparison of the nematode abundances between different identification methods, Pearson correlation was used.

As qPCR could only target seven nematode taxa, we focused the comparison of nematode communities identified by different methods on these seven nematode groups. Principal coordinated analyses (PCoA) with the Bray–Curtis distance matrix based on nematode relative abundances (vegdist function, VEGAN package [Oksanen et al., 2017]) was used to visualize the nematode community composition identified by different methods. To test the effect of the method, sampling origin, plant species and their interactions, PERMANOVA based on 999 permutations was used [adonis function, VEGAN package (Oksanen et al., 2017)]. The centroids and standard errors of nematode community composition were visualized using ggplot2 (Wickham, 2016).

3 | RESULTS

3.1 | Overview of individual methods

3.1.1 | Morphological identification

A total of 71 nematode taxa (families/genera) were identified. Total nematode abundance was significantly higher in the root zone of *C. jacea* than of the range expander *C. stoebe* ($F_{1,32}$ = 14.8, p < .001, Figure 1a). Absolute abundance of plant-feeding nematode was higher in *C. jacea* than in *C. stoebe* soils ($F_{1,32}$ = 21.1, p < .001, Figure 1b). More fungivorous nematodes were found in *C. jacea* than in *C. stoebe* soils, but this effect was dependent on sampling origin ($F_{1,32}$ = 5.0, p < .05). Similar to the absolute abundance data, relative abundance of plant-feeding nematodes was higher in the root zone of *C. jacea* compared to *C. stoebe* ($F_{1,32}$ = 10.2, p < .01, Figure 1c). In contrast, relative abundance of fungivores was affected by sampling

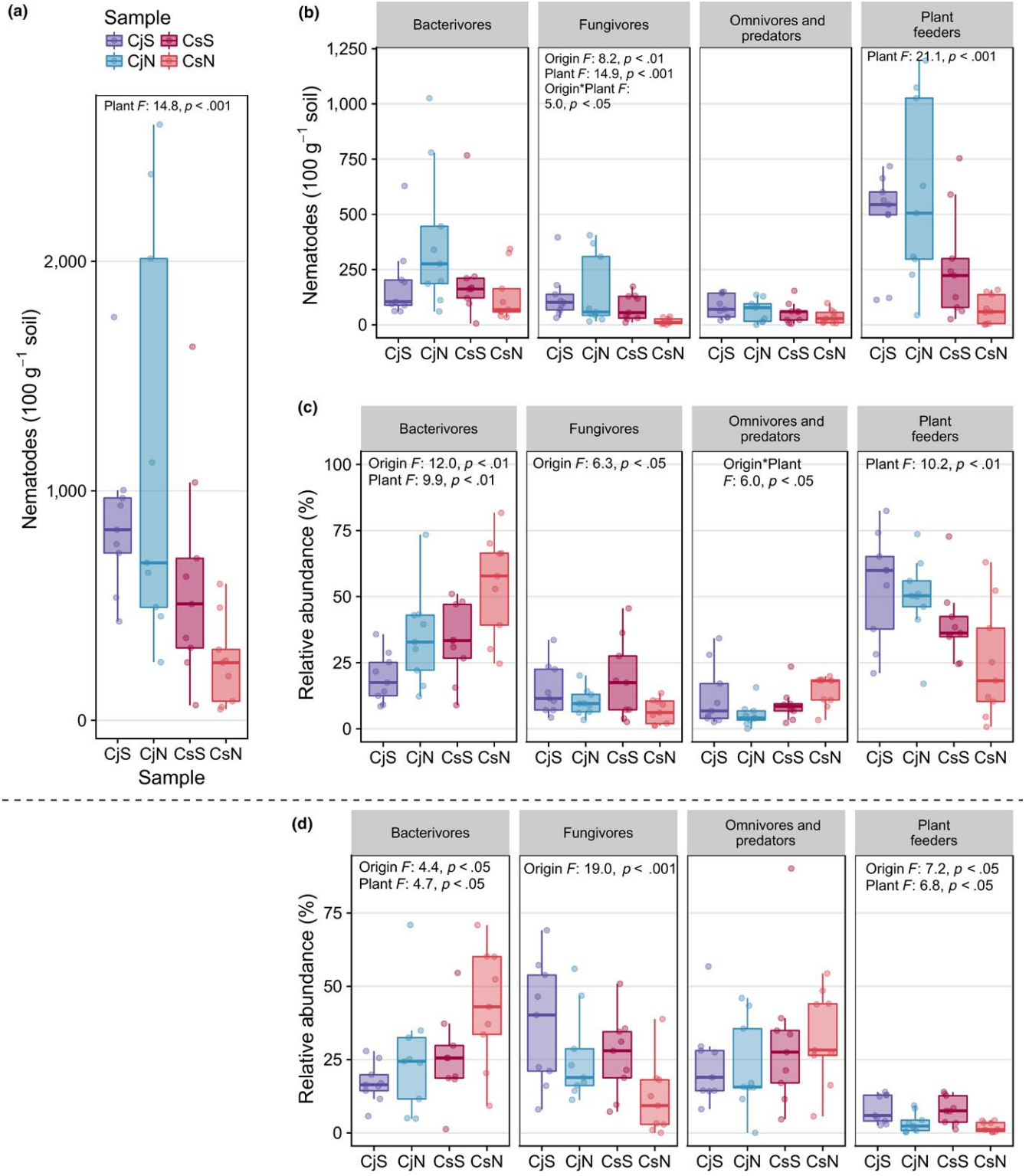

FIGURE 1 Total absolute nematode abundances quantified by microscopy (a); absolute (b) and relative (c) abundances of major nematode functional groups; (d) relative abundances of major nematode functional groups determined by high-throughput sequencing (HTS); Cj: *Centaurea jacea*, Cs: *C. stoebe*, N: Northern range, S: Southern range. In boxes observations within the 25–75 percentile range, points within the error bars representing observations within the 5–95 percentile range, the median is represented by a bold line inside the box. Significance of the effects of sampling origin, plant species and their interaction are based on ANOVA. Not significant effects are not shown

origin ($F_{1,32}$ = 6.3, p < .05), bacterivores nematodes by both sampling origin ($F_{1,32}$ = 12.0, p < .01, Figure 1c) and by plant species ($F_{1,32}$= 9.9, p < .01, Figure 1c) and relative abundance of omnivores+predators by their interaction ($F_{1,32}$ = 6.0, p < .05, Figure 1c). An overview of all results are presented in Table 1 and Supplementary Table S6. The analysis at highest taxonomic resolution revealed 36 taxa that were

TABLE 1 Overview of significant differences based on ANOVA with plant sampling origin (O), plant species (P) and their interaction (P × O) as revealed by the different identification methods

		Plant feeders			Bacterivores			Fungivores			Omnivores + Predators		
Method	Abundance	P	O	P × O	P	O	P × O	P	O	P × O	P	O	P × O
MorphoID	Absolute	Cj > Cs	–	–	–	–	–	Cj > Cs	S > N	CjS = CjN CsS > CsN	–	–	–
	Relative	Cj > Cs	–	–	Cj < Cs	S < N	–	–	S > N	–	–	–	CjS > CjN CsS < CsN
qPCR	Absolute	Cj > Cs	–	–	–	–	–	–	–	–	–	–	–
HTS	Relative	Cj > Cs	S > N	–	Cj < Cs	S < N	–	–	S > N	–	–	–	–

Cj, *Centaurea jacea*; Cs, *C. stoebe*; N, Northern range; S, Southern range.
qPCR based on the sum of 2–3 nematode taxa targeted per functional group.

significantly different between the treatments (see Supplementary Table S2).

3.1.2 | Molecular quantification of targeted groups using qPCR

Total nematode abundance was higher in *C. jacea* than in *C. stoebe* soils ($F_{1,32}$ = 6.7, p < .05, Supplementary Figure S1a). This effect was mainly caused by plant-feeding *Helicotylenchus* nematodes, which were highly abundant in *C. jacea*, but nearly absent in *C. stoebe* soils ($F_{1,32}$ = 9.9, p < .01, Supplementary Figure S1b). Abundance of plant-feeding Rotylenchus nematodes was significantly affected by the interaction between soil sampling and plant species, with higher numbers of nematodes in southern compare to northern *C. jacea* populations, while the opposite pattern was observed in the root zone of *C. stoebe* (Figure 1b lower panel). Fungivorous Aphelenchidae were significantly affected by soil sampling ($F_{1,32}$ = 4.3, p < .05) and plant species ($F_{1,32}$ = 6.2, p < .05, Supplementary Figure S1c). The abundance of bacterivorous nematodes was not significantly affected by plant species or sampling origin (Supplementary Figure S1d). An overview of all results are presented in Table 1 and Supplementary Table S6.

3.1.3 | Molecular determination of the entire nematode community using HTS

From all 268,950 quality-curated 18S rDNA reads (711 chimeras were removed), 215,326 were taxonomically assigned into 271 nematode OTUs within 101 genera. Forty OTUs within Chromadorea and Enoplea remained unassigned. Southern populations of both plant species hosted higher relative abundances of plant-feeding nematodes than northern populations ($F_{1,32}$ = 7.2, p < .05, Figure 1d), and *C. jacea* hosted relatively more plant-feeding nematodes than *C. stoebe* ($F_{1,32}$ = 6.8, p < .05, Figure 1d). There were also relatively more fungivores in southern than in northern soils ($F_{1,32}$ = 19.0, p < .001; Figure 1d). In contrast, overall relatively fewer bacterivores were detected in southern than in northern soils (main effect soil sampling: $F_{1,32}$ = 4.4, p < .05, Figure 1d) and *C. jacea* hosted relatively fewer bacterivores than *C. stoebe* (main effect plant species: $F_{1,32}$ = 4.7, p < .05, Figure 1d). Omnivores+predators were not affected by any treatment (p > .05; Figure 1d). An overview of all results are presented in Table 1 and Supplementary Table S6. The analyses at higher taxonomic resolution revealed that 41 nematode genera and 77 nematode OTUs were significantly affected by plant species, sampling origin or their interaction (see Supplementary Tables S3 and S4).

3.2 | Method comparisons

3.2.1 | Absolute abundances

Lower number of Plectidae (p < .001), Aphelenchoididae (p < .001), Monhysteridae (p < .05 and *Rotylenchus* (p < .05)

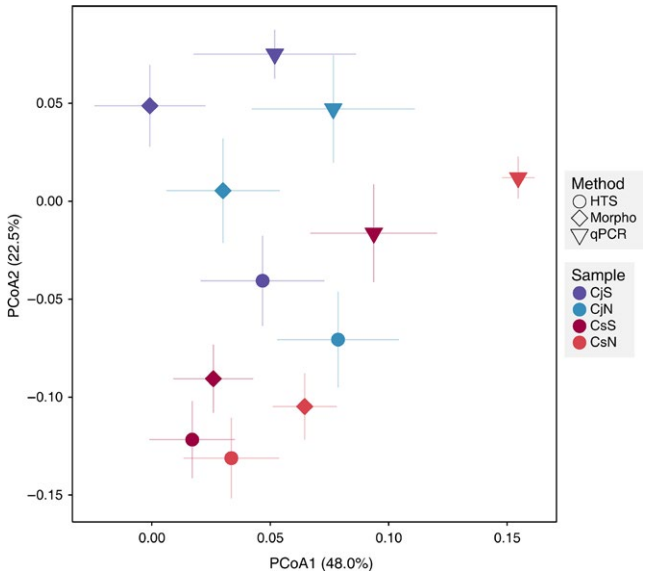

FIGURE 2 Principal coordinate analysis (PCoA) plot demonstrating centroids and standard errors of nematode community composition identified by microscopy (diamonds), qPCR (triangles) and HTS (circles) methods based on relative abundances of the seven nematode taxa targeted by qPCR. Percentages of total explained variation by PCoA axes are given in parentheses

nematodes was identified by qPCR than by morphological identification (Supplementary Figure S2).

3.2.2 | Relative abundances

Comparison between MorphoID, qPCR and HTS based on the seven nematode groups targeted by qPCR

Independent of methodology, ecological patterns (nematode taxa composition in the different treatments) were similarly represented by each identification method (Method*Plant species*Sampling origin; $F_{2,96}$ = 0.22, p = .99). Nevertheless, the identification method significantly explained most of the variation in nematode community composition ($F_{2,96}$= 11.4, p < .001; variation explained 15.1%; Figure 2). Largest differences in the nematode community composition were found between qPCR and HTS methods (variation explained 17.9%), followed by qPCR and MorphoID (variation explained 9.8%), and HTS and MorphoID (variation explained 7.3%, Figure 2). However, we also found a significant interaction effect between identification method and plant species ($F_{2,96}$ = 3.4, p < .01; variation explained 4.6%; Figure 2); in particular, the nematode community of *C. jacea* identified by MorphoID was significantly different from *C. stoebe*, while this separation was not found based on the other two methods (Figure 2). Similar to the abundance analyses, there was an interaction effect between plant species and plant origin ($F_{1,96}$ = 3.5, p < .01; variation explained 2.3%; Figure 2).

There were significantly positive correlations between most nematode taxa identified according to the different methods (Supplementary Table S6, Figure 3). In particular, relative abundances of Aphelenchidae, Cephalobidae, *Helicotylenchus*, Plectidae

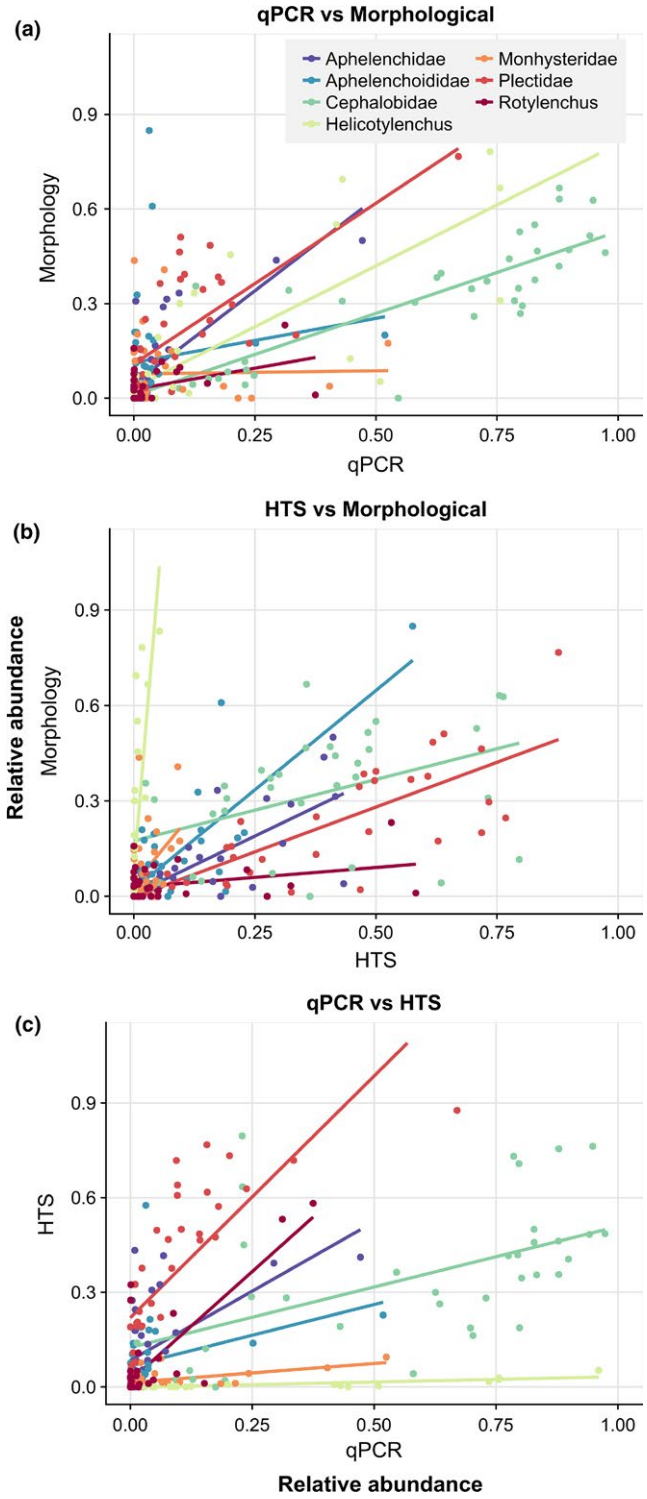

FIGURE 3 Correlations between the abundance of the seven nematode taxa targeted by qPCR that were quantified by microscopy and qPCR (absolute abundances, a) HTS and microscopy (relative abundances, b) and qPCR and HTS (relative abundances, c). See Supplementary Table S5 for detailed comparisons

and *Rotylenchus* were significantly positively correlated between all methods, with especially strong correlations between HTS and MorphoID (Figure 3, Supplementary Table S5).

Comparison between MorphoID and HTS

The community composition consisting of all 50 nematode taxa simultaneously identified by both methods showed the same ecological trends (Supplementary Figure S3). The majority of taxa was significantly positively correlated (18 exceptions based on $p < .05$, 21 based on $p < .01$, Figure 4). The ones that did not correlate significantly were mostly taxa low in relative abundances (lower than average in either MorphoID or HTS: 95.2% for $p < .05$, 94.4% for $p < .01$) and found only sporadically (in less than half of the samples) when determined by MorphoID (66.7% for both $p < .05$ and $p < .01$).

4 | DISCUSSION

In support of our hypotheses, all nematode community analyses including morphological identification (MorphoID), qPCR-based molecular identification and high-throughput sequencing (HTS) revealed changes in the nematode community structure between the range expanding plant *C. stoebe* compared with the native *C. jacea*, particularly reductions of plant-feeding nematodes with the range expanding plant species. However, ecological differences between other functional groups depended on methodology, and these differences were largely due to the representation in absolute or relative abundances, showing that overall our hypotheses have to be rejected.

4.1 | Method comparison

While morphological identification represents the most commonly applied technique to determine identities and abundances soil nematode communities, molecular analyses in form of high-throughput sequencing that are commonplace to study microbes (Geisen, 2016; Prosser, 2015) are now applied more often on animals (Bik et al., 2012; Capra et al., 2016; Eves-van den Akker et al., 2015; Gómez-Rodríguez, Crampton-Platt, Timmermans, Baselga, & Vogler, 2015; Griffiths et al., 2018). We show significantly positive correlations between all methods when focusing on seven functionally diverse nematode taxa targeted by qPCR and between most taxa recovered by HTS and MorphoID. Differences observed between morphological and molecular approaches, especially in relative representation within a community can be explained partly by biases inherent to molecular approaches including incomplete DNA extraction and PCR biases, resulting in over- and under-representation of certain (nematode) taxa (Darby et al., 2013; Geisen et al., 2015; Griffiths, Donn, Neilson, &

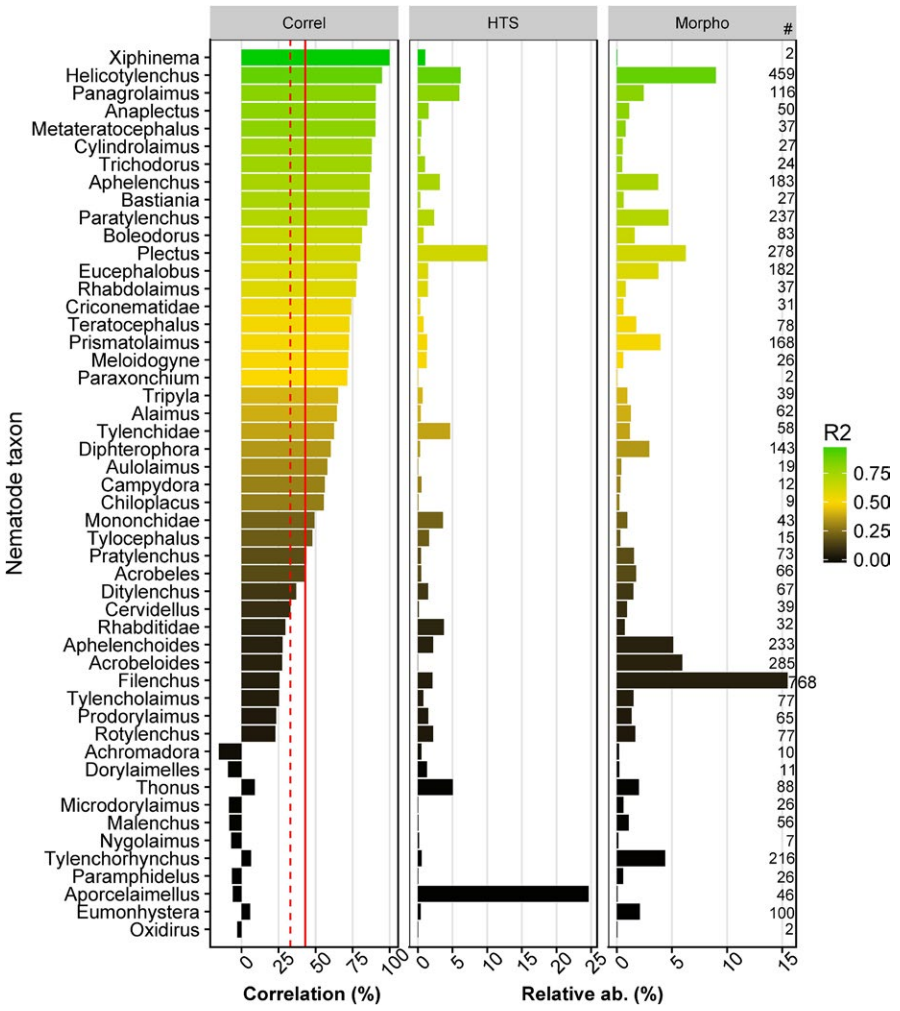

FIGURE 4 Correlation between the relative abundances of all nematode taxa that were identified by microscopy and HTS (left panel), and their relative abundance quantified by HTS (central panel) and microscopy (right panel). The colour of the bars indicates the strength of the relationship (Solid red line: $p < .05$; dashed red line: $p < .01$); #: total number of identified nematodes

Daniell, 2006; Porazinska et al., 2009; von Wintzingerode, Göbel, & Stackebrandt, 1997). For example, bacterivorous nematodes of the group Rhabditida, which are usually non-distinguishable based on morphological characters, and plant-feeding nematodes of the order Dorylaimida are more prominent in PCR-based than morphological approaches, while the opposite holds for other groups such as the plant-feeding Tylenchidae and Aphelenchoides (Darby et al., 2013; Porazinska et al., 2009). The low relative abundances of the root-feeding genera *Helicotylenchus* and *Rotylenchus* in HTS compared with morphological identification are in line with an under-representation in sequence data of Hoplolaimus also a genus within Hoplolaimidae (Porazinska et al., 2009), suggesting a general under-representation of members within this plant-feeding family in HTS approaches. In terms of absolute quantification, amplification biases especially in groups that contain a higher diversity of nematode taxa can be explained by differential amplification efficiency of closely related taxa (Darby et al., 2013; Porazinska et al., 2009). Furthermore, as we here reveal, analyses focusing merely on qualitative diversity data such as inherent to all HTS approaches can lead to different ecological conclusions than those based on the combination of quantitative and qualitative data (Vandeputte et al., 2017). Therefore, it seems unavoidable to count all nematodes linked to qualitative HTS approaches for in-depth community characterization.

Despite these limitations, several advantages are inherent only to molecular tools that could help at speeding up studies on nematodes similar to those on bacteria and fungi (Geisen et al., 2017). The increased taxonomic resolution and high coverage of nematode taxa using our HTS approach were likely possible due to the newly designed universal primer set that targets the most variable V4 region of the 18S rDNA that serves as the proposed barcoding region of eukaryotes (Pawlowski et al., 2012). Therefore, this primer pair, such as others that target a eukaryotic-wide conserved region, should amplify all nematode taxa. In combination with the sequencing depth provided by Illumina's MiSeq technology, we were able to identify a considerably higher diversity of nematodes than both morphologically and in than other HTS studies targeting nematodes before (Darby et al., 2013; Porazinska et al., 2009; Sapkota & Nicolaisen, 2015).

Molecular methods also allow high-throughput differentiation of morphologically indistinguishable taxa to species or even sub-species level (Eves-van den Akker et al., 2015). For instance, we identified eight genera with HTS within the morphologically hardly distinguishable family Rhabditidae (Floyd et al., 2002; Powers, 2004). The increased resolution of HTS is shown by a near doubling of taxa affected by treatments from genus to OTU level which might include ecological keystone taxa. While those taxa in theory could also be distinguished morphologically, this could only be done by few highly skilled taxonomists in a painstaking and therefore costly effort (Table 2) that would rule out higher sample throughput as needed in most ecological studies.

TABLE 2 Overview of total average costs (€) of morphological and molecular work on nematodes when conducted externally subdivided into materials and hours needed (time and office) for experienced technicians/researchers to perform the analyses

		Sample number		
		10	50	100
ALL				
Sample preparation and nematode extraction	Time	4.1	19	39
	Materials	0	0	0
	Total	415	1,939	3,861
MorphoID				
Nematode counting and identification (150 individuals)	Time	21	75	152
	Materials	5.5	28	55
	Office	0.5	1.0	1.5
	Total	2,176	7,648	15,357
qPCR & HTS				
DNA extraction	Time	2.9	4.7	6.7
	Materials	50	250	500
	Total	342	717	1,167
qPCR				
qPCR (7 primer pairs)	Time	1.3	1.7	2.4
	Materials	570	2,850	5,700
	Office	1.1	4.3	7.5
	Total	801	3,442	6,692
HTS				
HTS	Time	2.5	5.4	9.9
	Materials	1,000	1,800	2,550
	Office	4.1	4.5	4.8
	Total	1,663	2,792	4,017
Total costs (€)	MorphoID	2,591	9,587	19,218
	qPCR(7 primer pairs)	1,557	6,097	11,719
	HTS	2,419	5,447	9,044

4.2 | Method combination applied to study plant's range expansion

The distinct methods were applied to study nematode communities, especially plant feeders, as potential underlying drivers of successful plant range expansion. Plants that expand their range can become locally more dominant in the new than in their original habitat. This phenomenon is often attributed to a release from specialized natural enemies that normally control plant performance in the original range, also known as the enemy release hypothesis (Keane & Crawley, 2002). All three identification methods revealed that *C. stoebe* hosted fewer relative and absolute numbers of root-feeding nematodes than the common congener *C. jacea* across geographic locations. While differences in the nematode community composition between the plants studied might partly be due to differences in soil abiotics, controlled experimental studies using the same soil type also found differences in nematode communities such as lower numbers of plant-feeding nematodes with *C. stoebe* (Wilschut et al., 2016).

However, this and other experimental studies (Morriën et al., 2012) investigated *C. stoebe* and *C. jacea* only in soils from the expanded range under controlled (greenhouse) conditions. Our results show that *C. stoebe* generally has low numbers of plant-feeding nematodes than *C. jacea* and not only in northern soils partly rejecting Hypothesis 2. The production of nematode-repelling secondary chemicals might explain the reduced numbers of *C. stoebe* to plant-feeding nematodes (Wilschut, Silva, Garbeva, & van der Putten, 2017; Wilschut et al., 2016). Whether or not this points at local adaptation of root-feeding nematodes to *C. stoebe* in its original range will require further study. Reduced exposure to plant-feeding nematodes in northern soils suggests a benefit for *C. stoebe* to shift range, as can be demonstrated by plant–soil feedback experiments (Engelkes et al., 2008). However, plant-feeding nematodes may not necessarily be the prime cause of the observed variation in plant–soil feedbacks (Morriën et al., 2012).

Centaurea stoebe changes below-ground microbial communities (Callaway, Thelen, Rodriguez, & Holben, 2004) potentially by producing secondary metabolites repelling for instance root-feeding nematodes (Wilschut et al., 2017) explaining the differences observed here between *C. stoebe* and *C. jacea*. This might provide *C. stoebe* a growth advantage particularly in novel environments (Callaway & Ridenour, 2004; Callaway et al., 2004).

5 | CONCLUSIONS

We show that morphological, qPCR- and HTS-based nematode identification methods all reveal differences in the community composition of nematodes between the root zone of the range-expanding plant species *C. stoebe* and the congeneric native plant species *C. jacea*. Although morphological and molecular tools may be biased in distinct directions, most nematode groups targeted significantly positively correlate between all methods, promising feasible standardization and calibration between morphological and molecular approaches (Darby et al., 2013). Such calibrations preferably should be performed in different soils and ideally with known nematode communities (Darby et al., 2013; Griffiths et al., 2018).

We show that molecular tools, especially HTS, provide higher taxonomic resolution than morphological analyses (300 OTUs in more than 100 assigned genera compared with 71 genera/families) and are feasible to scientists with little expertise in morphological nematode identification. Furthermore, molecular tools allow identification of the entire nematode community in a sample and not a subset as analysed in morphological analyses, at an increased sample throughput resulting in profound cost-savings (Table 2), which is essential for large-scale ecological analyses. Therefore, and because taxonomic expertise to morphologically identify nematodes is declining (Neher, 2001), molecular tools are likely becoming more prominent in soil nematode analyses.

However, we reveal that relative abundance data partly provided different ecological patterns than absolute abundance data, which warrant careful interpretation of data obtained merely using HTS such as common in microbial ecology; relative data must be supplemented with absolute data to reliably interpret patterns in ecological studies. This calls for a future integration of morphological quantitative enumeration of all nematodes supplemented with a qualitative in-depth HTS community analysis that can be supplemented with targeted qPCR approaches to provide quantitative data on specific species or genera. This proposed combined methodology will provide a user-friendly, high-throughput and high-resolution analysis method combination to provide both quantitative and qualitative data on soil nematode communities that can be adopted to study aquatic nematodes and also other organisms.

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AUTHORS' CONTRIBUTIONS

S.G. and W.H.vdP. conceived and designed the study; F.C.tH. extracted the nematodes and isolated the DNA; H.D. performed the microscopic determination of nematodes; S.G. designed the new primers that were tested, optimized and applied by J.B. and H.M.; H.D., F.C.tH., L.B.S., O.K. and S.G. collected the data; L.B.S., O.K. and S.G. analysed the data; S.G. and W.H.vdP. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Raw data including final spreadsheets of the morphological identifications, qPCR analyses and OTUs of the sequence data are uploaded to DataverseNL (accession number hdl:10411/ZL0IIU) and Dryad Digital Repository https://doi.org/10.5061/dryad.f3n5ts4) (Geisen et al., 2018). The sequence data can be found at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number PRJEB24755.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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