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RESEARCH ARTICLE

Database and primer selections affect nematode community composition under different vegetations of Changbai Mountain

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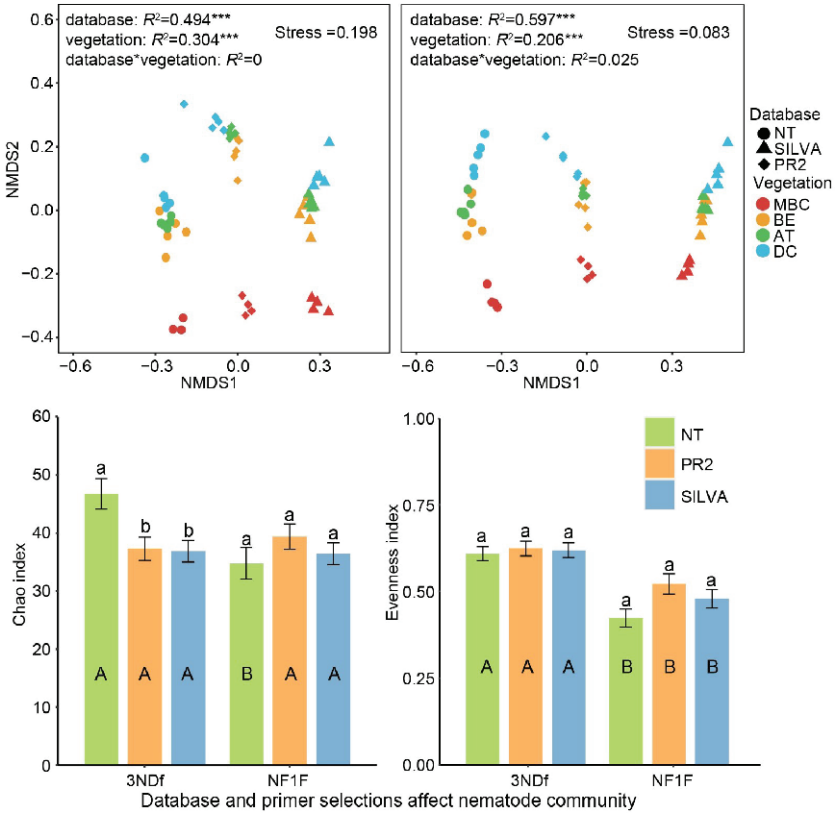
HIGHLIGHTS

- Different primers will affect nematode annotation at different taxonomic levels.
- Sequencing analysis with different primers cannot be compared directly.
- 3NDf primers with NT database could provide more taxa than other combinations.

GRAPHICAL ABSTRACT



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ABSTRACT

High-throughput sequencing technology is increasingly used in the study of nematode biodiversity. However, the annotation difference of commonly used primers and reference databases on nematode community is still unclear. We compared two pairs of primers (3NDf/C_1132rmod,

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NF1F/18Sr2bR) and three databases (NT_v20200604, SILVA138/18s Eukaryota and PR2_v4.5 databases) on the determination of nematode community from four different vegetation types in Changbai Mountain, including mixed broadleaf-conifer forest, dark coniferous forest, *betula ermanii* Cham and alpine tundra. Our results showed that the selection of different primers and databases influenced the annotation of nematode taxa, but the diversity of nematode community showed consistent pattern among different vegetation types. Our findings emphasize that it is necessary to select appropriate primer and database according to the target taxonomic level. The difference in primers will affect the result of nematode taxa at different classification levels, so sequencing analysis cannot be used for comparison with studies using different primers. In terms of annotation effect in this study, 3NDf/C_1132rmod primers with NT_v20200604 database could provide more information than other combinations at the genus or species levels.

1 Introduction

Soil nematodes represent a major component of soil communities in terrestrial ecosystems, and play an important role in regulating nutrient cycling and soil health (Kouser et al., 2021; Li et al., 2021; Swanepoel et al., 2021; Wang et al., 2021). In the past few decades, the rapid development of molecular techniques has the potential to replace traditional morphological analysis (Floyd et al., 2002; Griffiths et al., 2018; Gao et al., 2021). The emergence of next-generation sequencing (also known as high-throughput sequencing) has entirely transformed the field of molecular taxonomy, it can capture more details about biodiversity (Schenk et al., 2020b). High-throughput sequencing technology can generate large amounts of sequences in a short time at a comparatively low cost. Even though high-throughput sequencing technology has been in use for more than ten years (Porazinska et al., 2009), the choice of primers and databases will affect the sequencing results (Peham et al., 2017). Nonetheless, few studies have comprehensively evaluated the combined impacts of different primers and databases on the identification of nematode communities.

To successful characterization of biodiversity, primers should reliably amplify the target group, but they should not bind to non-target DNA in the sample (Peham et al., 2017). In comparison with extracting DNA directly from soil, elutriating nematodes before DNA extraction can provide more nematode reads (Griffiths et al., 2018). As far as we know, there are few universal primers that have targeted all groups of nematodes in the same way. From the widely used primers, NF1F/18Sr2bR primer pair is widely used in different ecosystems (Mullin et al., 2003; Porazinska et al., 2009; Du et al., 2020). The NF1F/18Sr2bR primer had a strong ability to amplify different target loci of nematodes (Porazinska et al., 2009). Recently, the primer pair 3NDf/C_1132rmod has been reported with higher taxonomic resolution and coverage of nematode taxa (Geisen et al., 2018). Researchers utilizing the primer pair 3NDf/C_1132rmod recovered a higher diversity of nematode community (Schenk et al., 2019) and identified more unclassified nematodes at the genus level than other primers (Schenk et al., 2020a). Consequently, these two pairs of primers NF1F/18Sr2bR and 3NDf/C_1132rmod were selected to compare their annotation effects on soil nematode communities in the present study.

In addition, nematode classification can also be affected by the accuracy or integrality of reference databases (Schenk et al., 2019). In-depth management of reference DNA sequence database is a basic requirement of metabarcoding studies (Abad et al., 2016). The choice of database may also impact nematode annotation. The NCBI NT database (https://www.ncbi.nlm.nih.gov/) is the commonly used database at present (Schenk et al., 2020b; Du et al., 2022; Li et al., 2022). A blastn search of shotgun reads against the NCBI NT database could result in false-positive and specificity issues (Borthong et al., 2018). The PR2 database (https://pr2-database.org/) provides exclusive access to the classification of eukaryotic small subunit (SSU) rRNA and DNA sequences. Even though this database is devoted to protists, such outgroup sequences are of high relevance for extracting these groups in further analyses of high-throughput sequencing data sets when universal eukaryotic primers are employed (Guillou et al., 2013). While the SILVA database (https://www.arb-silva.de/) provides information on SSU and large subunit rRNA genes (LSU) (Quast et al., 2013). But some genera have no known match in the SILVA database (Rzeznik-Orignac et al., 2017). Although molecular approaches have great potential in characterization of soil nematodes diversity, their acceptance as a standard method will depend on the improvements in primer generation and database integrity (Gao et al., 2021). Thus, it is worth investigating whether and how the selections of nematode database and primers affect the annotation of nematode community.

Changbai Mountain offers the largest altitudinal range of well-preserved forest habitats in north-east China (Shen et al., 2014; Sun et al., 2020). Along the altitude gradient, variable environmental conditions such as temperature, soil moisture and soil pH, have resulted in an obvious trend of biodiversity. For example, the Shannon diversity and genera richness of nematode assemblages in mixed coniferous-broadleaf forest are substantially higher than those in dark-coniferous spruce forest (Zhang et al., 2012). The vertical distribution pattern of above-ground vegetation will also have a significant impact on the composition of soil biotic communities (Shen et al., 2014; Sun et al., 2020). Dark coniferous forests may be dominated by fungivorous nematodes due to its comparatively difficult decomposed

litter, while broad-leaved forests may be dominated by bacterivorous nematodes (Guo et al., 2007). Thus, the differences in diversity and composition of soil nematode communities in Changbai Mountain provide an ideal platform to verify the influence of different primers and databases on the annotation of nematode sequencing.

In this study, we compared two pairs of primers (3NDf/C_1132rmod and NF1F/18Sr2bR) and three databases (NT_v20200604, SILVA138/18s Eukaryota and PR2_v4.5 databases) to investigate the nematode communities in different vegetation types along the elevational gradient of Changbai Mountain. We try to answer the following questions: 1) if different primers and databases influence the annotation of the nematode community composition; 2) Whether different databases and primers would affect the ecological patterns of nematode communities among different vegetations of Changbai Mountain.

2 Materials and methods

2.1 Study site and soil sampling

The research area is located at the Research Station of Changbai Mountain Forest Ecosystems of the Chinese Academy of Sciences, on the north slope of Changbai Mountain in Antu County, Jilin Province (128°28′ E, 42°24′ N), China. The climate in the region is temperate continental, with long, cold winters and warm summers. The average annual temperature in this area is 2.8°C and the mean annual precipitation is 700 mm. The soil is classified as dark brown forest soil. The north slope of Changbai Mountain is covered with different vegetation types along the altitude. Four different vegetation types were selected along the altitude of 800 m, 1600 m, 1900 m, and 2000 m, respectively, which represent mixed broadleaf-conifer forest (MBC), dark coniferous forest (DC), *betula ermanii* Cham (BE), and alpine tundra (AT), respectively. Five replicates were set in each woodland, and a total of 20 samples (4 vegetation types × 5 replicates) were collected at 0 − 10 cm depth. Soil samples were transported to the laboratory immediately after collection and were divided into two subsamples. One subsample was stored at 4°C for the extraction of soil nematodes, the other subsample was used to measure soil moisture by oven-drying for 8 h at 105°C.

2.2 Nematode DNA extraction, PCR amplification and sequencing

A total of 100 g fresh soil was used to extract soil nematodes using the cotton-wool filter method combined with Baermann funnel method (Oostenbrink, 1960; Townshend, 1963). The extracted nematodes were counted immediately under the stereoscopic microscope, then suspended in 2 mL water solution and retained at 4°C for less than 48 h before DNA extraction. The extraction of nematode DNA used the DNeasy Blood & Tissue Kit (Qiagen), details referred to Du et al. (2021). The extracted nematode DNA isolates were stored at −80°C. The DNA concentrations were determined by NanoDrop 2000 UV-Visible spectrophotometer (Thermo Scientific, Wilmington, USA), the average concentration of nematode DNA was 12.38 ng μL^{-1}. To compare the effects of different primers on sequencing results, two different primer pairs 3NDf/C_1132rmod (5′-GGCAAGTCTGGTGCCAG-3′/5′-TCCGTCAATTYCTTTAAGT-3′) and NF1F/18Sr2bR (5′-GCCTCCCTCGCGCCATCAGGGTGGTGCATGGCCGTTCTTAGTT-3′/5′-GCCTTGCCAGCCCGCTCAGTACAAAGGGCAGGGACGTAAT-3′) were selected to amplify target fragment, which target the V4 regions of 18S rDNA. The total volume of PCR amplification was 20 μL, which contained 4 μL 5 times FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL FastPfu DNA polymerase, 0.2 μL of bovine serum protein (BSA) and 10 ng template DNA. ddH_2O was added to achieve a volume of 20 μL finally. Thermal cycling conditions of 3NDf/C_1132rmod primers were as follows: the initial denaturation step was carried out at 95°C for 3 min and then followed by 30 cycles (denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s), and finally, extension at 72°C for 10 min, ending at 10°C. The PCR amplification of NF1F/18Sr2bR primers was performed as follows: an initial denaturation step was carried out at 95°C for 3 min and followed by 32 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s), and finally, extension at 72°C for 10 min, ending at 10°C. PCR products were detected by 2% agarose gel electrophoresis, loaded with 3 μL sample, and purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's protocol and quantified using QuantiFluor™ -ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.3 Bioinformatics

To obtain an annotated OTU table for nematodes, we used the following pipeline: FLASH was used to splice the double-endian sequence. Afterwards, the sequences were quality-controlled by applying FASTP. Subsequently based on 97% sequence similarity, USEARCH was used to cluster into OTUs (Edgar, 2013). All reads were clustered into OTUs using the UPARSE strategy by dereplication. The raw sequence reads were uploaded to the National Microbiology Data Center (NMDC) with accession number NMDC40017519.

Each sample was amplified by two pairs of primers 3NDf/C_1132rmod and NF1F/18Sr2bR to obtain 852 616 and 1 013 885 sequences, respectively, which were divided into 517 and 443 OTUs. Subsequently, nematode OTUs

were taxonomically assigned to genus level against the quality curated NT_v20200604, SILVA138/18s Eukaryota and PR2_v4.5 databases. Primers are subsequently simplified to 3NDf and NF1F. Databases are simplified to NT, SILVA and PR2 in the text. The proportion of nematodes annotated by 3NDf primers in three databases were 93.4% (NT), 94.8% (SILVA) and 94.4% (PR2). The proportions of nematodes annotated by NF1F primers were 77.6% (NT), 91.6% (SILVA) and 96.2% (PR2) in the three databases. These percentages refer to the proportion of nematodes in all species noted by these primers.

To compare the annotation performance of NT, SILVA and PR2 database, we retained all nematodes at the phylum and class level directly from the raw data, in order to reduce the impact of sampling on the nematode community, the same sequence of each sample was randomly selected from NT, SILVA and PR2 database for subsequent analysis (Supplementary Table S1). Due to the incomplete annotation of nematode taxa in SILVA and PR2 databases, we performed an integrated analysis based on selected species. The information of nematodes at the genus and family levels was integrated from the species level for these two databases. We summarized the annotation results of different primers in SILVA and PR2 databases and conducted a subsequent comparative analysis with the NT database.

2.4 Statistical analyses

The effects of primers and databases on the nematodes taxa at the family, genus, and species levels were analyzed by one-way ANOVA. Venn graph was used to analyze the share and unique taxa among NT, SILVA, and PR2 databases at different taxonomic levels. Heatmap graphs were made based on the shared taxa of three databases to show the differences at the genus level. The relative abundance of each shared genus was log-transformed and plotted. To characterize the alpha diversity of soil nematode community, four diversity indices were calculated, including Shannon–Wiener diversity, Simpson index, Chao index and Shannon evenness. The effects of vegetation types, primers and database on soil nematode alpha diversity were analyzed by one-way ANOVA. The effects of primers and databases on nematode community composition were determined by PERMANOVA analysis. Non-metric multidimensional scaling (NMDS) plots at the genus level were used to represent the differences in community composition between two pairs of primers and three databases. Statistical analysis was performed in R software, version 4.0.5 (R Core Team, 2019).

3 Results

3.1 Taxonomic identification and shared taxa of nematode communities

The annotation of nematode communities was significantly affected by the primers and databases. For 3NDf primer, the number of annotated taxa was different among the three databases at the genus and species levels; while for NF1F primer, significant differences were found at the family level among the three databases (Fig. 1). The combination of primer 3NDf and NT database annotated the most taxa at the family, genus, and species levels, whereas the combination of primer NF1F and NT database annotated the fewest number of nematode family (Fig. 1). Nematode sequences blasting against the database annotated more different taxa with the increase of taxonomic level from class to species (Table S2).

For the primer of 3NDf, the shared nematode taxa identified by three databases were 45 families, 58 genera and 35 species, respectively (Fig. 2A). While for the primer pair NF1F, the common taxa identified by three databases were 32 families, 36 genera and 15 species (Fig. 2B). NT database covered the largest percentage of the three databases, which can annotate more nematode taxa compared with SILVA and PR2 databases (Fig. 2). The heat map showed that *Tripyla*, *Clarkus*, and *Trichodorus* were the

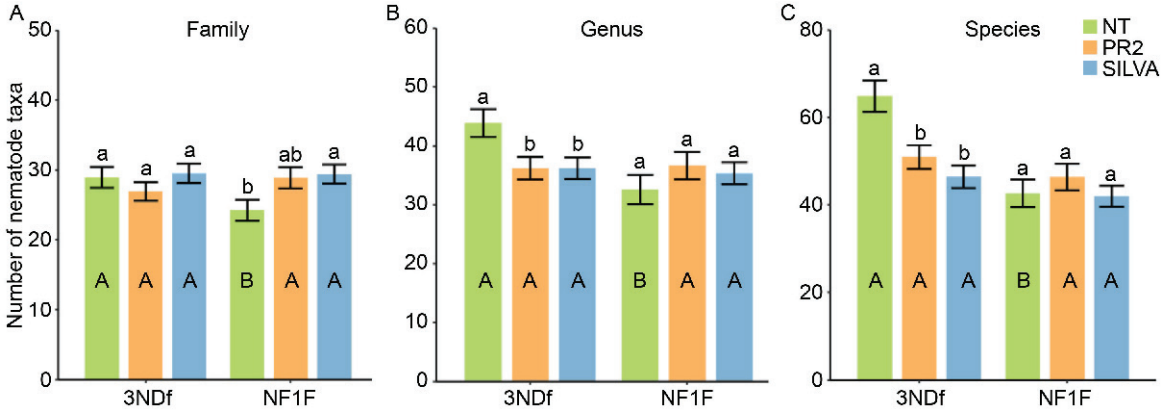

Fig. 1 The number of nematode taxa at the family (A), genus (B), and species (C) levels obtained from different primers and databases. Different capital letters indicate significant differences at $P < 0.05$ level between two pairs of primers in the same database and the lowercase letters represent significant differences among the three databases with the same pair of primers.

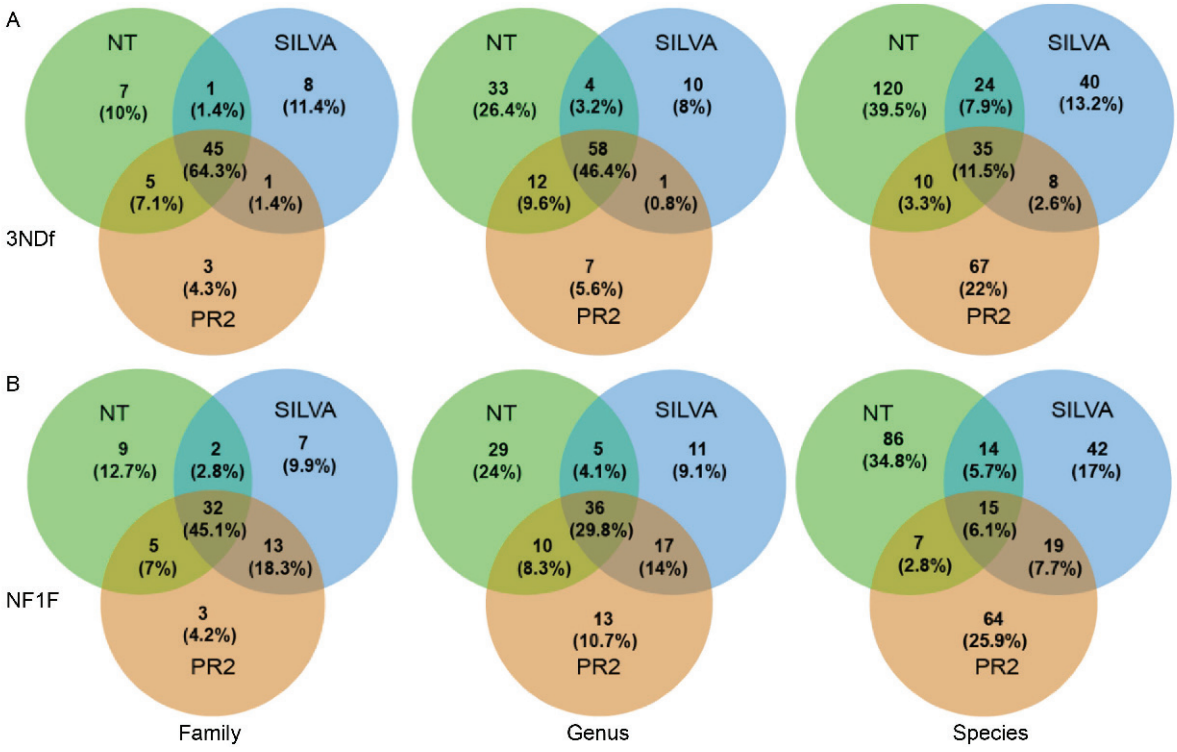

Fig. 2 Venn diagram of the sharing taxa obtained with primers 3NDf (A) and NF1F (B) in NT, SILVA and PR2 databases at different taxonomic levels.

most abundant genera in the three databases. For the 3NDf primer, the abundance of *unclassified_c_Enoplea* in NT database is lower than the other two databases and the abundance of *unclassified_p_Nematode* in NT database is higher than that in the other two databases (Fig. 3A). But *Tripyla* annotated by NF1F in SILVA database and *Clarkus* annotated by NF1F in NT database have lower relative abundance compared with other databases (Fig. 3B).

3.2 Diversity indices of nematode communities

The distribution patterns among different vegetations were similar in nematode diversity obtained by different primers and databases (Fig. 4). For two pairs of primers and three databases, the dark coniferous forest had the lowest value of nematode diversity and the mixed broadleaf-conifer forest had the highest diversity in comparison with other vegetations. Higher diversity were found in indices annotated by the primer 3NDf compared with the primer NF1F, especially for Shannon diversity and evenness indices (Fig. S1). For the primer 3NDf, the Chao indices obtained by the three databases were slightly different, with higher values observed in the NT database (Fig. S1).

The four vegetations were diagonally distributed along the first and third quadrant with the two primers in NT database (Fig. 5A). For the two pairs of primers, all the vegetation types were vertically distributed in SILVA database (Fig. 5B). The distribution trend of the four vegetations was consistent in the three databases, with obvious differences observed between mixed broadleaf-conifer forest and the other vegetation types (Fig. 5D, E). NMDS indicated that there were significant differences in the nematode communities obtained by different primers and different database annotations, while similar conclusion could be obtained when we compared the distribution patterns of nematode communities among different vegetations regardless of which primers or databases were used (Fig. 5).

4 Discussion

4.1 Effects of primers and databases on nematode community composition

Pevious studies have identified that nematode communities are not equally represented by different primer sets (Schenk et al., 2019; Schenk et al., 2020a) and different primers varied considerably in the nematode identification (Peham et al., 2017; Treonis et al., 2018). Our study further supports this finding that different primers had a significant influence on the composition of nematodes taxa. Problems associated with these biased results may be due to primer mismatches, gene copy numbers, and PCR bias, all of which can lead to misamplification and the under- or over-representation of nematode taxa. For example, Schenk et al. (2020a) found that intragenomic variation causes differences in gene copy numbers, which is also a factor leading to differences in nematode annotation. The difference can also be attributed to the known tendency of metabarcoding for missing

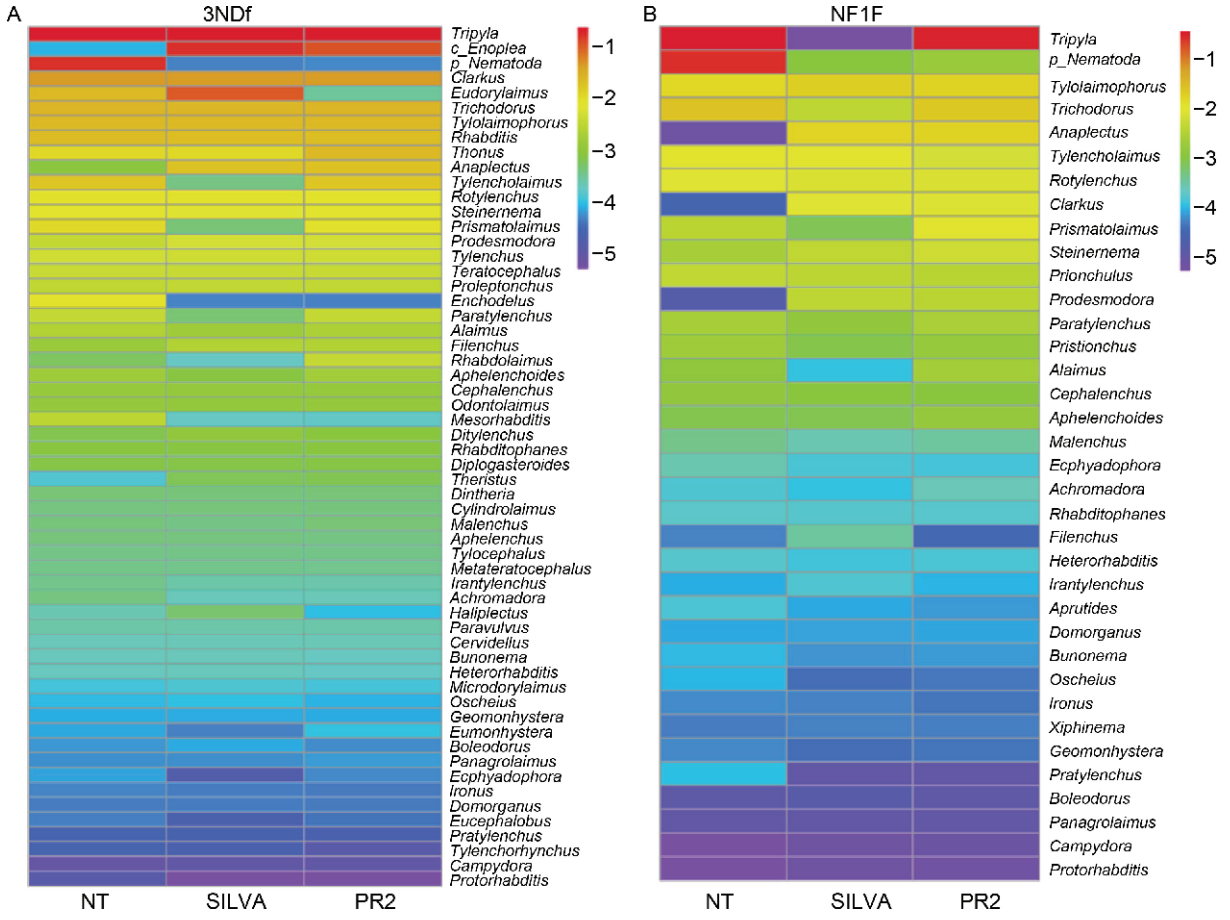

Fig. 3 The heat map of relative abundance of shared nematode taxa obtained with primers of 3NDf (A) and NF1F (B) in different databases. The legend from −5 to −1 represents the least to the most numerous genera.

species, either due to insufficient DNA or due to possible primer mismatches (Schenk et al., 2020a). Consequently, the selection of primers will affect nematode identification at different classification levels.

Among different databases, differences were also discovered in the nematode taxa. The finer the taxonomic level, the fewer taxa were shared by the three databases. These results indicated that every database is incomplete with some missing taxa. We did not compare nematodes annotation at the OTU level because the sequences obtained from various databases at the OTU level are not under the same criteria and this comparison makes no valuable information (Xue et al., 2018). In addition, at the genus level, SILVA and PR2 databases yielded names with meaningless letters, which made it impossible to quickly identify the nematode genus. Thus, for SILVA and PR2 databases, we need to integrate information at the genus and family levels to obtain conventional genus names, which is more complex than NT databases. This indicates that some databases are incomplete in nematode annotation at different classification levels. Further database management will improve the effective use of molecular species identification (Schenk et al., 2020b). The establishment of a complete gene expression database is also of great significance for understanding the pathogenic mechanisms of various parasitic nematodes (Wang et al., 2013). In fact, the parasitic nematodes in the database are much clearer than non-parasitic species. Studies have shown that the generation of well-organized and full-length sequence data sets is particularly crucial for improving molecular recognition of soil nematodes, especially for non-parasitic species (Gao et al., 2021).

In general, both primers and databases choices influence the nematode taxa identity. Therefore, the selection of primers should consider not only the characteristics of the primers themselves, but also the researcher's actual needs. For the assessment at a high taxonomic level (family or class level), there is a little difference between the two pairs of primers. While it is crucial to select the most representative database for further analysis, otherwise the annotation may be biased. Consequently, in terms of annotation effect, 3NDf primers with the NT database could provide more information than other databases from the genus or species levels in this study.

4.2 Effects of primers and databases on diversity of nematode community

The alpha diversity of nematode community in different vegetations were consistent between the two primers, but

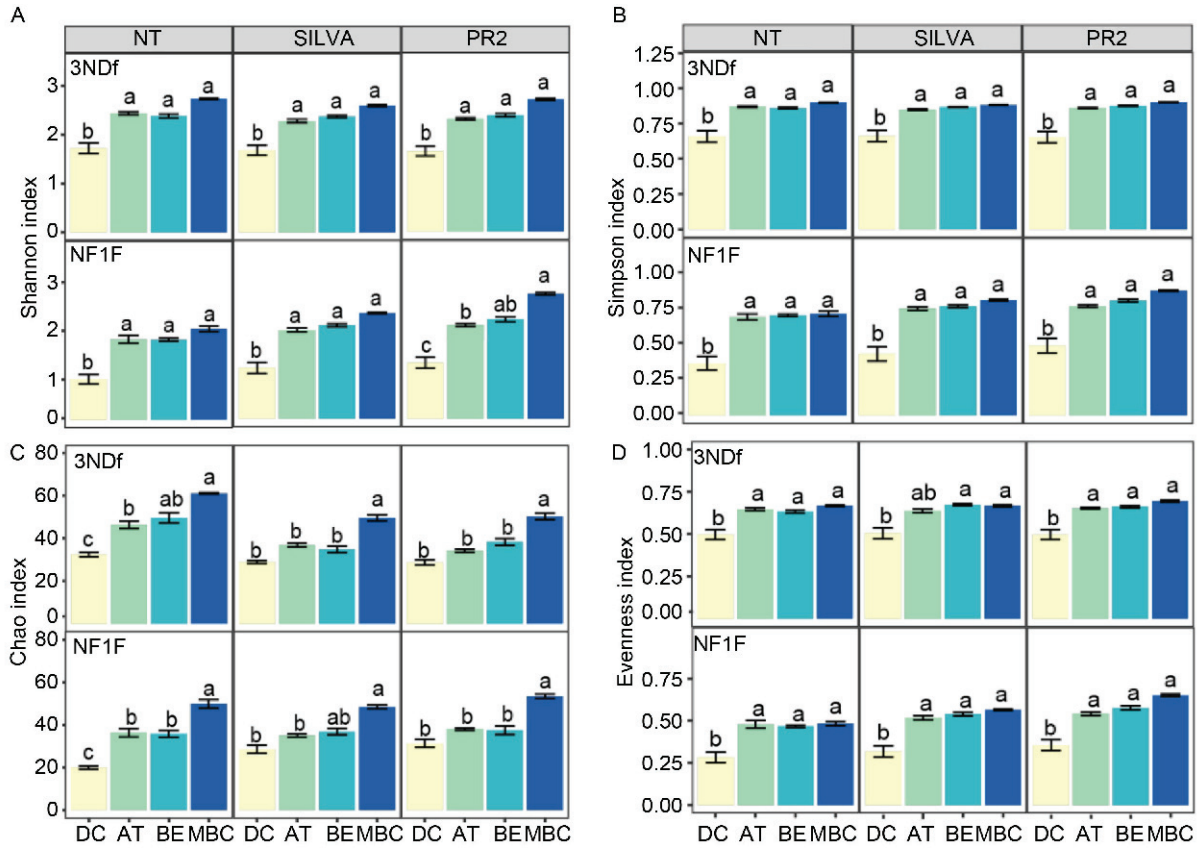

Fig. 4 Diversity indices of nematode community including (A) Shannon index, (B) Simpson index, (C) Chao index and (D) Evenness index. DC, AT, BE and MBC represent the dark coniferous forest, alpine tundra, *betula ermanii* Cham, and mixed broadleaf-conifer forest, respectively. Data are shown as mean ± S. E. Different letters indicate significant differences at $P < 0.05$ among four vegetation types.

the absolute values of taxa number obtained by the two primers were significantly different (Fig. 1). This indicates that while sequencing analysis may be accurate in inter-treatment comparisons, it cannot be used to compare with other studies using different primers. In addition, different databases had no effect on the nematode alpha diversity among different vegetations, and the nematode community composition indicated by NMDS also showed similar patterns in different databases and primers. Our findings indicate that the selection of database could have little influence on the ecological patterns of the different vegetations. However, in order to study the composition of nematode taxa, it is necessary to integrate the results of different databases and cross-verify with multiple primers. Among the three databases, we found that NT database annotated the most nematodes at different taxonomic levels compared with other databases. The information given by PR2 and SILVA databases is incomplete at the family level. Future research should combine information from different databases to obtain more detailed information which is more conducive to the thorough analysis of high-throughput sequencing. Although studies have compared the consistency of results obtained by morphological and molecular methods, no consistent conclusions have been reached (Du et al., 2020; Gao et al., 2021). Therefore, further validation and studies are needed in the future to compare the consistencies of the two methods in-depth and to improve the accuracy of molecular biological methods.

5 Conclusion

The selections of different primer and database could influence the composition of nematode communities at different taxonomic levels. Therefore, when choosing primer and database, we should pay more attention to the classification level according to our actual needs. When the main goal is the investigation of species composition and taxonomy, the sequencing method still suffers from poor consistency, so traditional microscopic identification is still indispensable. When the researchers focused on the ecological indicators or differences between communities, the annotation results of commonly used primers and databases have a good consistency for inter-treatment comparison. In addition, despite the rapid development of molecular techniques, some databases still have incomplete information. In the future, we should supplement more primers and database information to further improve the accuracy of molecular analysis in nematode ecological research.

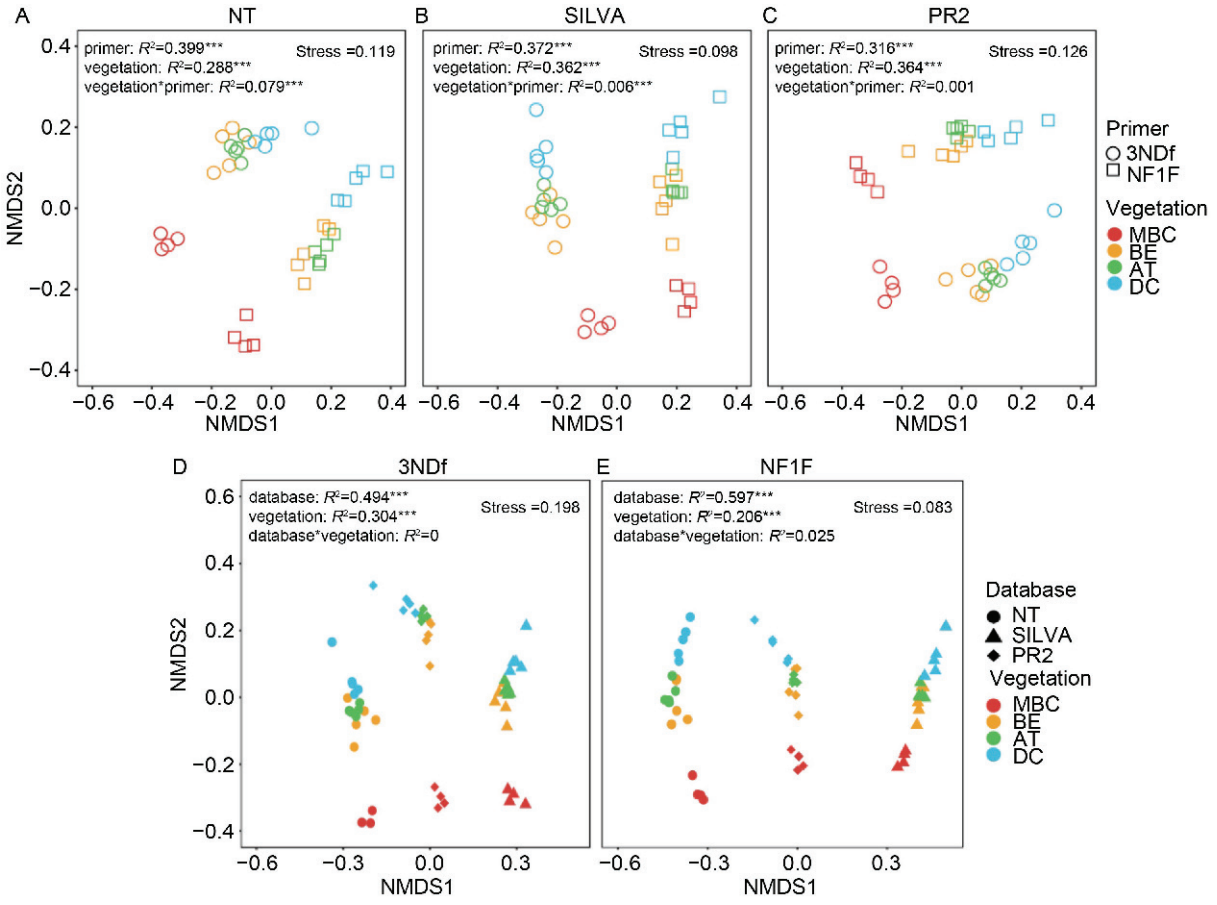

Fig. 5 NMDS plots based on Bray-Curtis distance compared the nematode genus composition of two primers in four vegetations among NT (A), SILVA (B) and PR2 (C) databases; and nematode community composition among the three databases for two pairs of primers with 3NDf (D) and NF1F (E), respectively. DC, AT, BE and MBC represent the dark coniferous forest, alpine tundra, *betula ermanii* Cham, and mixed broadleaf-conifer forest, respectively.

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Compliance and ethics

All authors report no conflicts of interest.

Electronic supplementary material

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