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Prevalence and Diversity of Plant Parasitic Nematodes in Irish Peatlands

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Abstract: The prevalence of plant parasitic nematodes (PPN) in the Irish peatlands was investigated in five different peatland habitats—raised bog, cutover scrub/woodlands, fens and peat grasslands, which were further sub-categorised into fourteen different sub-habitats. Within the raised bog habitat were healthy bog hummock (HBH), healthy bog lawn (HBL), degraded bog hummock (DBH) and degraded bog lawn (DBL) and the fen habitats were fen peat (FP) and rich fen peat (R-FP). Cutover scrub or woodland habitat included cutover scrub rewetted (C-RW), cutover scrub non-rewetted (C-NRW), woodlands rewetted (W-RW) and woodlands non-rewetted (W-NRW). Grassland included wasted peat (WP), rough grazing (RG-I) and improved fen peat grassland (IFPG-RW and IFPG-NRW). Soil samples from peatlands were all collected between July and December 2023 when the temperature ranged from 12 to 20 °C. One half of each sample was used for molecular nematode analysis and the other half for morphological identification of nematodes. For the morphological identification, a specific nematode extraction protocol was optimised for peatland soils, and the extracted nematodes were fixed onto slides to be studied under a high-power light microscope. Subsequently, the other part of the soil was processed to isolate total DNA, from which the 18S rRNA gene was sequenced for the identification of nematode taxa. The extracted DNA was also used for randomly amplified polymorphic DNA (RAPD) fingerprinting analysis to determine banding patterns that could classify different bog habitats based on PPN random primers. Compared to that in the climax habitats (HBH, HBL, DBH, DBL, FP, R-FP), PPN prevalence was recorded as being higher in grasslands (WP, RG-I, IFPG-RW and IFPG-NRW) and scrub/woodland ecosystems (C-RW, C-NRW, W-RW, W-NRW). The results indicate that nematode populations are different across the various bog habitats. Emerging and current quarantine PPN belonging to the families Pratylenchidae, Meloidogynidae, Anguinidae and Heteroderidae were noted to be above the threshold limits mentioned under EPPO guidelines, in grassland and wooded peatland habitats. Future actions for PPN management may need to be considered, along with the likelihood that these PPN might impact future paludiculture and other crops and trees growing in nearby agricultural lands.

Keywords: peatlands; healthy bogs; degraded bogs; paludiculture; wasted peat; plant parasitic nematodes

Citation: Pulavarty, A.; Klappauf, T.; Singh, A.; Molero Molina, P.; Godjo, A.; Molleman, B.; McMillan, D.; Kakouli-Duarte, T. Prevalence and Diversity of Plant Parasitic Nematodes in Irish Peatlands. *Diversity* **2024**, *16*, 639. https://doi.org/10.3390/d16100639

Received: 19 July 2024
Revised: 5 September 2024
Accepted: 4 October 2024
Published: 15 October 2024

1. Introduction

Climate change and biodiversity loss are two of the most significant problems posing threats to the stability of current and future human societies. The continuous increase in greenhouse gas emissions, particularly those of carbon dioxide (CO_2) and methane (CH_4), intensifies the greenhouse effect, contributing to the alteration of Earth's climate and leading to widespread climatic changes. Avoiding emissions and capturing CO_2 from the atmosphere in healthy natural peatlands is one of the most cost-effective ways to combat this.

Peatlands are a unique component of our natural and socioeconomic capital, offering a variety of ecosystem services and functions, such as vital wildlife habitats, recreational spaces, and land for farming and forestry [1]. Peatlands play a significant role as global atmospheric carbon sinks. Peat is a type of soil formed from partially decomposed organic matter, accumulating in waterlogged conditions. In peatlands, a waterlogged environment slows the decomposition rate of dead plant material, preventing the release of CO_2 into the atmosphere. Organic matter, in turn, gradually transforms into peat, locking away carbon over extended periods of time [2].

Europe is home to a large area of wetlands with a high concentration in the northwest, Nordic, and eastern regions. Nearly 10% of Europe's total surface area, or around 1,000,000 km^2, is occupied by peatlands, of which 241,812 km^2 are in the European Union (EU). Only about 320,000 km^2 are covered with 'active' mires or peatlands that are currently creating peat [1] and so are considered to be in good condition. Over half of the peatlands in many peatland-rich EU countries are degraded as the result of artificial drainage, mostly for forestry, agriculture, or peat extraction. In Ireland, over 80% or 1.2 million hectares of peatlands are degraded, but they still store over 2 billion tonnes or 70% of its terrestrial carbon, in only 21% of the land area [3]. Consequently, these areas emit up to 10 million tonnes of CO_2 annually, polluting waters, reducing the water storage capacity of landscapes and depleting the natural flora and fauna of these valuable ecosystems [4].

The Green Restoration Ireland (GRI) cooperative, the enterprise collaborator in this project, is restoring peat grasslands and other peatland habitats as a part of its Farm Carbon EIP (European Innovation Partnerships) project. Through their work, GRI helps fight climate change and biodiversity loss by restoring ecosystem services in a way that aids farmers in diversifying their incomes. To provide economic incentives for the restoration of farmed peatlands, GRI is developing new sustainable farming practices with elevated water tables, in collaboration with farmers, scientists and ecologists. For restoration of the damaged peatlands, alongside standard rewetting approaches, paludiculture (wetland agriculture) has been implemented as a new kind of agriculture in Ireland's first on-farm trials. Paludiculture exploits plants that can thrive in fully rewetted soils, including crops for grass and fodder, fruit, horticulture, vegetables, wood and other raw materials (from, e.g., *Sphagnum* mosses, cattails, etc.). The Intergovernmental Panel on Climate Change (IPCC) has recommended paludiculture as a way to reduce CO_2 and nitrous oxide missions. In recent years, paludiculture has attracted a lot of attention as a viable solution for agricultural peatlands [5]. As a result, many countries have initiated trials in paludiculture in an effort to restore degraded bogs [6,7].

The Molecular Ecology and Nematode Research Group of enviroCORE, at the South East Technological University (SETU), was invited to collaborate with GRI to evaluate the effectiveness of GRI's peatland productivity and restoration programme. This was undertaken by studying and analysing nematode diversity and communities in peatland habitats, before and after restoration. Nematodes are excellent environmental bioindicators because of their high abundance and diversity, being representative of their habitats and displaying well-defined responses to environmental change. In the process of studying nematode diversity, many families belonging to the PPN group have been identified in peatland habitats. The PPNs are well known to cause severe crop losses worldwide and thus are a major problem for crop producers in other parts of the world. Reporting the presence of these PPNs is essential and provides important information for policy makers, farmers and peatland owners, while diversifying Irish agriculture and helping select new crops for paludiculture. The overall results of this project, with details of all recorded nematode taxa (free living, predators, omnivores, herbivores, fungivores, bacterivores, insectivores) present in each peatland habitat, various indices calculated through NINJA, food web analysis, and metabolic footprints will be reported in a future publication.

Based on time availability, taxonomic expertise, financial project resources and accuracy constraints, it might be challenging for nematologists to decide on appropriate nematode identification methods. Each of the morphological, molecular (DNA sequencing)

and DNA fingerprinting techniques has its own advantages and limitations [8]. Implementing conventional morphological identification techniques is time-consuming and requires a lot of skill and expertise. Choosing just one molecular technique is not sufficient nor accurate enough for effectively studying nematode diversity. For nematode diversity assessments in the field using environmental samples, it is advisable to use one fingerprinting technique (such as RFLP, RAPD, AFLP, SSR, microarrays), and sequence analyses of a barcoding gene with bioinformatic analysis, and to complement these results with results from conventional morphological tools [9].

In the current study, the prevalence of PPNs in various peatland habitats in the Irish Midlands was reported using 18S rDNA amplicon sequencing, RAPD fingerprinting analysis, and nematode morphological data. The findings of this study can be used to predict nematode species that might pose a threat to future crop varieties and to inform policy makers to formulate agricultural management strategies in the peatlands so as to avoid or minimize the future potential effects of these pests. This article also discusses a basic description of the vegetation that grows in each peatland habitat to help understand the reasons behind the presence of specific PPN families or genera in specific peatland environments.

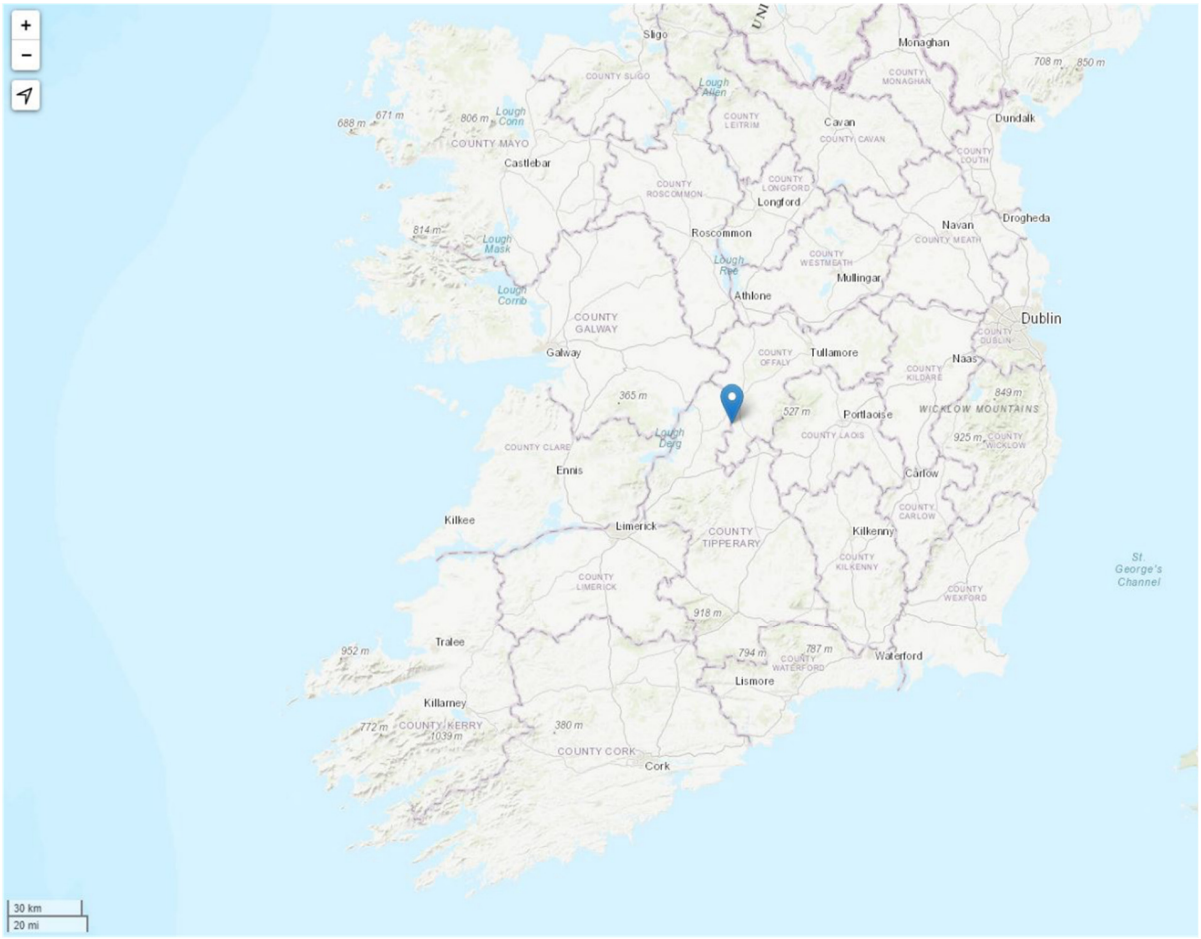
2. Materials and Methods

2.1. Peat Soil Sampling and Description of the Sampling Sites

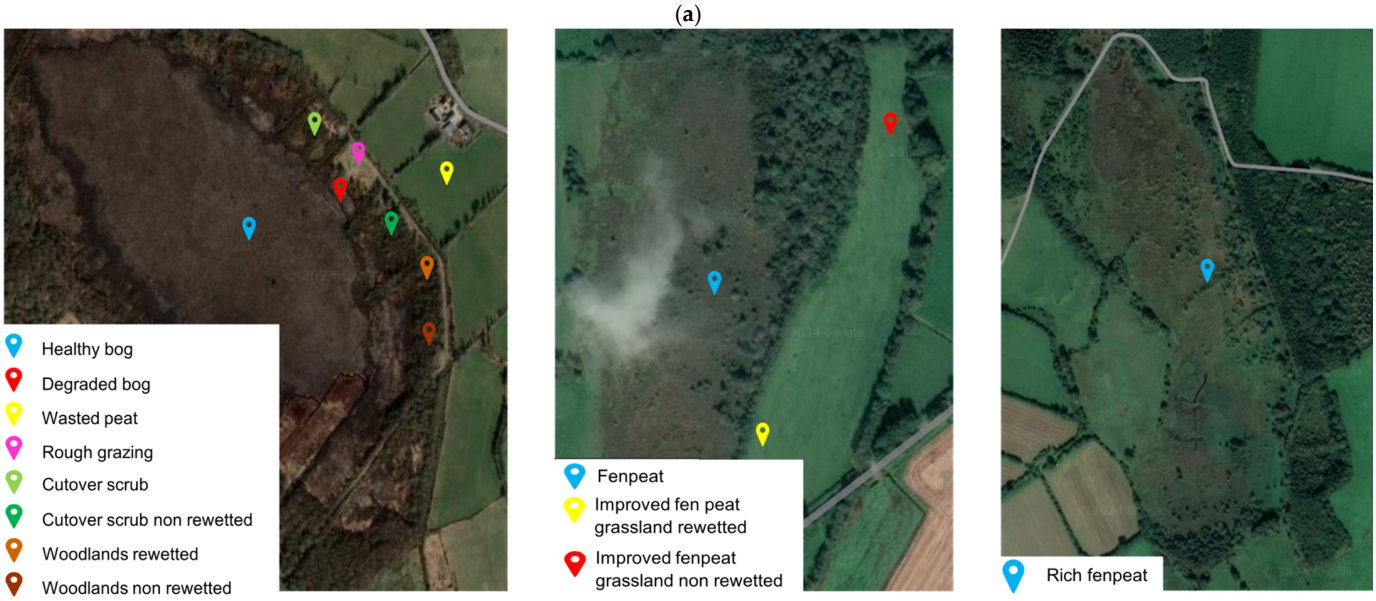
Sampling took place in the months of July up to December 2023, when the average temperature was in the range of 12 to 20 °C. The bog sampling sites are located in the Irish Midlands with Eircodes R42 H026 (site i), R42 F642 (site ii), R42 TW74 (site iii) in County Offaly (Figure 1a). The site co-ordinates were noted as (i) 53°01′14.2″ N and 7°57′15.5″ W, (ii) 53°05′14.01″ N and 7°87′69.96″ W, (iii) 53°06′08.4″ N and 7°80′08.4″ W, according to Google Maps version 11.148.0105 and SW Maps version 2.10.1.0 application. The different peatland habitats in the sampled farms are indicated in Figure 1b. The various peatland habitats investigated in this study were as follows: healthy bog hummock (HBH), healthy bog lawn (HBL), degraded bog hummock (DBH), degraded bog lawn (DBL), wasted peat (WP), rough grazing (RG-I), cutover scrub rewetted (C-RW), cutover scrub non-rewetted (C-NRW), woodlands rewetted (W-RW), woodlands non-rewetted (W-NRW), fen peat (FP), improved fen peat grasslands rewetted (IFPG-RW), improved fen peat grassland non-rewetted (IFPG-NRW) and rich fen peat (R-FP). These habitats were categorized as unmodified climax (raised bog and rich fen), modified or disturbed (grasslands) and successional (cutover scrub and woodland ecosystems) ecosystems. Within the climax ecosystems were healthy bog hummock (HBH), healthy bog lawn (HBL), degraded bog hummock (DBH) and degraded bog lawn (DBL) and fen habitats of fen peat (FP) and rich fen peat (R-FP). The raised bog and fens are climax communities that are relatively unmodified as the natural vegetation has not been removed, but they have been impacted by drainage. Modified, disturbed or grassland habitats included wasted peat (WP), rough grazing (RG-I) and improved fen peat grasslands rewetted and non-rewetted (IFPG-RW and IFPG-NRW). Successional habitats of cutover scrub or woodland ecosystems included cutover scrub rewetted (C-RW), cutover scrub non-rewetted (C-NRW), woodlands rewetted (W-RW) and woodlands non-rewetted (W-NRW).

Samples were collected in a 'W' manner (5 sub-samples per composite replicate), from the top 10–20 cm of the soil/benthos horizon with an auger, taking care to avoid roots and stones. To make three composite replicates, the sampling was performed two more times per site in the same 'W' manner as described above. Each sample was placed in a sealable plastic bag, with proper labelling of the site, peatland habitat and date on each of them, and they were then placed on ice packs within a thermally insulated portable bag. These bags were then transported to the enviroCORE laboratory at SETU, Kilkenny Road Campus, Carlow. In the laboratory, the soil samples were sieved and thoroughly homogenised to prepare three composite replicates (approximately 500 g), each containing 5 sub-samples. Three composite replicates were made in the same way for every peatland habitat. Every composite replicate was divided into two equal parts, with one

part being used for molecular analysis and the other part for morphological identification of nematodes.



(**a**)



(**b**)

Figure 1. (**a**) Approximate site location in the Republic of Ireland; (**b**) Bog sampling location and bog habitats in each location, (i) 53°01′14.2″ N and 7°57′15.5″ W, (ii) 53°05′14.01″ N and 7°87′69.96″ W, (iii) 53°06′08.4″ N and 7°80′08.4″ W; source Google Maps.

2.2. Processing of Soil for Morphological and Molecular Identification of Nematodes

The first half of each composite replicate was used for the extraction of nematodes following the methodology described by Pulavarty et al. [10]. For morphological analysis, nematodes were permanently fixed and mounted onto glass slides using a combination of 8.5% formaldehyde, glycerine and ethanol-based solutions [11]. The mounted nematodes were identified under a high-power light microscope (Euromex Delphi-X Observer, trinocular microscope DX.2153-PLPHi) using the keys, nematode pictures and illustrations from the manuals, books and research articles reported by Gharahkhani et al. [12], Mirbabaei Karani et al. [13], Smythe [14], Schmidt Rhaesa [15], Mekete et al. [16], Holovachov et al. [17] and Bongers [18]. Yeates et al. [19] referred to assigning feeders to the identified nematode families. Miro boards (https://miro.com/ (accessed on 19 July 2024)) were used to arrange the individual nematodes into families, genera and species, with different colour codes assigned based on the feeding habits of the identified nematode. This helped to produce a useful visual fingerprint of each peatland habitat.

The remaining half of each composite replicate was used for DNA extraction. Approximately, 20–25 g of soil from each replicate along with 5–10 mL of dH_2O was added on to a 1000 µm sieve. The filtered out wet soil was collected in a sterile mortar ensuring that it was devoid of roots and other soil debris. This soil collected in the mortar was left at room temperature overnight to let the water evaporate. The soil was then evenly mixed using a sterile pestle and subsequently, 100 mg of soil per replicate was used for total soil DNA extraction using the Qiagen DNeasy® PowerSoil® Pro kit, according to the manufacturer's instructions. The total soil DNA was quantified using both Invitrogen™ Qubit 4 Fluorometer and NanoDrop™ and its quality and integrity were confirmed by performing agarose gel electrophoresis. DNA extracted from each composite replicate was used for the following analysis:

(a) Randomly amplified polymorphic DNA (RAPD) reaction

Twenty-four random primers reported by Randig et al. [20], Chacon et al. [21] and Caswell-Chen et al. [22] were obtained from Metabion International (Germany). Among them, 14 primers (5′ to 3′) produced clear and unambiguous bands (A-5 (AGGGGTCTTG), A-6 (GGTCCCTGAC), A-7 (GAAACGGGTG), A-9 (GGGTAACGCC), A-10 (GTGATCGCAG), A-12 (TCGGCGATAG), A-13 (CAGCACCCAC), A-15 (TTCCGAACCC), A-16 (AGCCAGCGAA), A-18 (AGGTGACCGT), A-19 (CAAACGTCGG), A-20 (AGGTCACTGA), A-22 (CATTCGAGCC), A-24 (CCCGCTACAC)). These fourteen primers were used in this investigation to carry out a phylogenetic analysis and distinguish genetic polymorphism in DNA extracted from different peatland habitats in the Irish Midlands. The amplification was performed in 20 µL PCR reaction mixtures containing 1x QuantiTect SYBR Green PCR master mix (10 µL), 2 µM primers (Metabion International, Planegg, Germany), 50–60 ng DNA and 2.5 mM $MgCl_2$. A Bioer GeneExplorer Thermal Cycler was programmed as follows: 94 °C for 1 min, followed by 45 cycles of 94 °C for 1 min, 35 °C for 1 min and 72 °C for 2 min. The RAPD fragments were separated on a 2% agarose gel pre-stained with nucleic acid dye (Diamond™, Promega Corporation, Madison, WI, USA) solution using 1X TAE buffer. The gels were run for 4 h at 50 V and the RAPD fingerprint profiles were visualized under UV light and recorded with the Cell Biosciences Gel Documentation System. The size of the amplified fragments was determined using the Promega 1 Kb Ladder (G571A). All RAPD reactions were performed three times to confirm the reproducibility of the profiles.

(b) Amplicon sequencing of the DNA samples

The nematode18S V4 rRNA region was sequenced using the MN18F (5′CGCGAATRG CTCATTACAACAGC 3′) and 22R (5′GCCTGCTGCCTTCCTTGGA 3′) primer pairs [23,24], on an Illumina paired-end platform. This analysis was performed by Novogene (UK) Ltd., (Cambridge, UK) and the data obtained from them were studied and analysed to investigate nematode biodiversity in the peatland soil samples. Operational taxonomic units (OTU), relative abundance, heat maps, bar plots, and alpha and beta diversity results obtained from the sequencing company were studied and analysed, as detailed in Pulavarty et al. [25].

2.3. Analysis of Soil Nematode Survey

The abundance of the various PPN families was calculated from both the morphological and molecular data. Abundance was measured as the number of nematode (belonging to a particular family and genus) individuals present in each sample/habitat. The percentage of PPNs in each habitat was derived by the following equation: % PPN = PPN/Nematodes * 100. For morphological identification, the numerator (PPN) in the equation represents the total number of PPN individuals recovered in each peat habitat and the denominator (Nematodes) in the equation refers to the total number of nematodes extracted from each sample/habitat. For molecular results, the sequencing company provides information as OTU of individual nematode families/genera present in each habitat/sample. For the % PPN calculation with molecular data, the numerator (PPN) refers to PPN OTU (total added together), and denominator (Nematodes) in the equation refers to the total added OTU identified in each habitat/sample.

2.4. Recording Vegetation Growth in Various Peatland Habitats

During the sampling period (July–December 2023), the vegetation within each peatland habitat (trees, herbs, graminoids, mosses and shrubs) adjacent to sampling locations was noted manually through visual observations. Photographs of the shrubs, mosses, trees, herbs and graminoids were captured and recorded from each sampling site. The vegetation information noted from each site was verified by the GRI ecologist collaborator in the project and also through the Irish Vegetation Classification published by the National Biodiversity Data Centre, Botanical, Environmental & Conservation (BEC) consultants and the Department of Housing, Local Government and Heritage in April 2021 (https://biodiversityireland.ie/app/uploads/2021/08/BG.pdf, Accessed on 11 December 2023).

2.5. Statistical Analysis

RAPD data analysis: The RAPD fingerprint pattern information for the fourteen different peatland habitats was converted into binary data matrices by scoring the absence of a band as (0) and its presence as (1). The same scoring criterion was used for each primer–soil sample combination, which consisted of 14 primers and 14 Irish Peat habitat soil samples. The collective data were used to generate a RAPD Index using the following formula:

$$\text{RAPD Index} = \sum_{i}^{n} \text{MRW} \times \text{ScoreBand} / \sum_{i}^{n} \text{Total MRW}$$

where MRW refers to the molecular reference weight of any given band and Score Band refers to either 1 or 0.

The RAPD index data were used to generate a dendrogram by using IBM SPSS statistics software (version 29.0.1.0 (171)), which was based on a proximity matrix developed through hierarchical cluster analysis using squared Euclidean distance as the measurement criterion. The constructed dendrogram depicted the linkage between the various peatland habitats based on PPN diversity.

For the molecular data analysis, the data were analysed by the external company Novogene, UK, as described in Pulavarty et al. [25]. Novogene produced a certain proportion of "dirty data" in the raw data obtained by sequencing. In order to make the information analysis results more accurate and reliable, Novogene first merged the raw data and filtered them to obtain "clean data". Then, OTU clustering was carried out based on effective data. According to the OTU clustering results, taxonomic annotation was performed for the representative sequence of each OTU to obtain the corresponding taxa information and taxa-based abundance distribution. In order to analyse the explanation of grouping factor on the difference in samples and estimate the significance of grouping by permutation test, Novogene used ADONIS (also called permutational MANOVA or nonparametric MANOVA), which is a method of nonparametric multivariate variance test according to the Bray–Curtis distance matrix [26–28].

3. Results

3.1. Morphological Identification of Nematodes

For the morphological identification, stylet shape and length, mouth part, median and basal bulbs, tail shape, gonads, vulva/spicule, spermatheca, and the presence and absence of special features like setae and amphids were observed under the microscope. Based on all the above features, the PPNs identified were categorised into eight different families.

A total of 70–100 nematodes were identified from each peatland habitat. Out of the total nematodes extracted from each peat habitat, 26.0, 4.4, 50.5, 35.6, 17.7, 25.0, 79.2, 27.3, 24.2, 23.1, 8.0, 42.7, 45.8 and 6.7% were herbivores in HBH, HBL, DBH, DBL, WP, RG-I, C-RW, C-NRW, W-RW, W-NRW, IFPG-RW, IFPG-NRW, FP and R-FP, respectively (Table 1). The habitat C-RW (79.2%) had the highest PPN percentage compared to all other habitats, whereas PPN percentages were the lowest in HBL (4.4%) and R-FP (6.7%).

Table 1. Absolute abundance of PPN families in various peat habitats based on morphological identification. The numbers in the table represent the individual nematode numbers.

PPN Families/Peat Habitats	HBH	HBL	DBH	DBL	WP	RG-I	C-RW	C-NRW	W-RW	W-NRW	IGFP-RW	IGFP-NRW	FP	R-FP
Tylenchidae	20.0	4.0	40.0	26.0	4.0	8.0	9.0	8.0	1.0	1.0	2.0	11.0	6.0	8.0
Ecphyadophoridae	0.0	1.0	1.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0	0.0
Hoplolaimidae	0.0	0.0	0.0	0.0	0.0	9.0	51.0	13.0	1.0	0.0	3.0	37.0	12.0	2.0
Pratylenchidae	0.0	0.0	0.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0	2.0	40.0	1.0	0.0
Heteroderidae	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	2.0	2.0	0.0	0.0
Meloidogynidae	0.0	0.0	8.0	0.0	6.0	2.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0
Criconematidae	0.0	0.0	3.0	0.0	0.0	0.0	1.0	0.0	12.0	17.0	0.0	0.0	0.0	0.0
Hemicycliophoridae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	2.0	0.0	0.0	0.0
Total number of PPN	20.0	5.0	52.0	26.0	11.0	22.0	61.0	21.0	15.0	18.0	11.0	94.0	27.0	10.0
Total nematodes extracted	77	114	103	73	62	88	77	77	62	78	138	220	59	149
PPN Percentage (%)	26.0	4.4	49.5	35.6	17.7	25.0	79.2	27.3	24.2	23.1	8.0	42.7	45.8	6.7

Efforts have been made to identify the nematodes up to genus level and species level in some cases. The genera and species identified under each of the eight families are listed in Table 2.

Table 2. Genera identified under each PPN family in each peat habitat. Values in the parentheses indicate the number of nematode individuals of that particular genus.

Habitat	Family	Genus
HBH	Tylenchidae	*Basiria* (18) *Malenchus* (2)
HBL	Tylenchidae Ecphyadophoridae (1)	*Basiria* (3) *Coslenchus* (1)
DBH	Tylenchidae Meloidogynidae Ecphyadophoridae (1) Criconematidae	*Basiria* (11), *Boleodorus* (20), *Coslenchus* (8) *Meloidogyne* (8) *Macroposthonia* (3)
DBL	Tylenchidae	*Basiria* (12), *Boleodorus* (11), *Mirculenchus* (1)
WP	Tylenchidae Pratylenchidae Meloidogynidae	*Coslenchus* (4) *Pratelynchus* (1) *Meloidogyne* (6)

Table 2. *Cont.*

Habitat	Family	Genus
RG-I	Tylenchidae	*Basiria* (6), *Boleodorus* (2)
	Ecphyadophoridae (1)	
	Hoplolaimidae	*Rotylenchus* (1), *Helicotylenchus* (8)
	Heteroderidae	*Heterodera* (1)
	Meloidogynidae	*Meloidogyne* (1)
	Pratylenchidae	*Pratelynchus* (1)
C-RW	Criconematidae	*Macroposthonia* (1)
	Tylenchidae	*Tylenchus* (4), *Basiria* (3), *Mirculenchus* (1), *Coslenchus* (1)
	Hoplolaimidae	*Helicotylenchus* (49), *Rotylenchus* (2)
C-NRW	Tylenchidae	*Tylenchus* (5), *Basiria* (1), *Mirculenchus* (1), *Boleodorus* (1)
	Hoplolaimidae	*Helicotylenchus* (13)
W-RW	Tylenchidae	*Basiria* (1)
	Hoplolaimidae	*Helicotylenchus* (1)
	Criconematidae	*Mesocriconema* (8), *Criconema* (4)
	Hemicycliophoridae	*Hemicycliophora* (1)
W-NRW	Tylenchidae	*Tylenchus* (1)
	Criconematidae	*Mesocriconema* (9), *Criconema* (8)
IGFP-RW	Tylenchidae	*Coslenchus* (2)
	Hoplolaimidae	*Helicotylenchus* (3)
	Pratylenchidae	*Pratelynchus* (1), *Zygotylenchus* (1)
	Heteroderidae	*Heterodera* (2)
	Hemicycliophoridae	*Hemicycliophora* (2)
IGFP-NRW	Tylenchidae	*Basiria* (10)
	Hoplolaimidae	*Helicotylenchus* (37)
	Pratylenchidae	*Pratelynchus* (3), *Pratylenchoides* (37)
	Heteroderidae	*Heterodera* (2)
	Meloidogynidae	*Meloidogyne* (5)
FP	Tylenchidae	*Basiria* (5)
	Ecphyadophoridae (9)	
	Pratylenchidae	*Zygotylenchus* (1)
	Hoplolaimidae	*Helicotylenchus* (12)
R-FP	Tylenchidae	*Coslenchus* (2), *Basiria* (6)
	Hoplolaimidae	*Helicotylenchus* (2)

3.2. Molecular Analysis and Identification of Nematodes and Their Communities in Peatland Habitats

3.2.1. RAPD Analysis

The aim of RAPD analysis was to distinguish various peatland habitats based on PPN populations from various genera in each sample. In order to do so, the single linkage analysis approach was used in this study [29].

A total of 553 amplified bands (PCR product) were observed across all primers with the average number of bands per primer being higher in the range of 2000 bp to 250 bp for all the samples across habitats. The RAPD fingerprint patterns suggested high levels of variations among PPN populations present within the 14 Irish peatland soil samples. Some samples (i.e., HBL, HBH, DBH, DBL and C-RW) exhibited more variations among the amplified bands, which ranged between the sizes of 10,000 bp and 1500 bp while others (i.e., R-FP, FP, W-RW, W-NRW, IGF-RW, IGF-NRW, RG-I, WP) exhibited greater variation in the range of 1000 bp–250 bp. Examples of RAPD gel images of HBL and R-FP are shown in Figure 2.

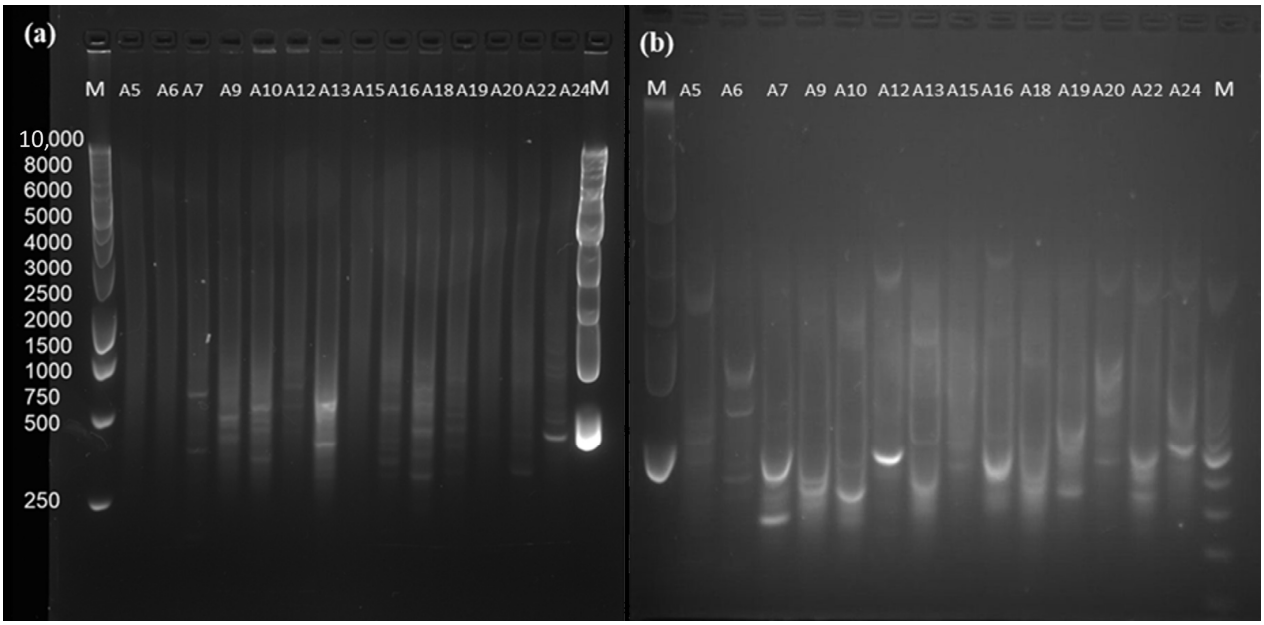


Figure 2. RAPD profile of peat habitats: (**a**) Healthy bog lawn (HBH), (**b**) Rich Fen peat (R-FP) obtained with primers A5, A6, A7, A9, A10, A12, A13, A15, A16, A18, A19, A20, A22, A24. M = Molecular weight marker (Promega 1 Kb Ladder (G571A)).

The genetic distances between the samples varied greatly, ranging from 3.745 to 0.001 (Table 3), with the PPN populations of samples HBL and W-RW showing the largest genetic distance (3.745) (Table 3). By looking at the dendrogram, it can be concluded that the R-FP, W-RW, W-NRW, IGF-RW, IGF-NRW, RG-I and WP habitats were clustered together, whereas, FP, C-NRW, DBH, DBL were closely related. Furthermore, HBL, HBH and C-RW were found to be more similar (Figure 3 and Table 3).

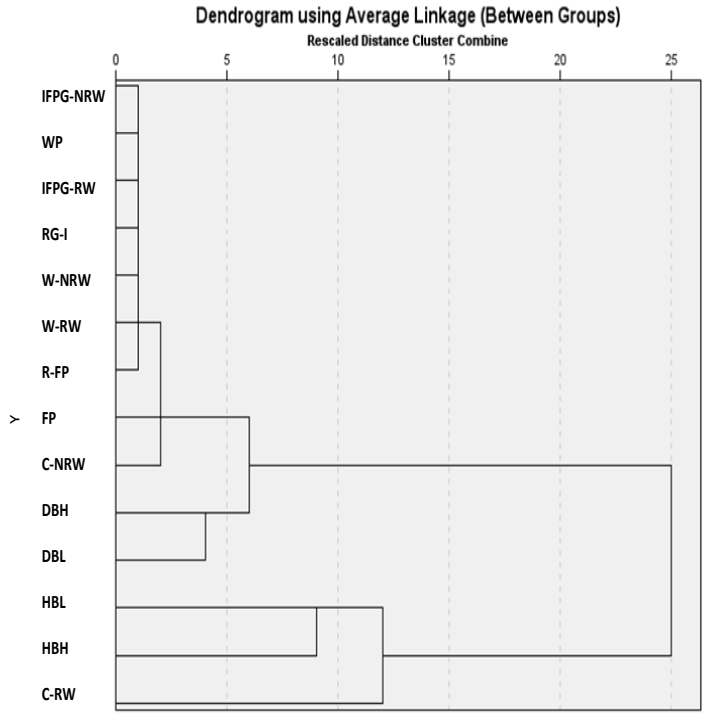


Figure 3. Dendrogram showing the proximity distance between various peatland habitats based on RAPD index data (constructed using IBM SPSS (version 29.0.1.0 (171)).

Table 3. Proximity matrix showing squared Euclidean distance.

Case	HBL	HBH	DBH	DBL	C-RW	C-NRW	R-FP	FP	W-RW	W-NRW	IFPG-RW	IFPG-NRW	RG-I	WP
HBL	0	0.883	2.021	2.328	0.961	2.652	3.203	3.198	3.745	3.74	3.651	3.651	3.671	3.651
HBH	0.883	0	1.259	1.1	1.316	1.589	1.961	2.265	2.461	2.446	2.388	2.388	2.397	2.388
DBH	2.021	1.259	0	0.395	1.343	0.233	0.424	0.481	0.606	0.601	0.592	0.592	0.586	0.592
DBL	2.328	1.1	0.395	0	1.389	0.297	0.509	0.661	0.664	0.664	0.642	0.642	0.639	0.642
C-RW	0.961	1.316	1.343	1.389	0	1.706	2.217	1.963	2.548	2.539	2.487	2.487	2.497	2.487
C-NRW	2.652	1.589	0.233	0.297	1.706	0	0.129	0.15	0.2	0.199	0.191	0.191	0.192	0.191
R-FP	3.203	1.961	0.424	0.509	2.217	0.129	0	0.127	0.083	0.083	0.083	0.083	0.076	0.083
FP	3.198	2.265	0.481	0.661	1.963	0.15	0.127	0	0.116	0.121	0.122	0.122	0.118	0.122
W-RW	3.745	2.461	0.606	0.664	2.548	0.2	0.083	0.116	0	0.002	0.003	0.003	0.001	0.003
W-NRW	3.74	2.446	0.601	0.664	2.539	0.199	0.083	0.121	0.002	0	0.001	0.001	0.001	0.001
IFPG-RW	3.651	2.388	0.592	0.642	2.487	0.191	0.083	0.122	0.003	0.001	0	0	0.001	0
IFPG-NRW	3.651	2.388	0.592	0.642	2.487	0.191	0.083	0.122	0.003	0.001	0	0	0.001	0
RG-I	3.671	2.397	0.586	0.639	2.497	0.192	0.076	0.118	0.001	0.001	0.001	0.001	0	0.001
WP	3.651	2.388	0.592	0.642	2.487	0.191	0.083	0.122	0.003	0.001	0	0	0.001	0

3.2.2. Amplicon Sequencing of the DNA Samples

The results obtained after amplicon sequencing were analysed, and the heat map shows that a total of 21 nematode families were detected in the peatland habitats, and among them, 9 families belong to PPN (Figure 4). The PPN prevalence was found to be the highest in the C-RW (47.1%) habitat followed by W-NRW (42.9%), W-RW (41.8%), C-NRW (39.3%), RG-I (24%), IFPG-RW (23.8%), WP (19.7%), IFPG-NRW (16.1%), F- RP (9.8%), DBH (8%), DBL (3.9%), FP (3.5%) and HBL and HBH (0.6%) (Figure 5). Based on the MANOVA results, the bars in the bar plot (Figure 5) were categorised into three groups.

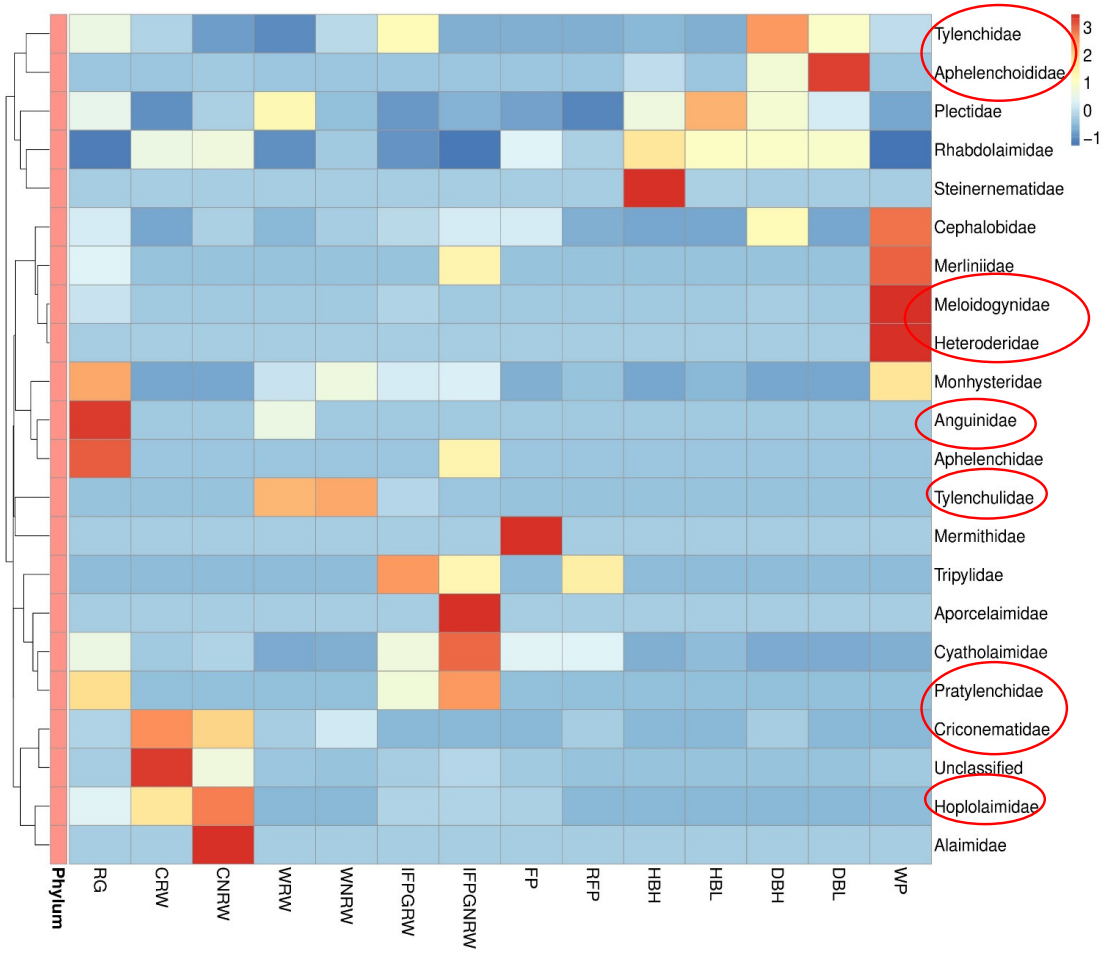


Figure 4. Heat map showing the abundance of different nematode families detected in various peat habitats. The PPN families are highlighted using red ovals.

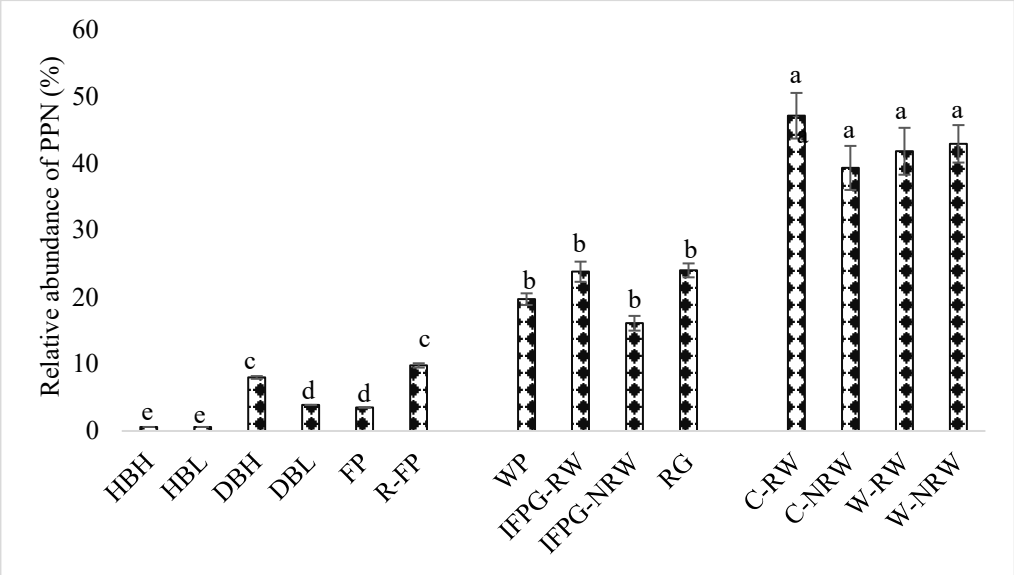


Figure 5. Relative abundance of PPN (%) in different peatland habitats (molecular data). Values represented by similar letters are not significantly different from each other in terms of PPN % ($p \leq 0.05$).

3.3. Vegetation Growth in Various Peatland Habitats

The healthier section of raised bog has a hummock–lawn–hollow complex and a high cover of >80% *Sphagnum* mosses with dwarf ling heather (*Calluna vulgaris*) on hummocks of red bog-moss (*Sphagnum capillifolium* var. *rubellum*), papillose bog-moss (*Sphagnum papillosum*) with some common cotton-grass (*Eriophorum angustifolium*) and pin cushion moss (*Leucobryum glaucum*). The lawns include lustrous bog-moss (*Sphagnum subnitens*) and soft bog-moss (*Sphagnum tenellum*) with hare's-tail cottongrass (*Eriophorum vaginatum*), deergrass (*Trichophorum caespitosum*), cross-leaved heath (*Erica tetralix*), white beak-sedge (*Rhynchospora alba*), bog asphodel (*Narthecium ossifragum*), bog rosemary (*Andromeda polifolia*) and round-leaved sundew (*Drosera rotundifolium*). The hollows include feathery bog-moss (*Sphagnum cuspidatum*) and cow-horn bog-moss (*Sphagnum auriculatum*). However, the hummocks are small (0.25–0.5 metres in diameter) and pools are scarce due to the partial degradation caused by the drying of the bog.

The degraded section of the bog is heath-like and is dominated by ling heather with a bryophyte layer of heath plait-moss (*Hypnum jutlandicum*) and abundant reindeer moss (*Cladonia portentosa*) and algae *Zygogonium ericetorum* with small amounts of cross-leaved heath, bog asphodel and round-leaved sundew. *Sphagnum* mosses are largely absent.

Wasted peat and RG-I are established grasslands used for grazing livestock (cattle and sheep). WP is reseeded improved pasture dominated by creeping bent (*Agrostis stolonifera*), meadow-grasses (*Poa* spp.) and the herbs white clover (*Trifolium repens*), docks (*Rumex* spp.) and nettle (*Urtica dioica*). The rough grazing is a diverse meadow of native grasses (cock's-foot (*Dactylis glomerata*), meadow grasses, creeping bent, false oat-grass (*Arrhenatherium elatius*), couch grass (*Elymus repens*), Yorkshire fog (*Holcus lanatus*), etc., and carnation sedge (*Carex panicea*) and herbs (creeping buttercup (*Ranunculus repens*), common knapweed (*Centaurea nigra*), bush vetch (*Vicia sepium*), meadow vetchling (*Lathers pratensis*), water mint (*Mentha aquatica*), thistles (*Cirsium* spp.) and nettle (*Urtica dioica*). Improved fen peat grassland- rewetted (IFPG-RW) and IFPG-NRW are in the same pasture and are composed of bent and meadow grasses, Yorkshire fog, creeping buttercup, docks and nettle.

The C-NRW has typical vegetation of cutover bog, which is undergoing ecological succession to cutover scrub and woodland. The cutover species include heather, cross-leaved heath, cotton-grasses, purple moor-grass (*Molinia caerulea*), tormentil (*Potentilla erecta*) and milkwort (*Polygala serpyllifolia*) with some Sphagnum and heath plait-moss.

Gorse (*Ulex europaeus*) dominates and includes some rusty willow (*Salix cinerea* ssp. *oleifolia*) and downy birch (*Betula pubescens*).

The C-RW includes hummocks of red bog-moss (*Sphagnum capillifolium* var. *rubellum*) and other species with some heather, heath plait-moss, purple moor-grass and bracken (*Pteridium aquilinum*).

The bog woodland areas are dominated by an abundant growth of downy birch (*Betula pubescens*), willows (*Salix* spp.), holly (*Ilex sps*.) and some sessile oak (*Quercus petraea*) with ivy (*Hedrea helix*) and honeysuckle (*Lonicera pericyclemum*) in the understorey.

The fen habitats (FP and R-FP) are dominated by rushes (*Juncus* spp.) and include lesser spearwort (*Ranunculus flammula*), water mint (*Mentha aquatica*), black bog-rush (*Schoenus nigricans*), purple moor-grass, sedges (*Carex* spp.) and fen mosses (*Campylium stellatum* and *Scorpidium scorpioides*).

4. Discussion

For this investigation, the PPN percentage calculations performed from molecular data are taken into consideration as the individual soil sample size, DNA extraction method and number of replications were kept consistent for all the different peatland habitats. The DNA extracted from these same samples was also used to perform RAPD analysis. In contemporary times, molecular approaches have gained popularity over traditional morphological methods because of their ease of use, technological advancements, reduced costs and accuracy. However, no single molecular technique on its own has the capability to provide a detailed and accurate analysis of any taxon [9].

The information about the genetic constitution nematode communities is crucial for the development of preservation methods and identification of new indicators of successful restoration of peatland ecosystems. Theoretical studies indicate that biological disturbances and habitat degradation lead to genetic variation among populations [30,31]. Other factors that affect genetic variation are the duration of habitat degradation, the size of the population, the vegetation of a habitat, generation length of organisms under study and gene flow between populations. Phylogenetic reconstructions can be a useful approach for comprehending the multitrophic interactions of nematodes with their hosts [30,31].

In this study, RAPD-PCR was used for the differentiation of various samples of peatland soil, and we tried to obtain a visual fingerprint of a peatland habitat and construct a dendrogram to understand proximity distances between the various habitats. The degree of genetic divergence among 14 peatland habitats was expected to be associated with the percentage of DNA fragments shared between them. Graphical hierarchical clustering is a stable type of systematic analysis that can represent important features of ecological conditions [32]. The similarities and dissimilarities in features of samples (i.e., habitat characteristics) result in high or low values of genetic distances in the proximity matrix (Table 3), which in turn affect the clustering of samples in the dendrogram. It is interesting to note that within the depicted dendrogram (Figure 3), healthy bog samples (i.e., HBH and HBL) are in proximity to degraded bog samples (i.e., DBH and DBL). Previous studies suggest that the main reason for the change in nematode communities and the increase in the proportion of PPN is human intervention [33,34]. In this instance, disturbance was not direct through the removal or clearance of vegetation, but it was rather indirect as a result of drainage. According to the Irish Vegetation Classification (IVC; https://biodiversityireland.ie/app/uploads/2021/08/BG.pdf, Accessed on 11 December 2023), the flora of healthy bogs mainly consists of *Sphagnum* mosses. *Sphagnum* may support the growth of moss-feeding nematodes, but it is generally devoid of any parasitic nematodes [35]. Thus, it can be concluded that the proximity between degraded bog samples and healthy bog samples is the result of the low genetic diversity of PPNs in these two ecosystems.

In the presented dendrogram, the grassland habitats (IFPG-NRW, WP, IFPG-RW, and RG-I) are clustered together. The sites of R-FP and FP are similar in terms of organic matter and vegetation, so they were therefore clustered separately from those of raised bog. The

woodland habitats (W-RW and W-NRW) were also noted to have similar vegetation and this explains their clustering together in terms of PPN.

In this study, while performing the morphological nematode identification, it was ensured that the total number of nematodes identified from each peatland habitat should be greater than 50 individuals, but the amount of soil and number of repetitions were not kept uniform across the peatland habitats. This was due to skill, effort, time and taxonomic expertise constraints while performing morphological identification. In practice, it was impossible to keep the sample size and replications identically uniform across the habitats while achieving a threshold of a minimum of 50 nematode individuals per habitat for morphological identification. Due to similar challenges, many reports in the past focused only either on morphological [36] or molecular [24] tools to identify nematodes, but did not utilise both. Recently, nematologists have arrived at a conclusion that based on the objective of their study, the methods adopted for identification should vary [8,9]. For nematode identification, a specimen should first be examined microscopically to determine the lowest possible taxonomic rank, followed by molecular techniques for species or subspecies identification. For quarantine purposes, species-specific molecular barcoding methods can be employed, while diversity assessments in field populations may use fingerprinting techniques, sequence analyses, or high-throughput sequencing with bioinformatic analysis to study environmental samples [8]. For this study, 18S rDNA amplicon sequencing and the RAPD fingerprinting technique were considered, while morphological identification was employed as a backbone and strong reference for understanding and interpreting the molecular results.

The molecular data analysis of PPN diversity relate very well to the ecological and vegetation status of the three peatland habitat groups as categorised in the Materials and Methods (Vegetation growth in various peatland habitats) Section (Figure 5). The WP, RG-I, IFPG-RW and IFPG-NRW peatland habitats were grasslands. Furthermore, the peatland habitats HBH, HBL, DBH, DBL, FP and R-FP are relatively pristine, unmodified and climax habitats. Moreover, the HBH, DBH, HBL, and DBL habitats also had a predominant growth of bog vegetation, including heathers and reindeer moss. Group 3 habitats can be categorised as wooded ecosystems with woody vegetation including gorse in the shrub layer and downy birch, willow, holly and sessile oak in the tree layer of these habitats.

Family Tylenchidae was common to all the peatland habitats (Tables 1 and 2). Nonetheless, nematode individuals were identified as belonging to the genera *Basiria*, *Coslenchus*, *Boleodorus*, *Malenchus*, *Tylenchus* and *Mirculenchus* based on morphological identification. Peatland habitats belonging to group 1, commonly had genus *Basiria*, which is described as composed of algal or moss feeders (http://nemaplex.ucdavis.edu/Taxadata/G019S1.aspx, Accessed on 15 January 2024) and has been reported earlier as being associated with plants growing in naturally acidic wetland soils [37]. The raised bog habitats (HBH, HBL, DBH, DBL) include *Sphagnum*, heathers and reindeer moss growing, and these habitats are highly acidic (pH 3.7) in nature and thus provide ideal conditions to encourage the growth of both *Basiria* and *Malenchus* spp. The next most commonly observed genus across the peatland habitats was *Coslenchus* (Table 2). The nematodes belonging to the genus *Coslenchus* feed and reproduce on seedlings (fescue tussock and rye grass), on mosses (*Tortula princeps* and *Bryum* sp.) and on the rhizosphere of wild grasses [38,39]. Mosses and sedges (graminoids with a grass-like morphology) are abundant in raised bogs and grasses in the grassland habitats belonging to group 2, which could explain the presence of *Coslenchus* spp. The common genus of the family Tylenchidae occurring in DBH and DBL is *Boleodorus* (Table 2). The presence and growth of heathers (*Calluna* spp.) and reindeer moss (*Cladonia* spp.) in this degraded bog habitat is a result of drainage, and this shift in vegetation influences the nematode community structure. *Boleodorus* nematodes were recorded on Amaryllidaceae and may have specific associations with the roots of bog asphodel, a member of the Liliaceae family, which becomes more abundant in drier conditions. However, this is just a projection and there is no specific reference in relation to this observation.

In the PPN family Hoplolaimidae, two genera were identified, *Helicotylenchus* and *Rotylenchus* (Table 2). Both molecular and morphological data show that the habitat C-RW has the highest abundance of PPN when compared to all the other habitats. Gorse plants (*Ulex* spp.) growing in the C-RW and C-NRW habitats have been reported to serve as hosts for PPNs, including those belonging to the family Hoplolaimidae [40]. Gorse, being a leguminous shrub, provides a suitable environment for various nematode species due to its root structure and the organic matter it contributes to the soil. Members of the family Hoplolaimidae, such as *Hoplolaimus* spp. and *Helicotylenchus* spp., are known to parasitize a wide range of plants, including woody shrubs and legumes like gorse [40]. These nematodes feed on plant roots, causing damage that can affect plant health and vigour.

The family Criconematidae, which includes the genera *Mesocriconema*, *Macroposthonia* and *Criconema*, is the other PPN family that was found to exist commonly in peatlands. These nematode genera were predominantly recorded in the peat habitats belonging to group 3 (C-RW, C-NRW, W-RW and W-RW). *Mesocriconema* and *Criconema* spp., belonging to the family Criconematidae, are ectoparasitic nematodes that feed on a wide range of woody and herbaceous plants, including several tree species such as sessile oak, birch, holly and other forest trees [41–43]. These species are present in the W-RW and W-NRW peatland habitats. The presence of *Mesocriconema* and *Macroposthonia* on the tree species has been documented in various studies, highlighting the ecological relationships and potential impacts on tree health and forest ecosystems [44].

Meloidogynidae is a PPN family that has many species that are currently quarantine pests in many countries worldwide. Both molecular and morphological results confirm the abundance of nematodes belonging to the genus *Meloidogyne* in all the peatland habitats belonging to group 2 (WP, RG-I, IGFP-RW and IGFP-NRW), which are all peat grasslands and thus modified ecosystems. All of these habitats are grasslands, but, in the vicinity of WP and RG-I sites, there are potential crops including blueberries, celery, cranberries, garden mint and lettuce. Nematodes of the genus *Meloidogyne* are root-knot plant parasites that cause severe crop losses worldwide [45,46] thus their presence could be a concern for future paludiculture efforts on such lands after rewetting. The growth of many crop varieties in proximity to these habitats could be a reason for the occurrence of the genus *Meloidogyne*. Alongside crops, several ranges of grasses have also been reported to serve as hosts for nematodes belonging to *Meloidigyne* spp. [47,48]. This explains the current finding of *Meloidogyne* spp. in these peatland habitats. Heathers that were growing in the DBH habitat have also been reported to serve as hosts for *Meloidogyne* spp. [49].

Both molecular and morphological data confirm that PPNs belonging to Pratylenchidae (root-lesion nematodes) were common in all the peatland habitats belonging to group 2 (WP, RG-I, IGF-RW and IGF-NRW). The genus *Pratylenchus* was identified as common with both molecular and morphological data. *Pratylenchus* parasitizes grass species and occurs in grasslands under restoration management [50]. This genus was also detected in the grasslands of Northern Ireland [51] and in other grasslands in the Republic of Ireland [52].

Bongers [18] previously classified the genus *Pratylenchoides* under the family Pratylenchidae, but eventually, this genus was grouped into a new family Merlinidae [53]. Nematodes belonging to Merlinidae were recorded in all the habitats belonging to group 2, but the genus *Pratylenchoides* was recorded morphologically only in the IFPG-NRW habitat. This habitat is a grassland and the nematodes belonging to this family (Merlinidae) and genus (*Pratylenchoides*) have been reported previously to grow in the rhizosphere of dune grass [54]. The other nematode genus detected (through molecular analysis) in all the group 2 peatland habitats is *Paratylenchus* in the family Tylenchulidae. This nematode is present in pasturelands of New Zealand [55] and in 35 wild and cultivated grasses and cereals of Canada [56]. Another quarantine PPN family that was detected in group 2 grassland habitats is Heterodoridae (genus *Heterodera*). This nematode is known to cause significant crop losses and has been categorised as one of the top ten most significant PPNs, based on scientific and economic importance [57]. This pest was also reported by Fleming et al. [51] in the grasslands of Northern Ireland. The nematode family Anguinidae has quarantine

pests that belong to the genera *Ditylenchus* and *Litylenchus*. Both of these genera have been identified as emerging pests in Ireland and the EU (EPPO Global Database) in general. In the studied peatlands, PPNs belonging to *Ditylenchus* have been detected in W-RW and in RG-I habitats. However, the exact plant that is serving as a host for this PPN in these habitats is not identified.

According to EPPO guidelines (EPPO Global Database), the current quarantine nematode pests in Ireland are Aphelenchoididae (*Bursaphelenchus* spp.) and Heteroderidae (*Globodera* spp.), while the emerging pests of concern are Anguinidae (*Litylenchus* and *Ditylenchus* spp.) and Meloidogynidae (*Meloidogyne* spp.). The optimum threshold limits for the PPN belonging to family Anguinidae (*Ditylenchus* spp.) is 1 nematode/gm of tissue [58] (McKenry, 1994), for the family Heteroderidae (*Globodera* spp.) is 5 eggs or juveniles/gm of soil [59] (Been et al., 2007), for the family Meloidogynidae (*Meloidogyne* spp.) is 1~2 nematodes/gm of root tissues [60], for the family Pratylenchidae (*Pratylenchus* spp.) is 200~1000 nematodes/kg of soil [61] and for the family Aphelenchoididae (*Bursaphelenchus* spp.) is as low as 1 juvenile/gm of soil [62] (EPPO Global Database). In the peatlands studied, both molecular and morphological results suggest that PPN belonging to the families Anguinidae, Pratylenchidae, Meloidogynidae and Heteroderidae were detected above the permitted threshold levels and have the potential to cause severe damage to future crops and grasslands if not managed efficiently.

The Intergovernmental Panel on Climate Change (IPCC) has recommended paludiculture (wetland agriculture) as a way to reduce greenhouse gas emissions. GRI is developing paludiculture in Ireland as an alternative way to diversify the income of farmers while aiding in the restoration and sustainable management of degraded bogs. Considering the scale of crop losses caused by PPN worldwide [57], it is very important for policy makers and growers in Ireland to take all of these findings in peatlands into account while aiming to establish this form of circular economy.

In this study, vegetation was recorded manually and samples were not intentionally collected to sample for PPN. It is, therefore, difficult to make accurate conclusions as to what plants or trees are serving as the exact hosts for these PPN in peatlands. Future research should be considered to investigate the exact PPN hosts and to employ effective management strategies to control the spread of these pests in Irish peatland soils.

Author Contributions: Conceptualization, A.P.; methodology, A.P., T.K., A.S., A.G. and P.M.M.; software, A.P. and A.S.; validation, A.P., B.M., D.M. and T.K.-D.; formal analysis, A.P., A.S. and T.K.; investigation, A.P.; resources, D.M. and T.K.-D.; data curation, A.P., A.S. and T.K.; writing—original draft preparation, A.P.; writing—review and editing, A.P., D.M. and T.K.-D.; visualization, A.P.; supervision, D.M. and T.K.-D.; project administration, A.P. and T.K.-D.; funding acquisition, D.M. and T.K.-D. All authors have read and agreed to the published version of the manuscript.

Funding: The project was jointly funded by Irish Research Council (IRC) and Enterprise Partner Green Restoration Cooperative Society Ltd. (GRI), Carlow, R93 K883, Ireland under the Enterprise based Postdoctoral Scheme. The Project grant number: EPSPD/2022/91.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data has been stored in the OneDrive folder of A.P., T.K.D., D.M. and library repository of SETU, Carlow campus, Ireland.

Acknowledgments: The authors acknowledge the support of the South East Technological University (SETU), the Irish Research Council (IRC) and Green Restoration Ireland Cooperative Society Ltd. (GRI). The authors would also like to thank the EU Erasmus Programme for funding the stay and study of the Erasmus interns, Tilman Klappauf and Patricia Molero Molina. Last, but certainly not least, the team appreciates the trust and good will by peatland farmers and landowners who allowed us to carry out our research in their fields.

Conflicts of Interest: Author Bastiaan Molleman and Douglas McMillan were employed by the company Green Restoration Ireland Cooperative Society Ltd. (GRI).

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