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Review

Advances in Nematode Identification: A Journey from Fundamentals to Evolutionary Aspects

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Abstract: Nematodes are non-segmented roundworms evenly distributed with various habitats ranging to approximately every ecological extremity. These are the least studied organisms despite being the most diversified group. Nematodes are the most critical equilibrium-maintaining factors, having implications on the yield and health of plants as well as well-being of animals. However, taxonomic knowledge about nematodes is scarce. As a result of the lack of precise taxonomic features, nematode taxonomy remains uncertain. Morphology-based identification has proved inefficacious in identifying and exploring the diversity of nematodes, as there are insufficient morphological variations. Different molecular and new evolving methodologies have been employed to augment morphology-based approaches and bypass these difficulties with varying effectiveness. These identification techniques vary from molecular-based targeting DNA or protein-based targeting amino acid sequences to methods for image processing. High-throughput approaches such as next-generation sequencing have also been added to this league. These alternative approaches have helped to classify nematodes and enhanced the base for increased diversity and phylogeny of nematodes, thus helping to formulate increasingly more nematode bases for use as model organisms to study different hot topics about human well-being. Here, we discuss all the methods of nematode identification as an essential shift from classical morphometric studies to the most important modern-day and molecular approaches for their identification. Classification varies from DNA/protein-based methods to the use of new emerging methods. However, the priority of the method relies on the quality, quantity, and availability of nematode resources and down-streaming applications. This paper reviews all currently offered methods for the detection of nematodes and known/unknown and cryptic or sibling species, emphasizing modern-day methods and budding molecular techniques.

Keywords: emerging methods; identification; meta-barcoding; morphology; PCR; nematodes

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1. Introduction

Nematodes are non-segmented roundworms that include a widespread group of metazoans with various habitats ranging from land to water. Nematodes are reported to be the most prevalent and copious in these habitats, with species ranging up to one million [1]. Nematodes are the least studied organisms despite such a vast distribution. According to studies, less than 0.01% of these species have been reported [2]. There have been roughly 26,000 species reported so far, out of which 4100 are reported as plant parasites. At the same time, insect-pathogenic nematodes can be used as beneficial organisms in crop production for biological plant protection [3].

Most soil nematodes contribute significantly to nutrient cycling in the natural environment. Some nematodes are reported to be very important in medical and veterinary sciences [4]. Precise detection is, thus, critical for understanding nematode biodiversity and developing important management practices. Primary identification and characterization are mainly based on morphological features, including external body structure; total length; differences in sex organs and stoma; organization of lips, mouth, and tail; and other visible morphological characteristics. This often leads to the absence of precise categorization among strongly linked taxa due to specific observable characteristics and a lack of trained taxonomists and thus becomes insufficient, especially when a large sample size is implicated [5]. Molecular and biochemical identification methods have recently been utilized to overcome the lacuna of morphological identification of nematodes. Other studies have also reported using nematode ribosomal DNA (rDNA) sequencing to evaluate nematode characterization and evolutionary relationships [6].

Consequently, it is important to correctly identify the nematodes and enhance their evolutionary lineages to understand and combat their adverse effects on animal health and plant yield, in addition to fruitful use in nutrient cycling and other related phenomena. *Caenorhabditis elegans* (*C. elegans*), a free-living nematode, has proved a good model organism that is preferred by many researchers [7]. Because of its simple structure, good heritable traits, the existence of a comprehensive molecular toolset, and an entire genome sequence, it is appropriate for immediate and efficient regulation of gene expression function. In this review, we aim to evaluate both the existing and modern methods to classify this group of important organisms for accurate identification, thus making a shift from traditional morphological methods to current and molecular techniques and paving the way for future applications that will help to further enhance and broaden their base. The various techniques viz morphological, DNA-based, protein/biochemical-based, and new emerging methods used to identify and classify nematodes are summarized in Figure 1.

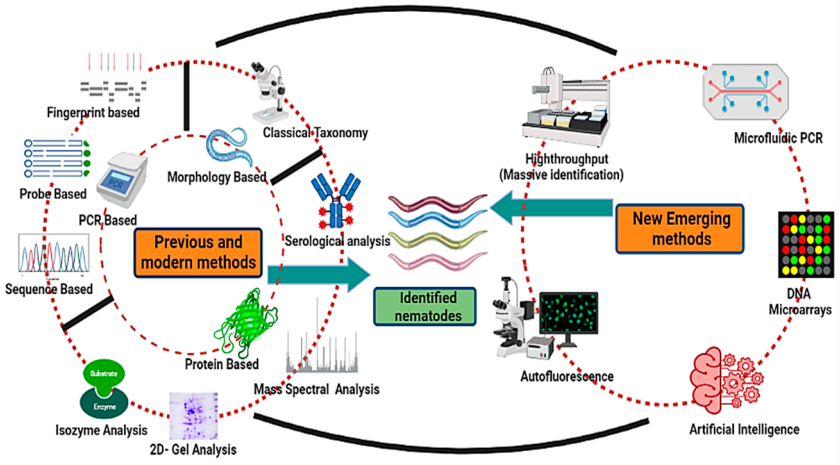

Figure 1. Different nematode identification techniques (previous, advanced, and emerging).

2. Conventional or Morphometric Method of Identification

Light microscopy has traditionally been used to diagnose and classify nematodes based on morphological and anatomical characteristics. It allows fast and reliable identification of specimens. It is cost-effective and allows a clear distinction between the function and morphological characteristics of the studied specimen [8].

Morphological Methods of Identification

Nematode identification methods vary from the detection of a single plant parasite in the soil or from the plants to the whole community observations [9]. Nowadays, different methods for defining and classifying nematodes are attainable, from basic morphological investigative techniques to much more complex high-performance sequencing methods [4,10,11]. Nathan Cobb (the father of nematology) has performed a lot of work identifying various free-living and parasitic nematodes, mostly based on their morphological characters [12]. The significant morphological identification parameters in nematodes include the body length, shape of the head, stylet length, shape of the stoma, number of annules, stylet shape and knob, lateral field structure, shape of the female tail terminus, shape and size of the spicule [13,14] and gubernaculum, and spermatheca shape [15]. Measuring such parameters and processes of nematodes requires trained taxonomists, which is on decline. Morphological parameters can also show variations due to different host plants, nutrition, geographical locations, and other environmental factors as is reported in few free-living and plant parasitic nematodes [16]. Therefore, it can become difficult for untrained individuals or researchers to identify and characterize a particular nematode species on morphology parameters alone [17] and thus, an integration of molecular analysis such as DNA sequences can be promising for accurate and proper identification [18].

3. Molecular Identification Methods

Molecular methods have advanced over classical or conventional taxonomic approaches for the characterization of nematodes [19]. Polymerase chain reaction (PCR) is widely established and efficiently employed to classify nematodes [20,21]. Good-quality research has been carried out using these molecular markers during the last two decades (Figure 2).

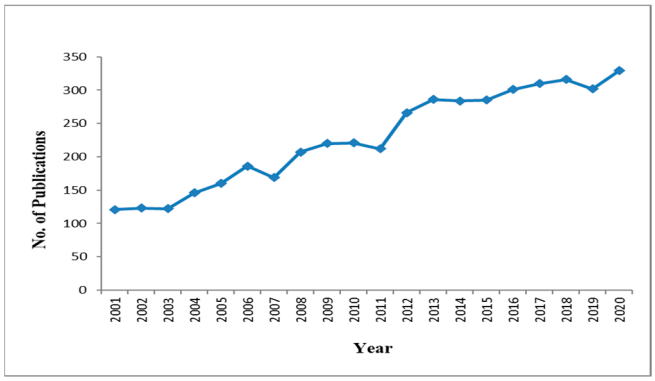

Figure 2. Publications on nematode identification as per PUBMED (Query: Molecular markers, DNA barcoding).

3.1. PCR-Based Methods

PCR-based markers are important and reliable sources, permitting the classification and characterization of new species in nematode taxa, such as Rhabditid, Meloidogyne, Pratylenchus, Globodera, and Heterodera [22–24]. DNA-based technologies such as real-time polymerase chain reaction and multiplexed tandem PCR are efficient in the preliminary screening of strongylid nematodes in farm animals, displacing conventional larval culture methodologies. Compared to traditional diagnostic processes, PCR-based detection

technologies have revolutionized the field of worm diagnostics [25]. These are used due to their improved qualities of sensitivity, specificity, speed, relative ease of use, and cost-effectiveness. Using this technique, many copies of a specified DNA template in vitro can be generated. During the last decade of the 20th century, some experiments have suggested the incorporation of effective methods apart from classical methods to decipher the nematode taxonomy [26,27]. The data generated through molecular analysis enhances our understanding of nematode systematics and its biology by exhibiting a particular target DNA sequence that will be favorable for the identification of nematode species [28]. When used to supplement conventional descriptive information, PCR-based molecular methods illustrate the improvements made in this field, providing sensitivity, correctness, and time savings. A significant accomplishment demonstrated that a PCR-based approach effectively elevates polymorphism patches that separate closely related worm species [29]. Identifying a particular species within a diverse array of nematodes from a soil community is indeed a breakthrough, and was first established using confined ratios of pure DNA and later verified by identifying single individuals from a potent mixture of worms within soil [30]. In PCR, amplification is performed using nematode DNA or the worm itself as a template [31]. Several researchers have suggested revised versions of the nematode classification and identification based on the 18S rRNA sequence similarities, which were again focused on using the PCR method [32]. Based on molecular biology and the need for taxa to be identified, emerging methods such as PCR techniques and sequence-based (ITS and COX), probe-based (qRT-PCR and multiplex PCR), and fingerprint-based (RFLP, AFLP, and RAPD) methods (Figure 3) have been developed. Table 1 summarizes some novel approaches to nematode identification that could be used in various situations and applications depending on the down steam, including both existing and emerging methods with mentioned pros and cons.

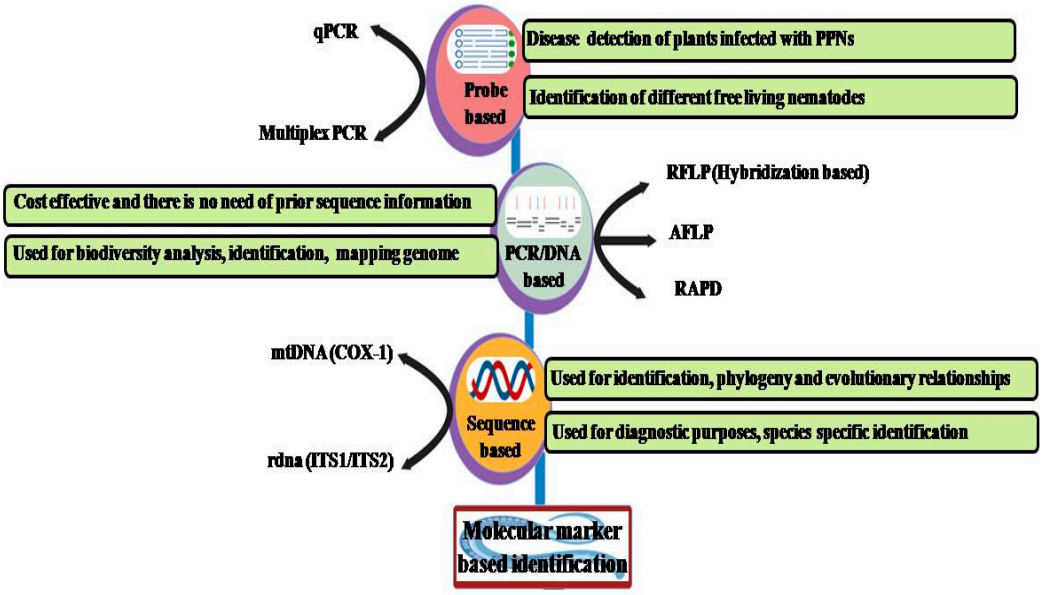

Figure 3. Different molecular markers for the identification of nematodes based on probe-, DNA-, and sequence-based methods.

3.2. Fingerprint-Based Methods

3.2.1. RFLP (Restriction Fragment Length Polymorphism)

This is one of the first molecular techniques that was used to differentiate different nematode species depending on the usage of other restriction enzymes that help in the digestion of whole genomic DNA or a particular amplified product. This results in the generation of banding patterns depending on the degree of divergence in sequences among different isolates. This method works on the principle of sequence polymorphism, forming

definite and distinct cleaving sites for restriction enzymes that produce variable fragments of different sizes [33]. Many authors used PCR-RFLP to improve Anisakis identification [34]. This method is simple to use to evaluate the H1 gene and second intergenic spacer of the lungworm Metastrongylus, which provided resistance to *Globodera rostochiensis*, a parasite of the potato cyst nematode, and distinguished between three populations of the *Meloidogyn earenaria* race while using this technique [35]. An individual study was carried out on 15 nematode isolates representing 6 species of Trichostronglus. RFLP was used to investigate the diversity of filarial parasite populations that were morphologically indistinguishable. When *Mbo* I and *Tag* I, two restriction endonucleases, were used in a combinatorial pattern with two probes *viz*, rDNA from *C. elegans* and pBM103 yielded fragments that were used to discriminate six filial species [36]. ITS-RFLP is an efficient tool for the classification of different nematodes, as in the case of *Bursaphelenchus*, where these nematodes were identified up to the species level. ITS-RFLP is used to discriminate between pathogenic and non-pathogenic isolates of *B. xylophilus.* A study was carried out to clarify the phylogeny and molecular discrimination in cereal cyst nematodes (CCNs) using restriction polymorphism of rDNA in different Heterodera and Gotland strain species. After digestion, this experiment differentiated *H. avenae*, *H. lapitons*, *H. filipjevi*, and the Gotland strain with the restriction enzyme (*Taq*I) [34]. Consequently, these reports suggest that RFLP-based characterization is a versatile tool for the examination of nematodes to understand their lineage.

3.2.2. Random Amplified Polymorphic DNA (RAPD)

This technique randomly amplifies genomic DNA in different genome sites, followed by traditional electrophoretic or "lab chip" analysis on any sequence of short DNA fragments/oligonucleotide primers [14,37]. It had been used for successful characterization of *Meloidogyne* spp. (plant-parasitic nematodes). For instance, two nematode isolates from northern Indiana with the same virulence for resistant soybean lines were separated from the other two southern Indiana isolates with different virulence activity, which led to the development of marker probes using dot blots [38]. These experiments demonstrated a diagnosis test using RAPD for different isolates of nematodes with some common virulence factors against resistant varieties.

In contrast, the differentiation of two races of devastating plant parasitic nematode *Globodera rostochiensis* (Roj and Ro2/3) was carried out using RAPD with similar conditions [39]. RAPD markers are suitable candidates for differentiating Meloidogyne species using isolated genomic DNA from nematodes, acting as a template [40,41]. RAPD characterization of the single female of the Meloidogyne species led to an observation that amplification patterns from a single female root-knot nematode are stable over three successive generations, leading to the assumption of the mitotic parthenogenetic reproductive mode of this nematode [42]. A shift from RAPD analysis was imperative as low polymorphism, the dominant mode of inheritance, is less ideal for differentiation patterns, and low reliability is the system's key drawbacks.

3.2.3. Amplified Fragment Length Polymorphism (AFLP)

AFLP is a strong DNA fingerprinting technique for any organism without prior sequence information. The process entails PCR amplification of restriction fragments from complete digestion of genomic DNA, commonly generated using a combination of two restriction enzymes. The AFLP technique was developed to create high-density linkage maps in positional gene cloning and molecular breeding. For instance, Höglund and co-workers employed this technology to uncover genetic differences in parasitic nematodes, including lungworms [43]. The principle of selective and accurate amplification underpins the AFLP approach, which was developed in response to challenges with endonuclease digestion of genomic DNA and adaptor ligation [40]. This method investigates gene expression to discover possible parasitic diseases, such as the potato cyst nematode (*Globodera rostochiensis*) [44]. It also identified the tobacco cyst nematode (TCN)

complex [41]. Even though AFLP and RAPD techniques have several similarities, the results of AFLP appear to be much more reliable under strict experimental parameters. In contrast to RAPD-PCR, AFLP is focused on a small quantity of DNA without requiring prior sequence information.

3.3. Probe-Based Detection Methods

Multiplex PCR and quantitative polymerase chain reaction (qPCR) are the two permitted probe-based detection methods of nematode species found in fish populations, such as *Anisakis*, *Pseudo terranova*, *Hysterothylacium*, and *Contracaicum* [45,46]. Multiplex PCR is efficiently applied in various biological and medical studies as it allows simultaneous amplification of many DNA fragments within one reaction [47]. A maximum of seven specific forward primers were merged with universal reverse primers that anneal to all species of DNA for the ITS region. Because the amplified species-specific primers were of varying sizes, a potential co-infection by multiple species was established [46]. qPCR is a polymerase chain reaction that incorporates a fluorescent label into every other amplified DNA copy, enabling exact real-time quantitative estimation of the number of multiple crafted copies for every PCR cycle. To identify and quantify *Anisakis* spp. and *Pseudo terranova* spp. nematodes parasitizing fish-based products, a TaqMan-based qPCR targeting the ITS-1 and 18S rRNA genes was established [48]. Correspondingly, ITS-2 was used to recognize *A. pegriffi* in fish via qPCR. An investigation into the danger of tomato injury was conducted using *Meloidogyne* spp. during an experiment [49]. Li and Co-workers (2014) developed a method for determining Heterodera glycine concentrations in soil samples taken from agricultural fields [50]. Utilizing this technique, researchers developed a real-time PCR assay to identify *M. hapla* in the root galls and surrounding soil. It enabled the differentiation of *M. hapla* DNA among 14 other *Meloidogyne* spp. except for *M. eminor*. The researcher detected DNA extracted from *M. hapla* from about 250 mg of soil, containing about one-third of an egg. Similar observations were carried out in other nematode species using the favorable TaqMan qPCR for detection and quantifications [51].

3.4. Sequence-Based Detection Method

Sequence-based molecular approaches involve the analysis of nucleotide sequences from particular segment(s) of the nuclear DNA, mitochondrial DNA (mtDNA), or whole genome [52]. The ribosomal DNA (rDNA) and mitochondrial cytochrome C oxidase subunit I (COX1) genes are usually preferred by most studies for diagnostic purposes as these have variable regions that remain conserved. The identification and PCR amplification is easy as both genes have multiple copy numbers in the nematode genome [53]. The sequence information generated is thus used to decipher the taxa's phylogeny [26]. The rDNA consists of conserved coding regions, such as the 28S, 18S, and 5.8S subunits with variable non-coding regions as an internal transcribed spacer (ITS) and external transcribed spacer (ETS) organized as tandem repeats, with intergenic spacers separating the repeating units [54]. ITS provides the sequence variability in the rDNA, interspersed by the 5.8S coding region in the rDNA cistron into ITS-1 and ITS-2, making the ITS applicable in molecular systematics for the discrimination of closely related species or sibling species [55]. For *Caenorhabditis* spp., diagnosis involves genetic crosses with unknown biological species, which has been characterized using ITS-2 markers alone [56]. ITS-1 and ITS-2 of nuclear rDNA have consistently been shown to be reliable genetic markers in parasitic nematodes of livestock and have helped to identify different strongylid nematodes, including species of *Haemonchus*, *Teladorsagia*, *Ostertagia*, *Trichostrongylus*, *Cooperia*, *Nematodirus*, and *Bunostomum* [57,58]. When ITS sequences from a variety of Strongylid nematodes were compared, it was shown that ITS-1 (364–522 bp) is usually larger than ITS-2 (215–484 bp). Because of an internal 204 bp region that is repeated twice, the ITS-1 region of *Ostertagia ostertagi* and *O. lyrata* (801 bp) is longer than that of other Trichostrongylids, including congeners [59]. The lengths of the ITS-2 sequences of Teladorsagia/Ostertagia species showed no significant differences. Furthermore, investigations suggest that the sequence variation in both ITS-1

and ITS-2 within a species is less (typically less than 1.5%) than the sequence differences among different species, laying the groundwork for strongylid identification and infection diagnosis. ITS-1 and ITS-2 are valuable genetic markers for the development of strongylid nematode diagnostic PCR-based techniques. They are brief (typically less than 800 bp), repetitive, homogenized, and species-specific [60].

4. Metabarcoding

Barcoding and metabarcoding are complementary approaches and comprehensive solutions to the development of biodiversity analysis. The two procedures are related because they always employ DNA-based accurate detection and have significant advantages due to their unique sequencers and specialized purposes. Metabarcoding has been touted as a ground-breaking tool, displacing standard DNA barcoding [61]. As DNA barcoding requires the sequencing of one well-curated individual at a time, the metabarcoding process has the potential to make use of the significant gain provided by second-generation sequencing technology, which can create millions of copies in a single run. Metabarcoding requires massive serial sequencing of intricate bulk materials for which exact recognition and curation are impractical. Metabarcoding has aided investigations of tiny eukaryotic cells, either whole populations or individual groups, with marine eukaryotes often being the focus [62]. It is used with morphometric evaluations, as evidenced in the research findings of estuarine plankton [63] and nematodes in aquatic habitats [64,65].

Meanwhile, molecular-based species identification is a successful approach, with various metabarcoding-based indices produced. Although nematodes have been extensively investigated at the molecular level [66], their suitability for a biomarker-based molecular approach has received little attention. Complex microscopic examination and metabarcoding studies on microorganisms in soil and marine resources are only carried out locally. Metabarcoding analysis of microbiological metazoan was concentrated on ribosomal RNA (rRNA) loci and the 18S rRNA gene [67]. The rRNA gene can be conveniently magnified from a wide variety of organisms using only a diverse range of universal PCR primers. Their chronological use in phylogenetic analysis has contributed to sizeable public sequence repositories containing curated benchmark datasets such as SILVA and PR2 [68]. Even though rRNA loci experience highly synchronized evolution inside higher eukaryotes, contrasting rRNA gene replicas can prevail inside individuals, populations, and species [69]. Polymorphic sequences repeatedly result in loads of molecular operational taxonomic units (MOTUs) being associated amongst each individual; intragenomic rRNA variants can affect alpha and beta diversity calculations in metabarcoding investigations. A new analysis discussed the diagnosis of gastrointestinal nematode parasites, and several PCR methods and DNA microarrays were investigated [70]. One major problem of eukaryotic metabarcoding studies is how to identify and circumvent the issue of intra-genomic rRNA variation. Bioinformatic tools for copy correction of rRNA metabarcoding datasets have been developed exclusively for prokaryotes since bacterial and archaeal species can also have multiple rRNA gene copies and exhibit some intra-genomic polymorphism [71].

Table 1. Emerging trends in nematode identification (different methods used for known and unknown nematode species).

Method		Target	Resolution	Aim	Cost	Pros	Cons	Reference
Morphological-Based								
Classical Morphometrics		Whole organism	Medium	Re-description of recognized species, and a description of new species	Low	Timeless and simple setup required; cost effective	No distinguishable morphological traits such as in larvae Paucity of trained taxonomists	[4,14,15,20]
Molecular/DNA-Based								
Fingerprint/Hybridization-Based	RFLP	DNA	Medium	Known species identification	Medium	Identification of related species	Time-consuming	[72]
	RAPD	DNA	Medium	Species identification. Phylogeny between species	Medium	Reproducibility. Prior information on the sequence is not required. A small amount of DNA is needed	Sensitivity Time-consuming	[14]
	AFLP	DNA	Medium	Species identification. Gene expression analysis and study	Medium	Prior information on the sequence is not required. A small amount of DNA is needed	Time-consuming. Individual identification not possible	[39,73]
Probe-Based	Multiplex-PCR	DNA	Low	Cryptic Species identification	Medium	Simultaneous study of several target genes. Time effective	Same primer but different target genes. Low-throughput identification	[42,43]
	Real time PCR (qPCR)	DNA	Medium	Cryptic Species identification	High	Polymorphism detection	Cost factor Low throughput	[45,46,48]
Sequence-Based	ITS	rDNA	High	Identification of cryptic or sibling species	High	Reference material and data	Automated processes limitation. Individual Identification	[29,74]
	COX	DNA	High	Identification of unknown species	High	Reference material and data	Automated processes limitation. Individual identification, standardization	[15]

Table 1. *Cont.*

Method	Target	Resolution	Aim	Cost	Pros	Cons	Reference
Protein/Biochemical-Based							
Isozyme Analysis	Intracellular enzymes	Medium	Identification of known species and description of new species	Medium	Better performance; cost- and time-effective	Processing on an individual basis. Only fresh or frozen samples required	[29,75]
2-D Gel Analysis	Protein	Medium	resolution of complex protein mixtures, identification of evolutionary relatedness	Low	Evolutionary inference of taxa; analysis of polypeptides	Dependency of polypeptides resolved and polymorphism on sample number	[76]
Mass Spectrometry (MALDI-TOF)	Protein	High	Identification of known species; diagnosis of PR proteins	High	High taxonomic resolution	Lack of genomic sequence	[77]
Serological Analysis	Antigen/Antibodies	Medium	Generation of antisera against nematodes	High	Requirement of a low amount of protein in some cases	Lack of cross-reactivity	[78]
New Emerging and Image-Based							
Machine Learning/A.I.	Image, annotation, and algorithm	Medium	Detection of phenotypes	Low	Nematode taxonomy and quantification. Fast and accurate identification	Multiple stages and requirement of expertise	[79,80]
Autofluorescence	Natural autofluorescenceof microorganisms	Medium	Utilization of natural autofluorescence of microorganisms to substitute traditional light microscopy	Low	Easy emission and excitation spectra studies	The sample should include the autofluorescent itself	[81]
Second-generation high-throughput sequencing	DNA	High	Nematode taxonomy (both known and unknown species) and quantification	Low	Cost-effective, depending upon the sample size	Requirement of field expert	[82]
Microfluidic PCR Technique	DNA	Medium	Nematode detection and identification of known and unknown	Low	High-throughput analysis; sensitivity, specificity, and cost-effective	Specific target requirement	Not applied yet to roundworms
Microarrays	DNA	High	Detection and identification and analysis of multiple genes	High	Isolation of pathogen not required; high-density probes for better analysis	Expensive and time-consuming	[83]

5. Biochemical- and Protein-Based Methods of Identification

The detection of microbial species using proteome-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) profiling has already reformed diagnostic microbiology. The molecular weight of proteins such as ribosomal and other abundant proteins is used to identify species. DNA-based approaches, mass-to-charge ratios, protein sequences, and immunological approaches highlight the utilization of different protein contents and shapes to characterize nematode species. Furthermore, protein structure and post-translational alterations extend the spectrum of nematode species that may be identified and make identification easier [84].

5.1. Analysis Based on Isozymes

Isozyme analysis is one of the early approaches employed to identify nematodes that did not rely on appearance. This approach involves the removal of soluble proteins from whole nematodes in buffer solutions, then running the extracts over a starch or polyacrylamide gel, and labeling certain enzymes. The electrophoretic technique, also called multi-locus enzyme electrophoresis, is based on isozyme migration patterns generated by the electrical load, molecular weight, and conformance due to changes in the amino acid composition. Esterases were the most often utilized enzymes, although additional enzymes such as malate dehydrogenase, superoxide dismutase, and glutamate-oxaloacetate transaminase were also used to various degrees. This strategy aided in improving and clarifying evolutionary relationships, notably among economically important Meloidogyne species. Despite this, the procedure was inefficient and time-consuming, with one of its shortcomings being the requirement to compare known samples [84]

5.2. Use of Two-Dimensional Gel (2-DGE) Analysis

Two-dimensional electrophoresis, also called 2-DGE, has been useful in nematode taxonomic studies. The approach allows for charge-based resolution of complex protein mixtures, followed by mass-based characterization in a dimension perpendicular to the first one. The resolution spectrum is then assessed between samples to check whether similarities or variations may be categorized as existing or absent for phylogenic or cladistic analysis of such a resultant data matrix. For instance, Navas and Co-workers (2002) employed 2-DGE to compare the proteomes of 18 root-knot nematodes from 4 strains [76]. According to the researchers, it was sometimes difficult to analyze spots. It was difficult to discern which reported alterations were fundamental due to gel anomalies and other issues. As a result, only 95 spots per nematode were scored, as revealed by the 2 replicates employed per nematode. The technique has advantages and downsides [76]; hence, 2-DGE allows for making evolutionary conclusions about the taxa.

5.3. Serological Analysis or Use of Antibodies

Immunoassays have great application in agriculture for the diagnosis of crop diseases, pesticides, and other naturally occurring compounds. Bird's first reported generation of antibodies against nematodes was shown in 1964 [85]. After that, several researchers have demonstrated promising outcomes using poly and monoclonal antibodies (mAbs). Meloidogyne, Heterodera, and Globodera genera have been classified using this approach. Lee (1965) attempted serological discrimination of Meloidogyne species [86]. Bird reported the probability of developing antiserum against nematodes. Various researchers have investigated the use of polyclonal and monoclonal antibodies against nematodes since Bird initially reported on the possibility of generating antiserum against nematodes with variable results. Lee observed that anti-serum generated against *M. incognita* did not form a typical arc-shaped precipitation band when coupled with antigens from another species within the same genus, with *M. hapla* demonstrating a lack of cross-reactivity in the Ouchterlony double diffusion assay [87]. The apparent specialty could thus be attributed to a small number of nematode samples. The cyst nematode Heterodera and Globodera

species have shown similarly mixed results [88]. The development of Kohler and Milstein's hybridoma technique raised expectations within the nematology community that mAbs could be developed for medical diagnostics [89]. The method involved fusing matured B cells from animals immunized with nematode antigens with mouse lymphoid tumor cells in vitro to form indefinite hybridomas for a continuous antibody yield. Using the hybridoma technique, immunoglobulins against many significant agronomic nematodes, such as *Meloidogyne incognita*, *Globodera rostochiensis*, *Heterodera glycines*, and *Globodera pallid*, have indeed been formed. Schots and colleagues discovered that only specific mAbs distinguished between *G. rostochiensis* and *G. pallid* isolates [90]. The hybridoma technique becomes more challenging as the number of nematode samples increases. Because the restructure of antigen-site-binding sequences for comparative observations is feasible through the single B cell sequence receptor (ScBCR-Secq), this line of nematode detection algorithms can be reconfigured using next-generation advanced technologies [91].

5.4. Use of Mass Spectrometry Analysis

Pathogenic nematode protein profiles can help researchers to understand how pathogenic nematodes interact with their hosts. Millares and co-workers (2012) classified proteins linked with antibiotic resistance in *Haemonchus contortus*, utilizing mass spectrometry and MALDI-ToF [92]. Without having access to a nematode's whole genome sequence, the MALDI-ToF technology can be used to classify them by investigating their proteomes. Although MALDI-ToF generates minor fragments, the ions generated are often single-charged and non-fragmented, meaning parental ion masses can be easily determined from mass spectra [93]. Figure 4 shows the basic setup required for nematode characterization and identification using proteins as markers, with some modern and day-to-day techniques followed for nematode identification. *Anguina tritici*, *A. funesta*, and *M. funesta* were separated using complete second-stage juveniles and/or proteins generated from these in a range of organic solvents [94]. These factors revealed that even a single *M. incognita* nematode (adult female or J2), cleaned or unclean, squashed or undamaged, may be utilized for MALDI-ToF-MS treatment investigations [95]. Juvenile and J2s had different protein profiles, with everyone possessing their diagnostic peak; larger mass and higher peaks were found when shattered and/or washed samples were employed. However, leading to a shortage of appropriate information in the database systems at the time, their efforts to categorize proteins using resemblance matches completely failed [96]. 2-DGE and MALDI-ToF-MS were used to examine the proteomes of two neuromorphic species, *Paragordius tricuspidate* and *Spinochordodes tellinii*.

In contrast, the species of hairworm shared 36.2% of the total protein spots in the 2-DGE analyses, where 38.0% were unique to *P. tricuspidate* and 25.8% to *S. tellinii*; a hereditary distance of 0.47 created 2 different species, verifying the strained relationship originally recorded for such species [97]. They have been engaged in the recognition of MS fingerprints of proteins obtained from digested gel plugs by conducting preliminary database searches.

Protein coding sequences in nematodes differ depending on their genetic makeup and environment. Furthermore, various variables can impact the results, the protein extraction procedure, the effectiveness of the 2-DGE activity, and the instrument design. These studies demonstrated that 2-DGE in conjunction with MALDI-ToF-MS is a dependable roundworm taxonomic classification method.

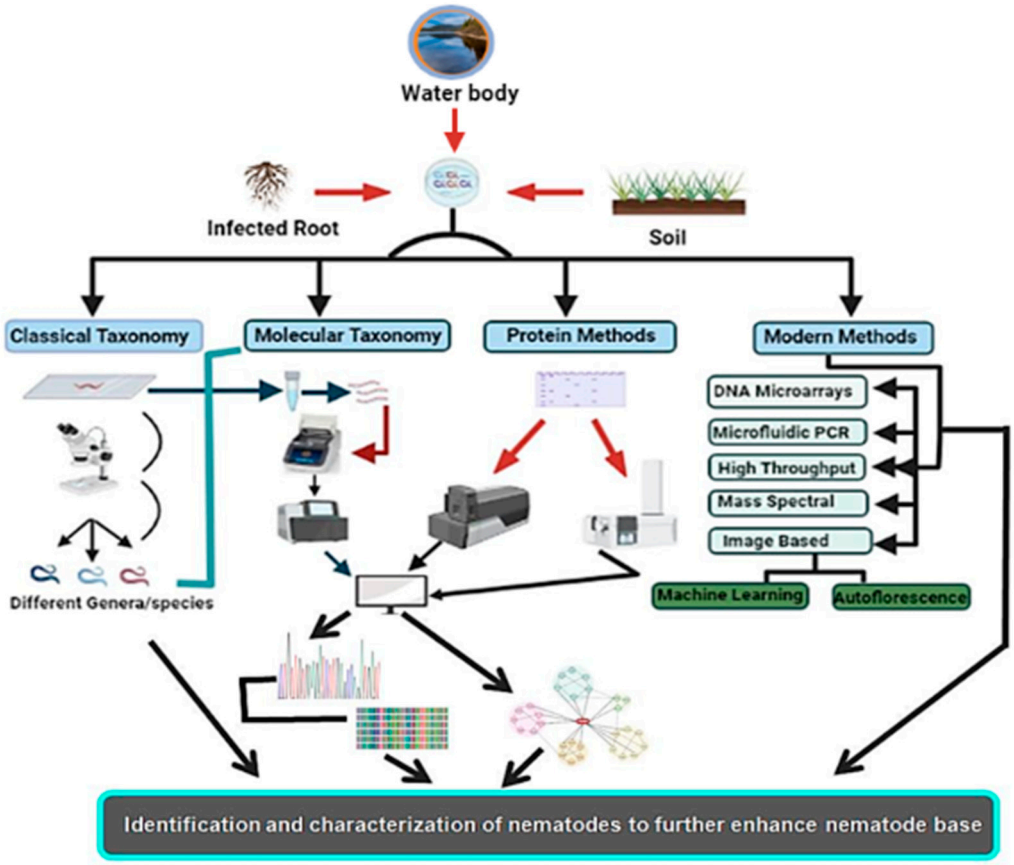

Figure 4. Different methods with their associated experimental setup, with the main focus on protein and modern approaches to decipher nematode identity and characterization.

6. Emerging Methods of Nematode Identification

With the growing demand and utility of nematodes (free-living nematodes) as a model for different studies, which include disease detection, aging, and other important physiological and developmental studies, the need to characterize nematodes with modern methods (Figure 4) has emerged, which could further enhance the already established base of nematode characterization. In 2006, Gasser and co-workers acknowledged that the genomic, genetic, and protein fields were recognized to define technological advances that provide several applications in veterinary parasitology research. Universal microarrays, nanotechnology systems, and MS frameworks have recently been established for about a decade. They are increasingly being employed in several studies, including parasitology and identification. These technologies could help develop or diagnose completely integrated methods [11,98,99]. These approaches could impact budding protozoan parasite research. However, by selecting or designing the latest technology, the biological consequence should be appropriately examined [99]. This technology is less sophisticated, complex, and cost-effective than HTS for a small number of samples. The Sanger worm classification or characterization method has sequenced PCR results for over 20 years. A practical method of reading DNA sequences was devised using molecular evolutionary biology and comprehensive analysis of a specific individual. Scientists utilized sequencing to distinguish among distinct harmonic populations and validate the development of a novel *Phaphid ascaris* species in marine fishes [100]. Ristau and colleagues used sequencing and phylogenetic analysis to describe the first strongly linked sister species of the freshwater nematode *Tobrilus gracility* [101]. Nowadays, this technique is precious since it enables the identification of new species or populations without prior information. The Sanger technique might be used to verify next-generation sequencing (NGS). It is easy to analyze because it requires essential bioinformatics and, therefore, can manage a short sequence. A

Sanger sequence's average duration is more significant than a second-generation sequence's average duration. However, this technique suffers from a critical bottleneck in large-scale detections [102]. The count of nematode species with sequenced and annotated genomes is increasing. This will aid researchers in understanding worms. This will most likely add research into the nematode genome evolution, comparative nematode genomics, and the biology of different parasitic nematodes. It is also helpful in the creation of new molecular markers for these species and population identification and characterization.

6.1. Image-Based Analysis and Identification

Accurate species identification is the foundation for all elements of taxonomic research and is an important part of biological research workflows. To address the need for identification, biologists have proposed more efficient approaches. Automated species identification is becoming an exceptionally crucial method for monitoring species occurrence across a broad taxonomic range. While there is a lot of research on automatic plant identification in general, little is known about how effectively automated identification algorithms recognize specific species and taxonomic groups and how this could be improved. When combined with modern machine learning approaches, this enormous growth in biological image data opens up possibilities for automatic species identification. A path-breaking insight into this technique could be its use in predicting the most uncommon and minute settings in nematode identification.

6.1.1. Use of the Deep Learning Approach

Deep learning breakthroughs, machine learning or artificial intelligence (AI), have paved the way for image-based nematode identification and quantitation. This approach is adapted to processing vast samples and identifying unique and minute items in complicated settings, such as nematode eggs. Machine learning for automated morphological detection involves several methods. A good proportion of images of nematodes, their eggs, or cysts are taken and independently assessed by a team of specialists to eliminate subjectivity. These are used to construct an algorithm that while masking/rejecting the background noise, captures the prominent properties of the objects in a layer-wise hierarchy from the photos. A supervised learning approach and a network model are then used to replicate the pattern of interest in the input photos. This method was used to build a unique end-to-end co-evolutionary selective autoencoder (CSAE) to classify soybean cyst nematode (SCN) eggs in different backgrounds [103]. The facts from various overlapping neighboring patches were incorporated to generate an inclusive representation and determine the occurrence of an egg in a given area. The model compares the pixel strength standards to reconstructed pictures to exemplify the assurance in anticipating that the item in the image is still an SCN egg. According to experiments using two sets of samples from unlike soil types, egg counts performed by proficient staff and this AI approach were analogous at the 95 % confidence level. A WorMachine using *C. elegans* was built with AI technology that combines the functionality of plentiful image analysis programs in an amalgamated platform for fully programmed and immediate review of specific morphological characters. The image processor on this platform bisects, detects, and trims free worms from still images obtained using pulsating strategies with interlacing fluorescence acquisitions [104]. The cropped worm masks are then utilized to extract morphological and fluorescence traits, separately assessed by the feature extractor, allowing for labeling of an assortment of worms. When using fluorescent reporters to determine sex-specific expression patterns in mutant *C. elegans*, the authors discriminated between males (XO), hermaphrodites (XX), and a range of phenotypes. The CB5362 strain, with alterations in sex-determining genes and quantification of intersex traits in worms maintained at different temperatures, may be utilized to assess unremitting morphological characteristics utilizing Wormachine. PCA and T-SNE were used to inspect the tail shape, gonad width (wider mid-width in egg-bearing worms), length and area of the body (males are smaller), and clarity of the head and tail for each worm (darker tail in males in the light field). Such results suggest that

nematode recognition, computation, and characterization play a significant role in artificial astuteness. On the other hand, adequate data may present a bottleneck in improving AI due to its plummeting taxonomy.

6.1.2. Use of Autofluorescence

The inherent autofluorescence of microorganisms might be utilized to augment traditional light microscopy in the future. Moreover, the emission and excitation spectra of the bacterial genera Lactobacillus and Saccharomyces vary [105]. Researchers have also shown that these spectroscopic fingerprints can differentiate Saccharomyces genus fungal species without fluorescent labeling. When eggs of various helminths were illuminated at multiple wavelengths ranging from white to infrared, they fluoresced. Variation in the fluorescence lifespan values (fluorescence intensity decay) was also indicative of the two species (*Ascaris lumbricoides* and *Ascaris suum*) under consideration. Thus, spectroscopic characteristics and lifetime value computation of autofluorescence in worms are valuable techniques for species identification [106].

6.2. Use of DNA Microarrays

DNA microarrays are almost made up of DNA molecules that have already been located or manufactured on glass microscope slides or silicon chips, such as cDNAs and nucleotide sequences. DNA, mRNA, or cDNA from cells, tissues, and organisms must be tagged and hybridized to microarray DNA using particular fluorescence molecules. The following fluorescent spot image was captured and statistically analyzed in a confocal scanner [107]. This theory is supported by the integration of DNA amplification and hybridization into several oligonucleotide-specific targeted sequences. Many genetic characteristics can be tracked in a single experiment. This method has a high-performance potential and can thus be applied to categorize unique and susceptible species. As sequenced samples grow, fundamental analysis's cost falls on a per-sample basis [108]. This method has been used to explore gene expression variation in worms, particularly *C. elegans*, and host–pathogen interactions, primarily from the host's perspective [109]. Even though the detection limit of current technology decreases environmental effects, it has been highlighted as a promising tool for bacterial identification [110]. The potential utility of DNA microarrays in identifying gastrointestinal worms has been described; however, no report of an analysis of nematode species has yet been published. Microarray has the potential to establish a standardized and uniform detection method for a wide range of pathogens [111].

6.3. Use of the Microfluidic PCR Technique

The microfluidic model is frequently utilized and can execute various PCR-based techniques, such as regular PCR, RT-PCR, and qPCR [112]. Presently, lesser studies are being conducted on this approach to identify environmental nematodes. Microfluidic digital PCR, which uses droplets to digitalize the PCR mix, may be effective for worm detection over microfluidic PCR approaches. This approach is based on the qPCR principle. However, the sample is added to 1000 separate bioreactors, each of which executes its amplification reactions. The fluorescent probe and primers are tailored to the quantifiable target. This development in technology expands the number of PCR reactions that can be performed, with either a significant number of samples being examined for fewer targets, or a smaller number of samples being checked for a more substantial number of targets. Its high sensitivity allows it to detect around one target out of a million. Because this method does not allow for posterior sequencing, the primers should be target-specific [113]. The optical microfluidic PCR approach uses droplets as different bioreactors, whereas the single-cell microfluidic PCR (or RT-PCR) technology uses fluid to separate single cells [114]. When studying all types of cells, a single-cell PCR or RT-PCR has the advantage of precisely quantifying and discriminating the genetic expression profile of every single cell from a sparse population of cells, without loss of data due to the average of the gene expression.

As a result, highly unusual biological events can occur. The possibility of high-throughput research is a fundamental benefit of digital microfluidics [115].

6.4. Use of the High-Throughput System for Massive Identification

This method has been used since 2005, which entails sequencing of the many samples in parallel or depth of the PCR items simultaneously [114]. The data from 10 mega-bases per cycle is meaningful to a few hundred giga-bases each cycle. The PCR will target the same area as the Sanger procedure to explain the taxonomy of a specimen population. Metogenetic approaches identify and quantify a specie's or group's relative abundance within a population of organisms. Meto-genetic techniques can also target 16S or 18SrRNA genes [115]. Different places in sequence databases are accessible depending on the family analyzed. However, the locus must comprise one or more variable areas and at least two generally well-conserved sections to differentiate between species. For annealing, the oligonucleotides that can amplify the sequence of all species in the targeted taxon, where the variable portions flanking the sequences are employed. There has been a surge in interest in meta-genomics and meta-genetics in the past few years. Sanger's and second-generation sequencing methods are now widely employed in meta-genetic research [116]. The word metagenetics may be used to define the taxonomic material by a high-performance sequencing application that focuses on the target nucleotide sequence. Now, there are two generations of HTS. Since 2011, various second-generation sequencers (PGM Ion Torrent TM, MiSeq, GS Junior) have been in service, potentially allowing institutions to benefit from this innovation at a lower cost. The library preparation (DNA barcoding), a barcode coupled with the targets for DNA fragments, clonal amplification, and HTS sequencing are the three primary phases in the second generation of HTS. This technology could pave the path for the development of a pooled DNA extraction method [117]. To prepare the library, there are two methods: one is known as "Amplicon-Seq", in which PCR is performed, and universal adaptors are then bound to a PCR product; and the other method is known as "Fusion-PCR", in which PCR primers with their 5′ universal adapters are employed. All sequencer lengths and effectiveness are computed. A technique was created upon the selection of two targets: the SSU and LSU rDNA loci. Samples of numerous nematode species were analyzed using both approaches to establish the sensitivity and accuracy. Following sequencing, some species remained unidentified due to PCR amplification failure. However, it was only confirmed qualitatively, and the authors suggested that the use of HTS to assess the relative abundance of organisms in a nematode population was premature. Multiple analyses of the same artificial sample were performed in a subsequent study to assess the reliability of this technique. Since some variations were discovered, this study determined that once interspecies resolution issues were resolved, their systems could provide a good estimate of the diverse species present in the sample [118]. High-throughput sequencing of rRNA genes was used to investigate the impact of management actions on nematode populations in the soil to classify worm groups and species as possible markers of soil nitrogen enrichment [119]. The researchers employed an iterative genetic algorithm to estimate the copy quantity of the rRNA gene in different situations and their findings on the classification of soil nematodes under various environments. The HTS alone allowed for quality estimation of the populations, but this enabled the quantification of the various populations. HTS was utilized as a schematic of the mitochondrial gene expressing cytochrome b for the variety and distribution of cyst nematodes in potatoes. This gene was useful in distinguishing between *Globodera pallid* mitotypes [92]. This investigation necessitated the development and testing of new primers. Among the significant findings were the capacity to identify new mitotypes through mutation or introduction, the wide distribution of the three major mitotypes, and the presence of at least two different mitotypes in 20% of the regions investigated. According to the researchers, this technique might be useful for tracking population dynamics across cultures or treatment regimens. The researchers then emphasized the need to include appropriate controls and powerful bioinformatics analysis to reduce the chance of inaccuracy. The second generation of sequencing technology

lengthens and complicates the experiment by including a wash sequence. Furthermore, the sequence reads have a smaller average size than the Sanger procedure reads. The number of combinations developed and even the quality of some platforms is significant. Additionally, compared to equivalent Sanger sequencing, the time necessary to execute the experiment is reduced. The cost of basic analysis decreases on a sample-by-sample basis as the count of sequenced samples increases. Following this, sophisticated analysis is essential to handle all the data, requiring a high degree of bioinformatics competence. Due to the sequencer's electronic reading system, arriving third-generation sequences can read unknown sequences over long distances at a lower cost. The sequencing matrix, a single DNA molecule that does not necessitate any prior PCR operations, also helps to reduce costs. Data analysis using third-generation sequencing methods, on the other hand, must be completed at a reasonably high speed. Although reads have a shorter playback time, there are currently only a few practical platforms on the market, and their accuracy has remained moderate. As a result of these upcoming advancements in second- and third-generation sequencing technologies, exploiting these massive amounts of data is a challenge for bioinformatics and may be a constraint for biologists. It is critical to be interdisciplinary for bioinformatics skills to meet the biologist's requirements, primarily for bio-analysis. It would be fascinating to incorporate analytical pipeline tools into user-friendly interfaces to prevent command-line systems from operating, increasing biologists' exposure to pipeline study. Doctors and veterinary surgeons are unlikely to frequently employ bioinformatics tools to diagnose infectious diseases in human or livestock. On the other hand, the MiSeqDx Illumina sequencer is the first FDA-approved equipment for bio-analysis and pulmonary microbiota diversity analysis. HTS methods are currently out of reach for all laboratories because of cost factors and sequence data processing. Bioinformatics pipelines were established concurrently with HTS development to help solve the problem of mega data analysis. Free access to bioinformatics pipelines allows non-experts to tailor various resources to their needs. If HTS remains isolated to specialized laboratories or platforms, it may become more extensively employed in the future, allowing for systematic research and possible hospital integration for diagnostic purposes [120].

7. Nematode Repositories and Databases

The database concept was developed with an eye on sequences, genome-related information, and gene ontology. Advancements in identification and characterization were made possible using molecular and other advanced methods with bioinformatics pipelines. There are various databases available for understanding and deciphering nematodes and, to a lesser extent, trematodes. Some of the most important databases and repositories are mentioned below.

7.1. NeMys

NeMys is a global nematode database linked to the UGent marine biology section. It is a generic system that stores relevant data on "species", which Tim Deprez designed in 1999 to house Nematodes, Mysida, Amphibians, and Pepperoni (NeMys). It documents independent marine, brackish, freshwater, and terrestrial nematodes, focusing on compartmentalization and ecological data. The website is linked to the Flanders Marine Institute's Worm Register of Marine Species (WoRMS).

7.2. Helminth.net

It is a web-based platform that started in 2000 as Nematode.net (www.helminth.net, accessed on 1 June 2022) to enhance nematode genome sequences research. Over the period, it was divided into two databases, namely, Nematode.net and Trematode.net. These two databases contain the data of about 73 roundworms and 17 flatworm species. This platform also provides data for numerous combinations of species' multi-omics features.

7.3. Nematode.net

It is a specialized database that contains the information and data regarding the sequence of nucleotides and related information from different nematode species across this growing phylum and is available to researchers worldwide.

7.4. NemaPath

NemaPath is a web-based examination of nematode metabolic pathways based on the Kyota encyclopedia of genes and genomes (KEGG) database. The NemaPath technique is divided into a web-based application that showcases compiled KEGG pathway maps based on a user-specified level of primary sequence similarity and a backhand layer for coordinating and analyzing nematode expressed sequence tags (ESTs) and contigs against illustrated KEGG protein information. Other tools include thorough NemaGene, EST group data, potential translations, GBrowse EST cluster views, and linkages from nematode data to different databases for synonymous *C. elegans* equivalents. So, the subject matches in the KEGG gene database and KO identification provide cross-referenced access to nematode genome information provided by NemaPath.

7.5. Helminth Control and Prevention (HelmCoP)

This database is dedicated to trematodes and parasitic nematodes of a plant, animal, and human nature. It provides comparative genomics and structural and functional data with a user-friendly interface that helps researchers to ask data-related questions. This database is used by researchers in drug, vaccine, and pesticide development in targeted roundworms, thus helping to target the pathogenicity of a particular parasite.

7.6. NEMBASE

This database (www.nematodes.org, accessed on 1 June 2022) was formed in lines similar to Edinburg-Wellcome Trust and Sanger Research Center, which deals with parasitic nematode ESTs. It is an open database that provides information pertaining to nematodes associated with meta-data that gives way to sequences. This database deals with the nematode transcriptome and provides significant inputs into nematode biology, drug discovery, and viral disease studies.

7.7. WormBase

This website can be accessed at www.wormbase.org, (accessed on 1 June 2022). It is a global organization dedicated to researchers and programming scientists who serve the scientific community worldwide by providing accurate, up-to-date, and easily accessible information on *C. elegans* genetics, genomics, developmental biology, and related worms. Few other databases attached to this central database, viz WoRMS database and marine species database, are accessible for personal use and provide more taxonomic literature.

7.8. WormBook

It is a massive, fully accessible repository of original, peer-reviewed chapters on the biology of *C. elegans* and other worms. Different techniques and protocols regarding worm research can be accessed through this website. All the major worm methods and collection of protocols for nematode research can be accessed using this database. *Worm History*, *Worm Breeder's Gazette*, and other biannual newsletters regarding *C. elegans* and other worms are communicated through this platform to exchange ideas and information.

7.9. USDA Nematode Collection

This collection is housed by the famous USDA Nematology Laboratory in Maryland, USA. It is one of the most significant nematode repositories with the most valuable worm collections. It accommodates different permanent slides and vials, with a number ranging over 49,000. It has a comprehensive repository, which is more than a million, and includes

the works of the most prominent nematologists, including Thorne, Cobb, Steiner, and other important works. It contains more than 38,000 species entries.

8. Conclusions and Future Perspectives

Taxonomy is important for the categorization of organisms and understanding of biodiversity and ecological aspects. The main prerequisite for taxonomy is naming the organisms in terms of the scientific name, considering the morphological information available for the organism. Morphology-based classification forms the backbone of taxonomy. However, it becomes compulsory to use molecular information to address this limitation due to the inconsistency in or lack of sufficient morphological characteristics to describe the taxonomic status of species or organisms. Consequently, several methods are considered when selecting and identifying a species that all depend on the query to be addressed in terms of whether the result should be quantitative, qualitative, fundamental, applied, or targeted. Therefore, different tools must be chosen, keeping in mind the desired application. No single method can always provide the best answer, so the samples' nature and availability force the researcher to choose a particular set of techniques. For instance, for identification of the nematode sample, the first step is to use a microscope or morphological studies provided if a trained taxonomist is available. The molecular technique can also be used here to identify the specimen to the genus or species level. If the purpose is to identify the diversity within the population, then the best tool is to the use of any fingerprinting technique (RFLP, RAPD, or AFLP). Multiple copy number genes are more helpful for amplification than single-copy genes when individual analysis is carried out, as with DNA barcoding, using the rDNA/rRNA or Cox1 gene to identify the genus or species level. Some methods such as HTS are helpful in both qualitative and quantitative studies. However, HTS, microfluidic PCR, and qPCR are quantitative methods only. The qPCR targets the sequence to be studied using primers and probes. Additionally, protein-based or biochemical methods help decipher the complex proteome identification and help understand nematode identification and pathogenesis. The methods described owe a peculiar property to others in identifying the nematodes. Some are devoted to fundamental research while others are dedicated to basic and applied sciences. On top, some methods help when a greater sample size needs to be processed simultaneously such as HTS, which helps add significance to the results. It is pertinent to mention that the morphometric approach does not lose its shine despite the advancement of molecular methods. It helps to confirm the taxonomical identification combined with the molecular approach and provides deep insight into biodiversity. It has been advanced with the induction of machine learning or AI and image processing approaches, which allow fast and accurate identification. The new emerging methods help circumvent the obstructions faced by the scarcity of trained taxonomists and allow rapid and precise identification. Machine learning or AI helps in accurate diagnostics for identification purposes while autofluorescence using spectroscopic features and lifetime value measurements can further enhance the identification process. In this review, we focused on both current and emerging techniques. The latter is very promising, but one must be aware of every method's pros and cons. The emerging techniques are promising and highly effective. All the described methods help form databases and repositories, which become a source of high-level information and form a data retrieval system. As a result, open access to these databases and bioinformatics algorithms should be promoted and used for further research. With the emergence of different methods, the need to enhance the databases requires proper data availability.

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