

1 **Titles, authors and addresses**

2 *Molecular diagnosis of trichodorid species from Portugal*

3 **PCR-RFLP identification of trichodorid nematodes**

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5 **Molecular diagnosis of trichodorid species from Portugal**

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18

1 **Abstract**

2 A PCR-RFLP assay was developed for the identification of trichodorids belonging to  
3 *Nanidorus*, *Paratrichodorus* and *Trichodorus* genera. Exploring the variability of the  
4 18S SSU rDNA gene this method provides for the first time a molecular diagnosis tool  
5 alternative to the difficult and time consuming phenotyping especially of quite similar  
6 species, enabling selectivity in mixed samples with non-trichodorid species, and also the  
7 differentiation of juveniles. Based on the alignment of previously obtained 18S rDNA  
8 nucleotide sequences of trichodorids from Portugal, a pair of selective primers was  
9 designed in conserved regions to allow the amplification in all known Portuguese  
10 species of a variable region located at the 3' end of the gene. The PCR product, 615bp in  
11 length, exhibits nucleotide variability to generate restriction fragment patterns which  
12 were consistent among populations of the same species and allowed to discriminate  
13 trichodorids at the species level. The proposed protocol was tested and proved effective  
14 with twelve trichodorid species from Portugal (*N. minor*, *P. allius*, *P. anemones*, *P.*  
15 *divergens*, *P. hispanus*, *P. pachydermus*, *P. porosus*, *T. beirensis*, *T. lusitanicus*, *T.*  
16 *primitivus* and two other *Trichodorus* species, A and B) and six non-indigenous  
17 trichodorid populations (*N. minor*, *P. allius*, *P. anemones*, *P. pachydermus*, *P. porosus*,  
18 *T. primitivus*).

19

20 **Keywords** – identification, intraspecific variation, PCR-RFLP, ribosomal DNA, trichodorid,  
21 18S sequence.

22

23

## 1 **Introduction**

2

3 Didelphic *Nanidorus*, *Paratrichodorus* and *Trichodorus* nematodes of Trichodoridae family are  
4 economically important polyphagous ectomigratory plant root ectoparasites. More than 90  
5 species are described (Almeida & Decraemer, 2005), some being known to cause direct damage  
6 by feeding, resulting in a reduction of root development, and a few (13) are vector specific  
7 strains of TRV, *Pea early-browning virus* and *Pepper ringspot virus*, that have a deleterious  
8 effect upon several economically important crops, such as ornamental bulbs, potatoes and  
9 tobacco (Harrison & Robinson, 1978, 1986; Taylor & Brown, 1997). Their distribution is wide  
10 in North America and Europe, but they have been reported from different parts of the world  
11 (Taylor & Brown, 1997). Up to now, sixteen trichodorid species have been reported in Portugal  
12 (Almeida *et al.*, 1989; Almeida & Santos, 1997), some known as TRV vectors (Brown &  
13 Weischer, 1998; Duarte *et al.*, 2001, 2002, 2010).

14 Tobraviruses have a wide host range, basically annual plants that can perennate the virus  
15 (Cooper & Harrison, 1973) and vector nematodes, in which can be retained for months or years  
16 (van Hoof, 1970). Infected roots and vectors act as virions reservoirs providing inocula to be  
17 gradually transmitted, by a compatible vector, to the roots of healthy susceptible adjacent plants.  
18 These features play an effective role in the tobnavirus dissemination, bridging the infection  
19 between successive susceptible crops and enable the virus to be exposed to their host range,  
20 finding potential hosts in different plants (Harrison, 1977; Harrison & Robinson, 1978, 1986).

21 The commercial activities of plant material have favoured the dispersal of both agents,  
22 conducting to more diverse encounters between trichodorid species and virus isolates. When  
23 field populations of trichodorids become viruliferous, they can persist infected for many years  
24 (Harrison, 1977, Weingartner, 2001), being known to cause high economical losses and in  
25 growing areas of susceptible crops (Mojathedi *et al.*, 2000, 2001; Weingartner, 2001), mainly  
26 due to its difficult control (Ploeg & Decraemer, 1997). Presently, the difficulties of disease  
27 management in fields already contaminated are related with the efficient virus survival strategy,

1 the inexistence of diverse crop cultivars TRV resistant, and the lack of efficient chemical  
2 treatments, as nematicides do not eradicate the problem and have high environmental  
3 containments upon their use.

4 Given the complex relationship that exists between trichodorids and the various TRV strains  
5 (Brown & Weischer, 1998) and the limitations of the currently available control tools, it is  
6 essential to prevent the introduction of TRV to virus-free areas and to minimise the problem in  
7 infected regions, for which it is crucial a rapid and accurate identification of trichodorids to the  
8 species level. Currently, this identification is based on morphobiometrical characterization,  
9 which requires the drawing and measurement of body structures under x1000 amplification of  
10 several adult specimens of each population, which is a laborious and time-consuming task that  
11 requires expertise services. The differentiation of some species, mainly in *Paratrichodorus*, is  
12 difficult due to high intraspecific variability. Very frequently in the growing season, *e.g.* at  
13 sampling time, small numbers of specimens, or juveniles are the only stage present, commonly  
14 in a complex mixture of vector and non-vector species. Without the reproductive structures,  
15 which are main diagnostic features for morphometrical identification, immature specimens are  
16 difficult or impossible to identify. For crop protection management, it is however, important to  
17 identify those immature stages at species level. Furthermore, with the decline of skill-base  
18 nematode taxonomists within Europe, there is an urgent demand for the development of an  
19 alternative tool for a routine detection and clear identification of trichodorids.

20 Direct-analysis of the genetic material is potentially a powerful method to identify nematodes.  
21 Classification of plant parasite nematodes based on the PCR amplification (Saiki *et al.*, 1988) of  
22 specific DNA regions is nowadays a routine. For these purposes, mitochondrial DNA (Powers &  
23 Harris, 1993; Adam *et al.*, 2007; Kumari & Lišková, 2009), satellite DNA (Castagnone-Sereno  
24 *et al.*, 1998) and the ITS region of ribosomal DNA (Zijlstra *et al.*, 1995, 1997; Iwahori *et al.*,  
25 1998; Wang *et al.*, 2003; Hübschen *et al.*, 2004a, b; Berry *et al.*, 2007, 2008) are widely used.  
26 The ITS and 18S regions have been also exploit to resolve nematodes phylogenetic relationships  
27 (Blaxter *et al.*, 1998; Megen *et al.*, 2009; Duarte *et al.*, 2010), for DNA barcode-based nematode

1 detection and biodiversity analysis (Floyd *et al.*, 2005) and successfully applied to identification  
2 of *Xiphidorus* spp. (Oliveira *et al.*, 2004), virus vector nematodes, *Longidorus* spp.,  
3 *Paratrichodoros* spp., *Trichodoros* spp. and *Xiphinema* spp. (Vrain *et al.*, 1992; Molinari *et al.*,  
4 1997; Boutsika *et al.*, 2004; Holeva *et al.*, 2006; Riga *et al.*, 2007).

5 The aim of this work was to develop a simple and inexpensive molecular method in order to  
6 allow the identification of trichodorid species present in Portugal. An rDNA-PCR-RFLP  
7 (restriction fragment length polymorphism) was designed as a diagnostic technique that explores  
8 the variability of the 18S rDNA region to discriminate trichodorid species belonging to  
9 *Nanidoros*, *Paratrichodoros* and *Trichodoros* producing clear species-specific diagnostic  
10 patterns. The method was tested with trichodorid populations of different geographic origins.

11

## 12 **Materials and methods**

13

### 14 **PCR-RFLP design**

15 The selection of a genomic region within the SSU rDNA sequences, to allow a clear species  
16 differentiation, was based on the analysis of a set of nucleotide sequences previously obtained in  
17 2010 by Duarte *et al.* (Table 1), and their deduced restriction enzymes (RE) maps, resorting to  
18 the Package programmes MAP, MAPLOT and MAPSORT, GCG version 10.0, Wisconsin  
19 Package programmes. A computer programme was build to assist the *in vitro* design of the PCR-  
20 RFLP for this conserved region. Aiming the design a simple technique, the selection criteria  
21 obliged the use of a unique pair of trichodorid-specific primers to amplify a small region,  
22 containing nucleotide variability detectable by restriction endonuclease cleavage. The  
23 programme was run either to the entire 18S sequence or to variable sub-regions, corresponding  
24 to viable/pragmatic PCR products to select RE cleavage sites able to differentiate the set of  
25 trichodorid species under study. The REs were selected aiming the production of species-specific

1 digested products, differentiable in size from each other by over 80-100bp to allow their easier  
2 visualisation by electrophoresis, through agarose gels.

3

#### 4 **Nematode species**

5 Twenty-one populations comprising 12 *Trichodorus*, *Paratrichodorus* and *Nanidorus* species  
6 were collected from different geographical regions in Portugal and abroad. *Trichodorus* sp. A  
7 and *Trichodorus* sp. B are designated following Almeida (1993). *Paratrichodorus* sp. C and sp.  
8 D refer to two different unidentified trichodorid species (Table 1). All nematodes were extracted  
9 directly from infested field soil (Brown & Boag, 1988) and used without subsequent cultivation.  
10 Representative adult specimens from each species were collected, morphological and  
11 morphometric identified and kept in 1M NaCl at -20°C. Five species (*N. minor*, *P. divergens*, *P.*  
12 *pachydermus*, *P. porosus* and *Trichodorus* sp. A) are represented by two populations from  
13 different geographic origins while *T. primitivus* is represented with three populations.

14

#### 15 **DNA extraction and PCR amplification**

16 Total DNA was extracted from a single adult trichodorid (Stanton *et al.*, 1998) after  
17 morphological and morphometric confirmation and photomicrograph recording.

18 PCR reactions were performed with 10mM-Tris/HCl (pH 8.8), 50mM KCl, 1.5mM MgCl<sub>2</sub>,  
19 0.2µM of each primer, 0.2mM of dNTP, and 0.5-1µl of nematode total DNA (0.05 to 0.5ng  
20 DNA template) and 2.5 units of Taq DNA polymerase. The reactants for amplification were  
21 supplied by *Invitrogen, Life Technologies*. The primers used in the PCR reaction, 1091-F (5'-  
22 AGGAATTGACGGAAGGGCAC-3') and 1671-R (5'-TCCTCTAAGTAAATCCCATTTGG-3')  
23 were synthesised and supplied by the same manufacturer.

24 The thermal cycling parameters included an initial denaturation step at 94°C for 2 min, followed  
25 by 40 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 30 s, with an extension at

1 72°C for 2 min, and an additional final extension step at 72°C for 10 min. Negative control  
2 without DNA template and a positive control with *T. primitivus* DNA were included in all  
3 experiments. Separate specimens of each species were used to produce the PCR products. All the  
4 nucleotide sequences in this study were therefore obtained from one unique individual. All  
5 sequence analysis were done with GCG version 10.0 (Genetics Computer Group), Wisconsin  
6 Package programmes.

## 7 **RFLP**

8 Approximately 1.5-3µg of each PCR product (*ca.* 615bp of the amplicom) were single digested  
9 with 5U of *AatII*, *AleI*, *AvaI*, *BsrI*, *Fnu4HI*, *TaqI* or *TspRI*, according to the manufacturers  
10 recommendations, in a final volume of 12µl. The digestions with *BsrI*, *TaqI* and *TspRI* were  
11 conducted for 2 h at 65°C, while the remaining four were carried out at 37°C.

12 The hydrolysed PCR products were size-separated in a 2% agarose gel (Agarose I, *Stratagene*)  
13 in 1X TBE buffer (89mM Tris/HCl, 89mM boric acid, 2mM EDTA), containing 0.5µg/ml of  
14 ethidium bromide and run at *ca.* 7 V/cm at RT in 1X TBE buffer. A 100bp ladder (*Invitrogen*,  
15 *Life Technologies*) and a XIII (50-750bp), *Roche, Diagnostic GmbH* DNA were used as size  
16 markers. The DNA fragments were visualised by gel observation in a 300nm wavelength UV  
17 transilluminator, photographed with a Polaroid Instant Camera System and the band pattern  
18 analysed visually.

## 19 **Evaluation of the molecular diagnostic method (PCR-RFLP)**

20 Total cellular DNA was extracted from 42 separate individuals, representing 21 populations (two  
21 individuals of each population) and subjected to the proposed PCR-RFLP protocol to evaluate  
22 the technique as a species differentiator tool. Patterns obtained with DNA prepared from two  
23 individuals of each species prepared in two occasions were compared to evaluate the possible  
24 variation in PCR-RFLP patterns when obtained in different experiments.

1 Two parameters were used to evaluate the PCR-RFLP as a differentiation system for trichodorid  
2 species. The typability, as the percentage of organisms to which was possible to obtain a positive  
3 and non-ambiguous result with the system; and the reproducibility, as the percentage of  
4 organisms, which produce the same result, when repeatedly tested with the system.

5 The specificity of this technique and the putative occurrence of false positive results were tested  
6 *in silico* by resorting to several species, which sequences were published in the GenBank, at that  
7 time: sequences of *Longidorus elongates* (AF03659), *Prismatolaimus intermedius* (AF036603),  
8 *Pungentus silvestris* (AY284788), *Pungentus* sp. (AJ966501), *Thornia steatopyga* (AY284787),  
9 *Tylencholaimellus striatus* (AY284837) and *Xiphinema rivesi* (AF036610). This selection  
10 included taxonomically related genera, *Prismatolaimus*, *Longidorus* and *Xiphinema* and also  
11 those that, under the stereoscopic microscope, could be more easily confounded with  
12 trichodorids, when picking the specimens from the soil suspension for DNA extraction, namely,  
13 *Diphtherophora*, *Doryllium*, *Lepthonchus*, *Pungentus*, *Proleptonchus*, *Thornia* and  
14 *Tylencholaimellus*. The specificity of the primers, that confer the specificity of the PCR phase,  
15 was assessed against the NCBI nucleotide sequence database (05.08.2010)  
16 (<ftp://ftp.ncbi.nih.gov/blast/db/FAST>). Furthermore, the specificity of the restriction enzyme of  
17 the referred sequences was also investigated searching for identical restriction patterns, using  
18 MAPSORT programme.

19 Strictly for the purpose of this work, molecular type species were defined referring to the 18S  
20 rDNA nucleotide sequences corresponding to morphobiometrical well characterized trichodorid  
21 specimens of twelve species (Table 1). The molecular type species of *P. anemones* (AF036600)  
22 and *P. pachydermus* (AF036601) (Blaxter *et al.*, 1998) were considered as reference sequences.  
23



## 1 Results

### 2 Identification of a candidate region for PCR-RFLP design

3 The selected genomic region of *ca.* 615bp, located at the 3' end of the gene, fulfils the stated  
4 requirements. The BLAST search with the selected primers mach the DNA of no other  
5 organisms, being therefore, apparently trichodorid-specific, but the amplicons yielded are size  
6 undifferentiated.

7 Due to the low rate of variable positions in this region, the discrimination of all species was only  
8 achieved by the analysis of combined results obtained with several different simple restriction.

9 As shown in Table 2 and Figure 1, the *AatII*, *AleI*, *AvaI*, *BsrI*, *Fnu4HI*, *TaqI* and *TspRI*  
10 restriction enzyme maps, of the selected region within the 18S rDNA of trichodorid sequences,  
11 yield enough variability to distinguish the species under analysis.

### 12 PCR-RFLP

13 Amplification of the rDNA-18S region was successful to all the 42 specimens collected,  
14 generating the expected fragments with *ca.* 615bp. The detection of DNA polymorphisms by  
15 PCR-RFLP allowed the identification of all populations, confirming the predictions made from  
16 the nucleotide data analysis (Figure 1).

17 The technique did not discriminate populations of the same species, collected from different  
18 geographic origins, mainly from continental Portugal, which reinforces the species specificity of  
19 the method. As a matter of fact, identical patterns were observed in two different Portuguese  
20 populations of *P. divergens* and *Trichodorus* sp. A; two different populations of *N. minor* and *P.*  
21 *porosus* (one Portuguese and one Brazilian) and two different populations of *P. pachydermus*  
22 and *T. primitivus* (one Portuguese-continental and one Scottish). These results indicate that  
23 despite their origin, at least the 3' end of the 18S gene is totally conserved. *T. primitivus*  
24 population from Azores was included in the PCR-RFLP analysis but was not sequenced due to  
25 sample limitations. The *Fnu4H* restriction pattern of *T. primitivus* population from Azores Island

1 differed from both the continental and the Scottish ones by an additional band of *ca.* 270bp. As  
2 the size of the PCR fragment is conserved in this species (615bp) this difference is probably the  
3 result of partial digestion of the *Fnu4HI* site located between the contiguous fragments 192bp  
4 and 83bp.

5 Through *AatII*, *Fnu4HI* and *TaqI* restriction, this technique also allows a clear distinguishing of  
6 the morphobiometrical similar *P. pachydermus* and *Paratrichodorus* sp. A populations, both  
7 from the same geographical region of Vila Real (North of Portugal) (Figure 1). The juvenile  
8 specimens of *Paratrichodorus* sp. D were collected in Chaves, where a mixed population of *P.*  
9 *anemones* and *P. pachydermus* had been found. Application of the PCR-RFLP technique did not  
10 generate a pattern similar to any of the patterns obtained with those other species. In fact, its  
11 RFLP pattern is identical to *P. pachydermus*, except for the *TaqI* restriction products that  
12 resemble *Paratrichodorus* sp. C (Figure 2). Further studies of that population will provide  
13 additional molecular and morphological data to clarify if these individuals are from a different  
14 molecular species, or constitute a molecular variant of *P. pachydermus*.

15 No single enzymatic restriction could distinguish all the species in this study, although some  
16 have much more taxonomic potential resolution than others. *BsrI* restriction allows  
17 distinguishing *Nanidorus* and *Paratrichodorus* from *Trichodorus*, generating also a *P. porosus*  
18 specific pattern. Differentiation between *Nanidorus* species requires one additional restriction  
19 with *Fnu4HI*, while up to three subsequent cleavages are necessary for the identification of  
20 *Paratrichodorus* and *Trichodorus* species. The combination of the patterns obtained by up to  
21 four of seven individual enzymes allowed however, a clear differentiation of all species under  
22 analysis (Table 2).

### 23 **Evaluation of the molecular diagnostic method (PCR-RFLP)**

24 The repetitions performed with DNA extraction and the PCR-RFLP technique showed a high  
25 reproducibility of this technique. The restriction patterns of repeated samples processed in two  
26 occasions were indistinguishable.

1 The typability capacity of populations, previously morphobiometrically characterised, was  
2 100%, regardless their geographic origin. However, two morphologically unidentified species  
3 (*Paratrichodorus* sp. C and D) were not clearly typified as any of the molecular type species.  
4 These two unidentified *Paratrichodorus* populations may represent distinct morphological  
5 species from those under analysis, and for which no molecular type species were included in the  
6 selected set of sequences.

7 The specificity of the test is mainly determined at the PCR step, due to lack of similarity  
8 between the 1671-F reverse primer and the selected sequences (*Longidorus elongates*,  
9 *Prismatolaimus intermedius*, *Pungentus silvestris*, *Pungentus* sp., *Thornia steatopyga*,  
10 *Tylencholaimellus striatus* and *Xiphinema rivesi*), that abrogate the amplification.

11 Sequences from other two genera, available in GenBank are incomplete not including the region  
12 that matches the reverse primer sequence: being therefore, impossible to foresee the specificity  
13 of the test applied to the complete 615bp sequences.

14

## 15 **Discussion**

16 In the present work we described a PCR-RFLP method that generates patterns for the clear  
17 identification of trichodorid species important for agriculture, including virus vector species. The  
18 restriction pattern produced by up to four of seven different enzymes segregated the twelve  
19 analysed species, nine of which are known to be TRV vectors.

20 The 18S rDNA region was selected as the target DNA to propose a molecular diagnostic  
21 technique for identification of trichodorids at species level. This region appears to be conserved  
22 within the populations of a trichodorid species, presenting however enough variability among  
23 species that generates a clear species identity profile. This complies with the known sequence  
24 variability higher among species, than among populations within species, due to concerted  
25 evolution of repeated DNA sequences (Dover, 1982). Other rDNA non-coding regions, such as  
26 the internal transcribed spacers, that are subjected to a lower selection pressure are frequently

1 used for diagnosis purposes. Non-coding regions have been reported as containing molecular  
2 polymorphism to allow distinguishing populations, for example, cyst nematode (Bekal *et al.*,  
3 1997, Szalanski *et al.*, 1997, Subbotin *et al.*, 2000, 2001 and Aimiri *et al.*, 2002), *Pratylenchus*  
4 (*Waeyenberg et al.*, 2000), and trichodorids (Boutsika *et al.*, 2004). These authors differentiated  
5 four species, including *P. pachydermus* and *T. primitivus* from Scotland, UK, using species  
6 specific primers exploring the ITS variability.

7 The higher conservation of 18S region increases the difficulty of producing an easily  
8 differentiable band pattern required to facilitate the non-equivocal identification of the species.

9 A single restriction enzyme does not allow the characterization of all investigated species;  
10 however, the discriminative power combined restriction enzymes, *AatII*, *AleI*, *AvaI*, *BsrI*,  
11 *Fnu4HI*, *TaqI* and *TspRI*, resolved genomic groups among the trichodorid populations studied,  
12 although these species belong to three genera and differ by less than 6.6 % in their G+C content.

13 The use of well-characterized trichodorid populations by morphobiometry and by nucleotide  
14 sequence analysis allowed the validation of this PCR-RFLP for identification of trichodorid  
15 species. In the absence of previously defined and agreed molecular species types described by  
16 any of the present International Group, we define here, within the scope of the present work, the  
17 molecular type species analysed. The definition of molecular type species needs, in our opinion,  
18 future clarification to avoid potential confusions. This definition would also allow a credible  
19 comparison of nucleotide data, within populations of a morphological species.

20 When designing the PCR-RFLP, the priority was the proposal of a simple technique, easy to  
21 operate and inexpensive, which are basic requirements for a routine diagnosis technique. It was  
22 chosen a universal PCR reaction, to amplify a fragment with the shortest possible size (615nt)  
23 that, after a set of single digestions, produced bands easily differentiable in size. Single RE  
24 digestions can be performed successively, according to the flowchart of Figure 2, which allows  
25 the reduction of the number of digestions to a maximum of four, turning therefore the procedure  
26 less expensive. After the second restriction nearly half of the species may be identified and after

1 the third one nearly 84%. Furthermore, if time is a priority, the seven digestions can be set up  
2 simultaneously. Interpretation of results is quite straight forward.

3 The PCR-RFLP technique here described is the first molecular tool for trichodorids  
4 identification, discriminating populations of different species, but recognising identical  
5 populations of the same species. Such a method was long required for trichodorid and TRV  
6 management. Trichodorids do not move quickly or far, but once established in a soil they usually  
7 persist for many years and the virus they transmit tend to reappear at the same sites in successive  
8 plantings (Harrison & Robinson, 1986). Thereby, TRV management can be based on the  
9 assessment of vector trichodorid populations and their infectivity in soil samples collected before  
10 planting. The task of collect and processing soil samples and identifying trichodorid species  
11 requires trained staff, consequently, the existence of rapid, sensitive and species-specific  
12 nematological diagnostic methods is of the utmost importance for the effective risk assessment.  
13 This technique does not have to be performed by nematologists and is particularly adequate  
14 when only a few immature specimens are present in soil samples. In these cases, the incorrect  
15 identification can have major economical implications, as a few vector specimens are able to  
16 spread a compatible TRV isolate (Ploeg *et al.*, 1992, Brown & Weischer, 1998). The method  
17 may be also important in the authentication and legal quarantine crop protection schemes as well  
18 as for other scientific studies.

19 The application of this PCR-RFLP method to different trichodorid populations originated from  
20 vaster geographic regions, or its bioinformatic simulation using new sequences when available in  
21 the GenBank, will allow further testing of the method. It will be then possible to verify if the  
22 proposed patterns are conserved, not only within the analysed populations, but also in others,  
23 from different countries or continents; or if, conversely, population variability will be detected,  
24 as it could be the case of *T. primitivus* from Azores.

25 The results here presented were very consistent. If the technique proves to be species-specific in  
26 laboratorial tests with vaster groups of soil-inhabitant nematodes, the method appears promising

1 to be performed directly with soil suspensions, avoiding the time consuming task of specimens'  
2 collection for DNA extraction.

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1

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2 **Table 1** Trichodorid populations and respective geographic origin, accession numbers of  
 3 the 18S rDNA trichodorid sequences obtained from the EMBL/GenBank/DDBJ  
 4 database used to design the PCR-RFLP method, and references of the populations  
 5 assayed

Trichodorid populations	Geographic origin	Accession number of sequences	References <sup>c</sup> of tested populations
<i>Nanidorus</i> spp.			
<i>N. minor</i> <sup>a</sup>	Nm-Pt	DQ345526	Nm-Pt
<i>N. minor</i>		AM269897	
<i>N. minor</i>	Nm-Br		Nm-Br
<i>N. nanus</i> <sup>a</sup>		FJ040485/6	
<i>Paratrichodoros</i> spp.			
<i>P. allius</i> <sup>a</sup>		AM269895	
<i>P. allius</i>	Pall-US		Pall-US
<i>P. anemones</i> <sup>a</sup>	Pa-Gb-Eng	AF036600	Pa-Gb-Eng
<i>P. divergens</i>	Pd-Pt-a		Pd-Pt-a
<i>P. divergens</i> <sup>a</sup>	Pd-Pt-b	DQ345528	Pd-Pt-b
<i>P. hispanus</i> <sup>a</sup>	Ph-Pt	DQ345527	Ph-Pt
<i>P. pachydermus</i> <sup>a</sup>	Ppa-Gb-Sct	AF036601	Ppa-Gb-Sct
<i>P. pachydermus</i>	Ppa-Pt		Ppa-Pt
<i>P. porosus</i> <sup>a</sup>	Ppo-Pt	DQ345524	Ppo-Pt
<i>P. porosus</i>	Ppo-Br		Ppo-Br
<i>P. teres</i> <sup>a</sup>		AM269896	
<i>Paratrichodoros</i> sp. C <sup>b</sup>	PspC-Pt	DQ345523	PspC-Pt
<i>Paratrichodoros</i> sp. D <sup>b</sup>	PspD-Pt		PspD-Pt
<i>Trichodoros</i> spp.			
<i>T. beirensis</i> <sup>a</sup>	Tb-Pt	DQ345530	Tb-Pt
<i>T. primitivus</i> <sup>a</sup>	Tp-Pt	DQ345533	Tp-Pt
<i>T. primitivus</i>	Tp-Pt-Az		Tp-Pt-Az
<i>T. primitivus</i>	Tp- Gb-Sct	AF036609	Tp- Gb-Sct
<i>T. lusitanicus</i> <sup>a</sup>	Tl-Pt	DQ345532	Tl-Pt
<i>Trichodoros</i> sp. A <sup>a</sup>	TspA-Pt-a	DQ345534	TspA-Pt-a
	TspA-Pt-b		TspA-Pt-b
<i>Trichodoros</i> sp. B <sup>a</sup>	TspB-Pt	DQ345531	TspB-Pt

6 <sup>a</sup> Molecular type species for the purpose of the present work.

7 <sup>b</sup> Unidentified species.

8 <sup>c</sup> Abbreviation criterion: first capital letter refers to genus, followed by one or two small letters referring to  
 9 species-country of origin-additional letter to distinguish different sites.

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3 **Table 2** Restriction Fragment Length Polymorphism of the *ca.* 615 bp region of rDNA-  
 4 18S for *Nanidorus*, *Paratrichodorus* and *Trichodorus* species. Expected sizes (base  
 5 pairs) based on sequence information obtained from the EMBL/GenBank/DDBJ  
 6 database

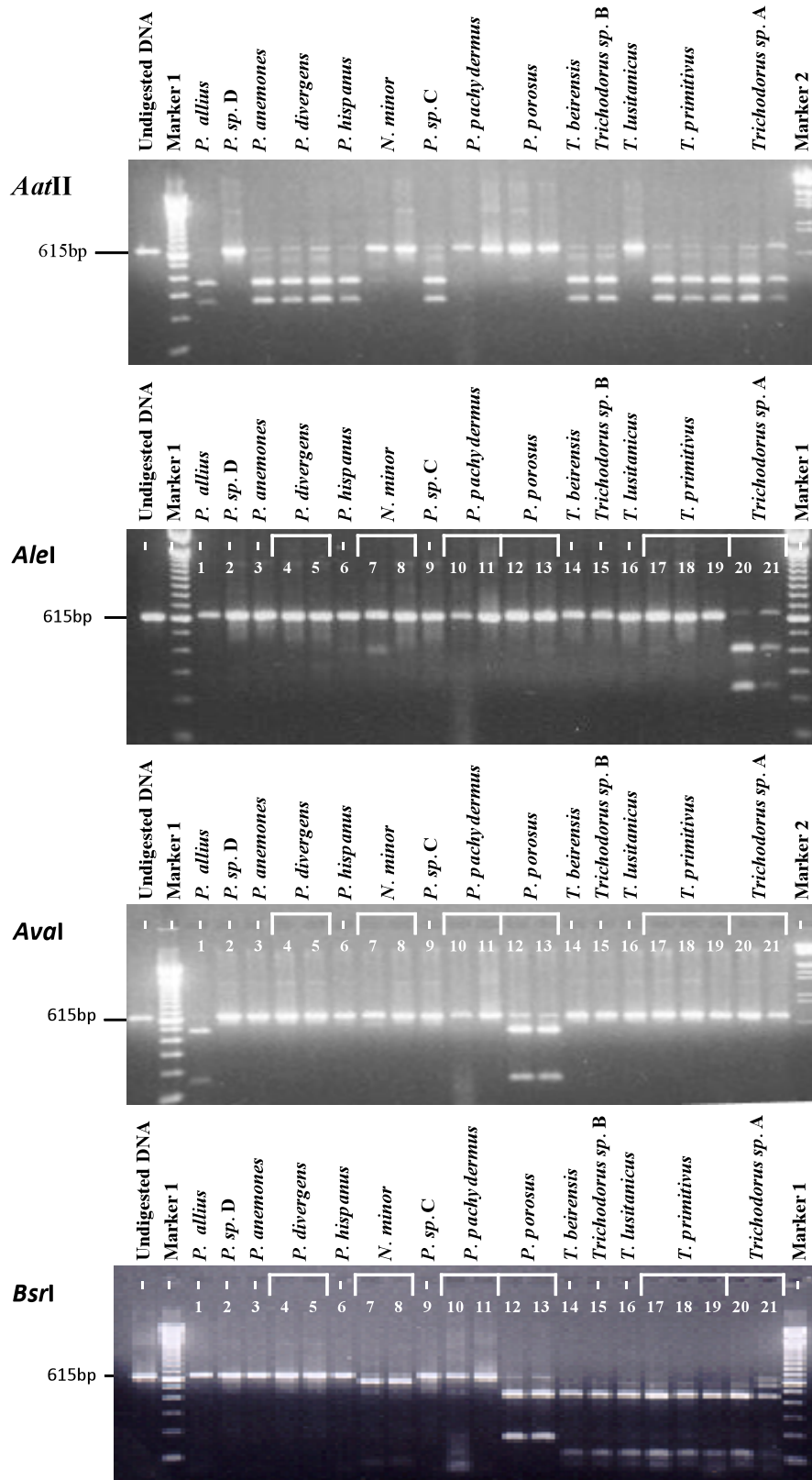
Trichodorid populations	<i>AatII</i>	<i>AleI</i>	<i>AvaI</i>	<i>BsrI</i>	<i>Fnu4HI</i>	<i>TaqI</i>	<i>TspRI</i>
<i>Nanidorus minor</i> – Pt <sup>a</sup>	615	615	615	548, 67	275, 240, 40, 40, 17, 3	615	280, 220, 115
<i>N. minor</i> – Gr	615	615	615	548, 67	275, 240, 40, 40, 17, 3	615	280, 220, 115
<i>N. nanus</i> – NI	615	615	615	548, 67	240, 215, 60, 57, 40, 3	615	280, 220, 115
<i>Paratrichodorus allius</i> – US <sup>a</sup>	358, 256	614	461, 153	614	302, 275, 37	614	282, 246, 86
<i>P. anemones</i> – Engl <sup>b</sup>	356, 256, 1	613	612, 1	612, 1	300, 275, 37, 1	529, 83, 1	332, 280, 1
<i>P. divergens</i> – Pt <sup>a</sup>	357, 257	614	614	614	301, 275, 38	360, 254	246, 220, 87, 61
<i>P. hispanus</i> – Pt <sup>a</sup>	358, 257	615	615	615	302, 275, 38	615	282, 246, 87
<i>Paratrichodorus</i> sp. C – Pt	358, 255	613	613	613	275, 241, 50, 36, 8, 3	246, 255, 112	282, 246, 85
<i>P. pachydermus</i> – Sct <sup>a</sup>	615	615	615	615	302, 275, 38	615	282, 246, 87
<i>P. porosus</i> – Pt <sup>a</sup>	614	614	461, 153	440, 174	302, 275, 37	614	282, 246, 86
<i>P. teres</i> – Gr <sup>a</sup>	614	614	614	614	302, 275, 37	556, 58	282, 246, 86
<i>Trichodorus beirensis</i> – Pt <sup>a</sup>	357, 258	615	615	442, 105, 68	301, 275, 39	615	334, 281
<i>T. lusitanicus</i> – Pt <sup>a</sup>	615	615	615	442, 105, 68	301, 275, 39	615	334, 281
<i>T. primitivus</i> – Pt <sup>a</sup>	356, 259	615	615	443, 105, 67	300, 192, 83, 40	615	280, 246, 89
<i>T. primitivus</i> – Sct	356, 259	615	615	443, 105, 67	300, 192, ( <sup>b</sup> ), 40	615	280, 246, 89
<i>Trichodorus</i> sp. A – Pt <sup>a</sup>	357, 258	392, 223	615	442, 105, 68	301, 275, 39	615	334, 281
<i>Trichodorus</i> sp. B – Pt <sup>a</sup>	357, 258	615	615	442, 105, 68	301, 192, 83, 39	615	334, 281

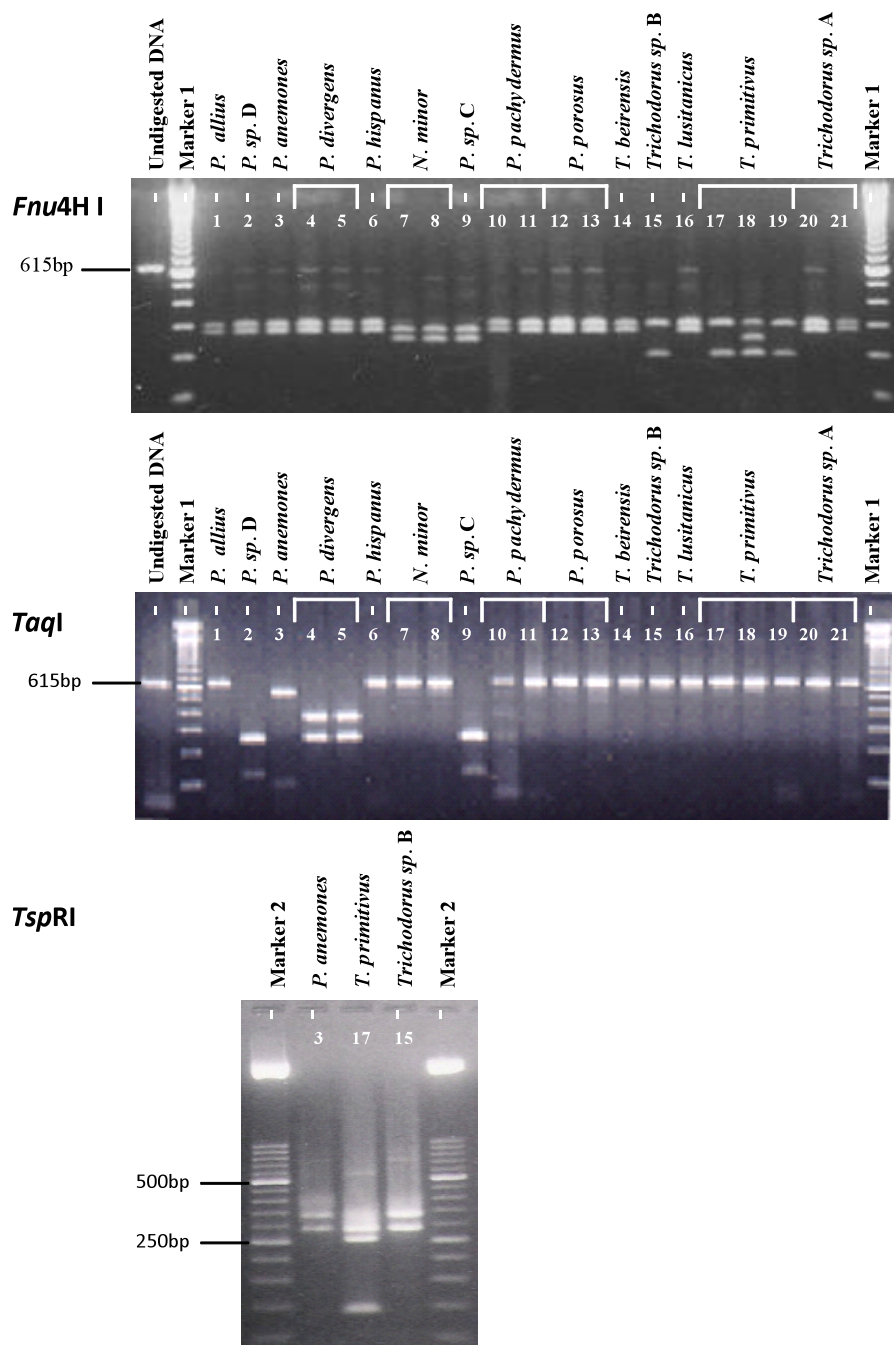
7 Engl, England; Gr, Greece; Pt, Portugal; Sct, Scotland; US, USA; <sup>a</sup> Molecular type  
 8 species to the purpose of the present work; <sup>b</sup> a fragment of 83 nt is absent from this  
 9 expected restriction fragments, when compared with the Portuguese sequence. This  
 10 difference is due to the presence of 12 undetermined nucleotides in the Scottish *T.*  
 11 *primitivus* sequence (accession number, AF036609), abrogating the identification of a  
 12 putative *Fnu4HI* restriction site.

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2 **Figure 1** Restriction patterns of the PCR products enclosing the 3' end of 18S rDNA  
3 region of trichodorids obtained by simple digestion with *AatII*, *AleI*, *AvaI*, *BsrI*, *Fnu4H*  
4 I, *TaqI* and *TspRI*. Code of the lanes: (1) *P. allius*, US; (2) *Paratrichodorus* sp. D, Pt; (3)  
5 *P. anemones*, Engl; (4) *P. divergens*, Pt-b; (5) *P. divergens*, Pt-a; (6) *P. hispanus*, Pt; (7)  
6 *N. minor*, Pt; (8) *N. minor*, Br; (9) *Paratrichodorus* sp. C, Pt; (10) *P. pachydermus*, Pt;  
7 (11) *P. pachydermus*, Sct; (12) *P. porosus*, Pt; (13) *P. porosus*, Bs; (14) *T. beirensis*, Pt;  
8 (15) *Trichodorus* sp. B, Pt; (16) *T. lusitanicus*, Pt; (17) *T. primitivus*, Pt; (18) *T.*  
9 *primitivus*, Pt-Az; (19) *T. primitivus*, Sct; (20) *Trichodorus* sp. A, Pt-a; (21) *Trichodorus*  
10 sp. A, Pt-b. *TspRI* patterns identical to those shown in lanes 3 and 17 were obtained

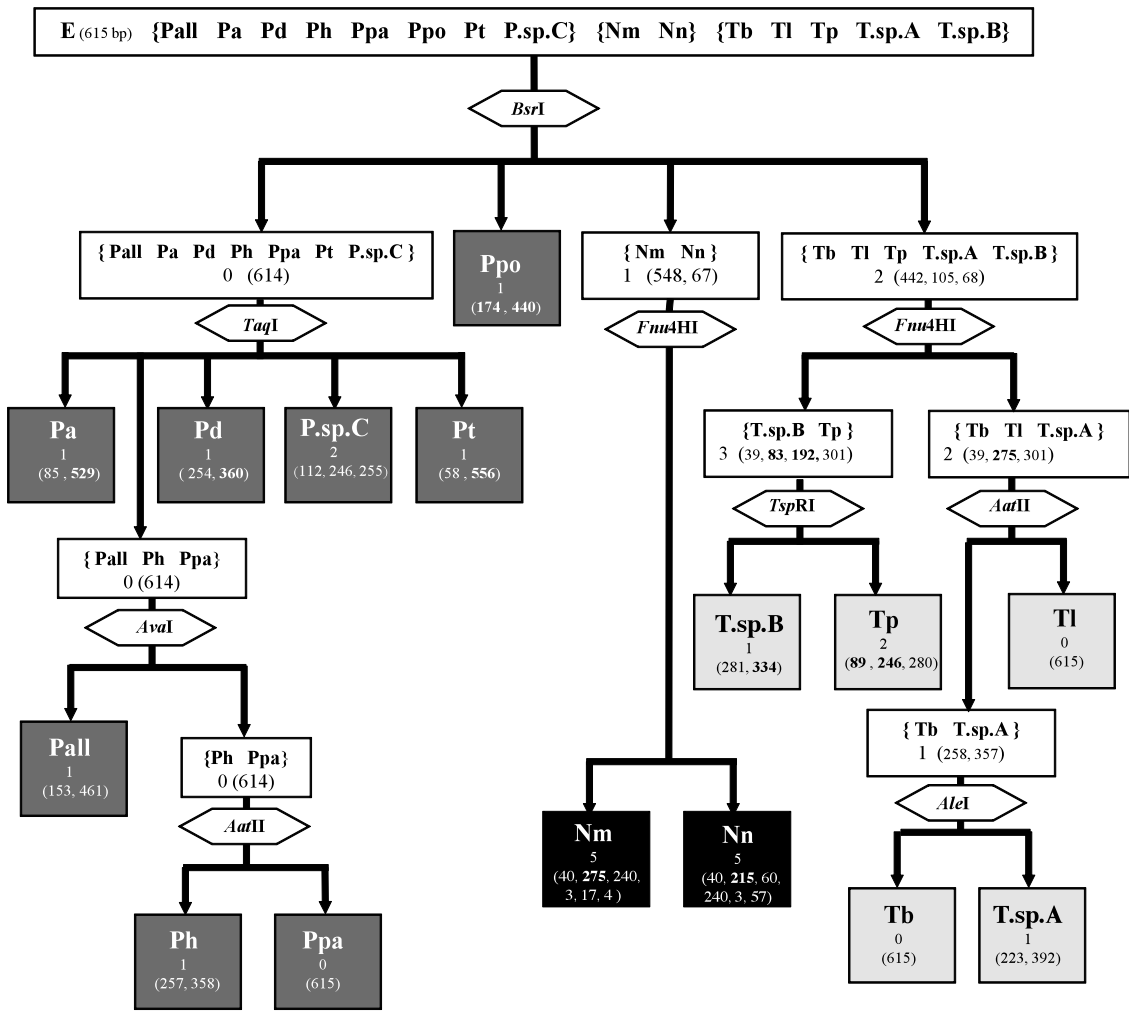


1 respectively with *Paratrichodorus* sp. D, Pt; *T. beirensis*, Pt; *T. lusitanicus*, Pt;  
2 *Trichodorus* sp. A, Pt-a; *Trichodorus* sp. A, Pt-b and *T. primitivus*, Pt-Az; *T. primitivus*,  
3 Sct; *P. allius*, US; *P. hispanus*, Pt; *Paratrichodorus* sp. C, Pt; *P. pachydermus*, Pt; *P.*  
4 *pachydermus*, Sct; *P. porosus*, Pt; *P. porosus*, Bs (results not shown). DNA molecular  
5 weight markers: Marker 1, 100 bp Ladder (Invitrogen, Life Technologies); Marker 2,  
6 XIII (50-750 bp), Roche, Diagnostic GmbH.

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3 **Figure 2** Flow chart summarizing the Restriction Enzyme digestions required for  
 4 trichodorid identification to species level with PCR-RFLP technique. Rectangles,  
 5 represent a group of indistinguishable species; hexagons, represent the digestions with  
 6 restriction enzymes; Species identified are included in black (*Nanidorus*), dark grey  
 7 (*Paratrachodorus*) and light grey (*Trichodorus*) squares: top line, species identified;  
 8 middle line, number of targets for the RE indicated above; bottom line, sizes (bp) of the  
 9 fragments produced. Nm, *N. minor*; Nn, *N. nanus*; Pall, *P. allius*; Pa, *P. anemones*; Pd,  
 10 *P. divergens*; Ph, *P. hispanus*; Ppa, *P. pachydermus*; P.sp.C, *Paratrachodorus* sp. C;  
 11 Ppo, *P. porosus*; Pt, *P. teres*; Tb, *T. beirensis*; Tl, *T. lusitanicus*; Tp, *T. primitivus*;  
 12 T.sp.A, *Trichodorus* sp. A; T.sp.B, *Trichodorus* sp. B.

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LEGENDS OF FIGURES

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**Figure 1** Restriction patterns obtained by simple digestion of the PCR products enclosing the 3' end of 18S rDNA region of trichodorids.

**Figure 2** Flow chart summarizing the Restriction Enzyme digestions required for trichodorid identification to species level.

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