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Morphological and molecular characterization of two isolates of *Paratrichodorus porosus* from Shenzhen, China

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Summary

Studies were conducted to characterize morphological and molecular profiles of two isolates of *Paratrichodorus porosus* (SZ1 and SZ2) which were recovered from *Acacia mangium* in Tianxinshan and *Gleichenia linearis* in Yangmeikeng environmental monitoring sites in Shenzhen, China, respectively. Analysis of morphometric, morphological and molecular characters revealed these two Shenzhen isolates are identical to *P. porosus.* Measurements of both study isolates lie within the ranges for *P. porosus.* It is typologically characterized by possessing a clearly swollen body cuticle after fixation, an onchiostyle ventrally curved, 46 – 58 μm long, a pharyngeal bulb usually with a well developed anterior-dorsal intestinal overlap, a secretory-excretory pore opening between the nerve ring and anterior end of pharyngeal bulb, 90 – 110 μm from the anterior end, a reproductive system with didelphic, amphidelphic, without spermathecae, a pore-like vulva in ventral view and occupying 52.0 % – 59.5 % of total body length from anterior end, a short and barrel-shaped vagina with small sclerotizations, a pair of ventromedian advulvar body pores located prevulvar and postvulvar, a rounded tail and a subterminal anus in females. The sequence analysis based on partial rDNA 18S gene and 28S D2/D3 expansion segment confirm its identity as *P. porosus*. This is the first report of *P. porosus* associated with *A. mangium* and *G. linearis*.

Keywords: *Paratrichodorus porosus; Acacia mangium*; *Gleichenia linearis;* 18S small subunit rDNA; 28S large subunit rDNA; PCR; stubby-root nematode; taxonomy

Introduction

Paratrichodorus Siddiqi, 1974 is a cosmopolitan stubby-root nematode genus occurring mainly in tropical and subtropical regions (Hunt, 1993). *Paratrichodorus* species are migratory ectoparasites of roots. *Nanidorus minor* (Colbran, 1956) Siddiqi, 1974, which was formerly known as *Trichodorus christiei*, was first ectoparasitic nematodes shown to damage plants (Christie & Perry, 1951). Significant crop losses due to *Paratrichodorous* and other trichodorids, both as plant parasites and as vectors of plant-pathogenic viruses, is a worldwide problem (Decraemer, 1991). Thirty-three nominal species in *Paratrichodorus* have been described. Among those, *P. porosus* (Allen, 1957) Siddiqi, 1974 is a widespread and economically important species that causes damage to sugarcane, camellia, maize, sorghum and grapes etc. It is distributed in 24 countries around the globe (CABI, 2009). In China it was found in Fujian, Yunnan, Zhejiang, Guangdong provinces (Zheng *et al*., 2004; Zhao *et al*., 2005; Liao *et al*., 2011).

P. porosus is designated as a quarantine pest by many countries (Seed Association of Thailand). It is known to transmit tobacco rattle virus (TRV) (Ayala & Allen, 1968; Taylor & Brown, 1997) and causes black-rot disease of Chinese yam (*Dioscorea batatus*) (Nishizawa, 1973). It also has a broad host range of up to 100 plant species (Decraemer, 1995; Sheedy *et al*., 2010) and is an important pest of many crops (Plantwise). Feeding by *P. porosus* on cells of root tips causes growth and elongation of roots to cease, and results in stubby-root symptoms. *P. porosus* causes extensive damage to the root system of *Camellia* (Barriga, 1965) and affects forage production in North Carolina, USA, by damaging maize and sorghum crops along with *Tylenchorhynchus claytoni* and *Pratylenchus zeae* (Chévres-Román *et al*., 1971). Heavy parasitism of them affected the uptake of nutrients and water in plants from the soil (Chévres-Román *et al*., 1971).

Accurate identification of trichodorids to the species level is crucial to implement appropriate control measures for these nematodes. Typically, trichodorid identification is based on analysis of morphological and morphometrical

characters. However, differentiation of some species, mainly in *Paratrichodorus* is often difficult due to high intraspecific variability. Recently, DNA-based approaches have been successfully employed for the molecular diagnosis of trichodorids (Boutsika *et al*., 2004; Riga *et al*., 2007; Duarte *et al*., 2011; Kumari & Subbotin, 2012). Kumari & Subbotin (2012) reported that analysis of the D2/D3 of the 28S rDNA sequence data set revealed four unidentified species of *Trichodorus*, and the partial 18S rDNA sequence data set distinguished four unidentified species of *Trichodorus* and two unidentified species of *Paratrichodorus*. Duarte *et al*. (2011) developed a PCR-RFLP assay based on the 18S rRNA gene for rapid identification of 12 trichodorid nematodes belonging to *Trichodorus*, *Paratrichodorus* and *Nanidorus*.

During a survey of nematodes in five environmental monitoring sites in Shenzhen, China in 2013, two *Paratrichodorus* isolates, SZ1 and SZ2, were recovered from soils around the roots of *Acacia mangium* Willd. in Tianxinshan and *Gleichenia linearis* (Burm. f.) Clarke in Yangmeikeng, respectively. Through this study, both nematode isolates were identified as *P. porosus* (Allen, 1957) Siddiqi, 1974, representing the first report of this nematode from *A. mangium* and *G. linearis*.

The main objectives of this study were to: (i) confirm the identity of two *P. porosus* isolates based on morphological and molecular approaches; and (ii) investigate their phylogenetic relationships with other *Paratrichodorus* species based upon sequence analysis of the 18S and 28S D2/D3 rDNA.

Materials and methods

Morphological Characterization

Nematode isolates SZ1 and SZ2 in this study were recovered from rhizospheric soils in *A. mangium* and *G. linearis*, respectively. Nematodes were extracted by a sieving and decanting method (Brown & Boag, 1988). Specimens were heat-killed, fixed in 3 % formaldehyde and processed to glycerin by the formalin-glycerin method (Hooper, 1970; Golden, 1990). Specimen preparation and measurements were as described in Golden and Birchfield (1972). Measurements of nematodes were performed with the aid of a camera lucida and a stage micrometer. The morphometric data were processed using Excel software (Ye, 1996). Photomicrographs were taken with a Leica video camera (DFC490) attached via a C-mount Adapter fitted on a Leica microscope (DM4000B), and edited using Adobe Photoshop CS5. Morphological identification of specimens was done using keys provided by Decraemer (1995) and Decraemer and Baujard (1998), with corresponding species descriptions.

Molecular Characterization

For each isolate, three females were hand-picked into distilled water for DNA extraction, amplification, and sequencing. They were placed into 50 µl of worm lysis buffer (WLB) containing Proteinase K for DNA extraction (Williams *et al*., 1992). DNA samples were stored at – 20 °C until used as a PCR template.

The primers used for polymerase chain reaction (PCR) and DNA sequencing are presented in Table 1. Primers SSUF 07/SSUR26, 18S965/18S1573R and 18SnF/18SnR were used for PCR amplification and DNA sequencing for small subunit 18S and D2A/D3B for large subunit 28S rDNA.

The 25 µl PCR was performed using TaqMix DNA polymerase (Guangzhou Dongsheng Biotech Ltd., Guangzhou, China) according to the manufacturer's protocol. The thermal cycler program for PCR was as follows: denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C with 30 s; annealing at 55 °C for 45 s, and extension at 72 °C for 2 min. A final extension was performed at 72 °C for 10 min (Ye *et al*., 2007).

PCR products were cleaned using an EZ Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, Ontario, Canada) according to the manufacturer's protocol before being sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China) using an ABI PRISM 3730 sequencing system.

The nematode sequences from this project were deposited in genBank. We used DNA sequences with the highest matches with our isolates from the genBank database for phylogenetic analysis. DNA sequences were aligned using ClustalW (San Diego Supercomputer Center). The model of base substitution in the 18S and 28S sets was evaluated using MODELTEST version 3.06 (Posada & Crandall, 1998). The Akaike-supported model, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) running the chain for 10^6 generations and setting the 'burn in' at 1000. We used MCMC (Markov Chain Monte Carlo) methods within

Table 1. Primers used for polymerase chain reaction and DNA sequencing

Primer	Marker	Sequence (5' to 3')	Reference
SSUF07	18S	AAAGATTAAGCCATGCATG	Floyd *et al*. (2002)
SSUR26	18S	CATTCTTGGCAAATGCTTTCG	Floyd *et al*. (2002)
18S965	18S	GGCGATCAGATACCGCCCTAGTT	Mullin *et al*. (2005)
18S1573R	18S	TACAAAGGGCAGGGACGTAAT	Mullin *et al*. (2005)
18SnF	18S	TGGATAACTGTGGTAATTCTAGAGC	Kanzaki and Futai (2002)
18SnR	18S	TTACGACTTTTGCCC GGTTC	Kanzaki and Futai (2002)
D2A	28S	ACAAGTACCGTGAGGGAAAGTTG	Nunn (1992)
D3B	28S	TGCGAAGGAACCAGCTACTA	Nunn (1992)

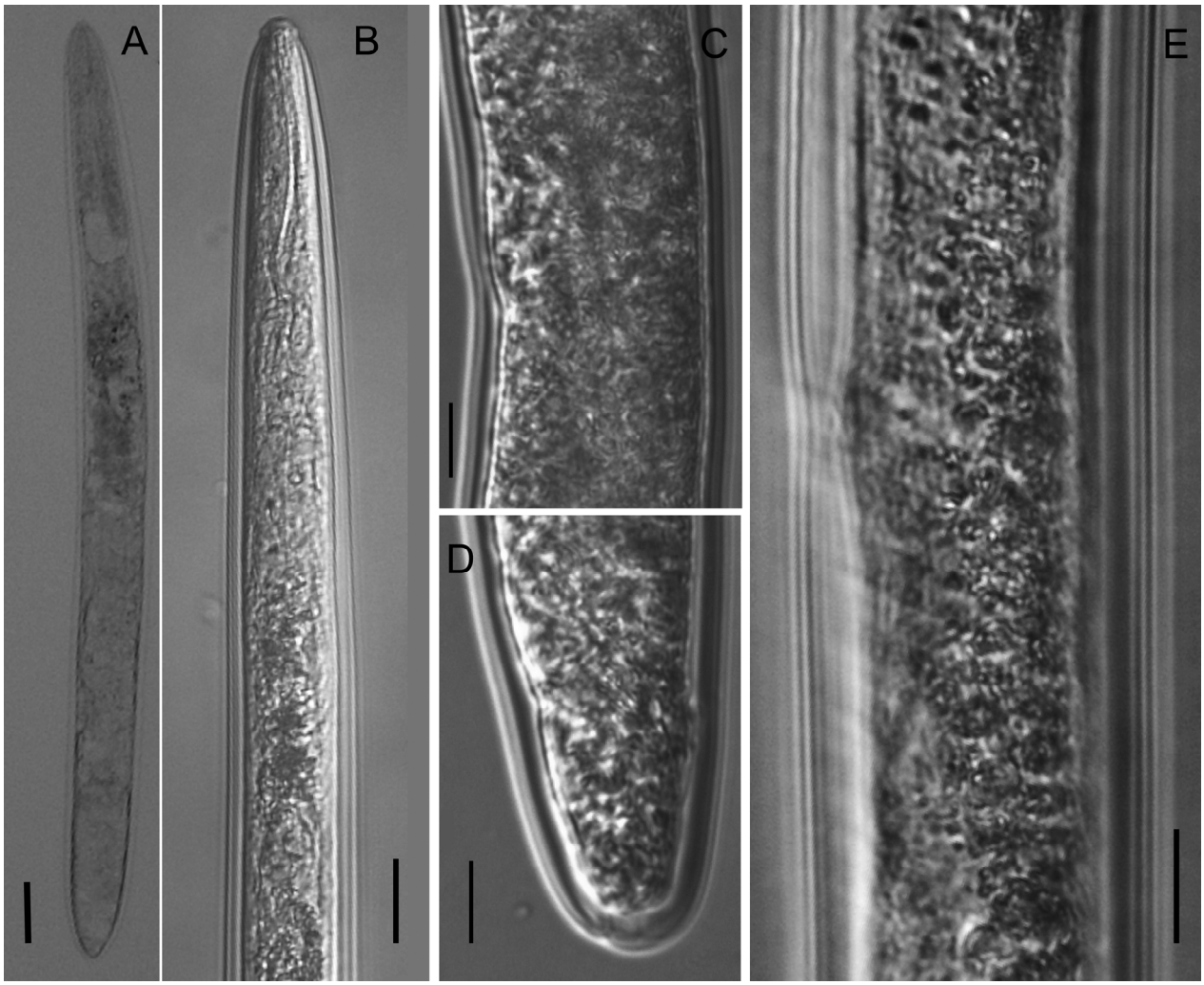

Fig. 1. Light micrographs of *Paratrichodorus porosus* from *Acacia mangium and Gleichenia linearis*. A: Female entire body; B: Female anterior body; C: Female onchiostyle; D: Female tail; E: Reproductive system of female. Scale bars: A=50µm; B=20µm; C – E=10µm

a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using the 50 % majority-rule.

Results

Morphological description

Morphometrics of females of two isolates of *P. porosus* are presented in Table 2. Measurements of both study isolates lie within the ranges previously reported for *P. porosus* (Decraemer, 1995; Zheng *et al*., 2004; Zhao *et al*., 2005; Liao *et al*., 2011). These two isolates are identical based on morphology and molecular characteristics, thus they are treated as the same species (but different geographical isolates) in the following description.

Female: General appearance typical for the genus. Body configuration usually rounded at both ends and straight when heat-killed (Fig. 1). Body cuticle clearly swollen after fixation, 3.5 – 5.0 µm thick at mid-body. The onchiostyle ventrally curved, guide ring around or less than distal third of onchiostyle, at 15 – 20 µm from anterior end. Pharyngeal bulb usually with a well developed anterior-dorsal intestinal overlap, bulb rarely offset. Secretory-excretory pore between nerve ring and anterior end of pharyngeal bulb. Reproductive system didelphic, amphidelphic, without spermathecae. Vulva pore-like in ventral view. Vagina short, barrel-shaped. Vaginal sclerotizations small, inconspicuous. One pair of ventromedian advulvar body pores, located prevulvar and postvulvar (one anterior and one posterior to vulva), lateral body pores absent. Rarely sperm in uteri. Tail rounded. Anus subterminal.

Male: Not found.

Morphometric and morphological analysis:

Morphometrics of *P. porosus* SZ1 did not differ from those of *P. porosus* SZ2, fitting in the ranges of those reported in the previous description except for a lower b value from both SZ1 and SZ2 than that reported by Liao *et al*. (2011) (4.3 – 4.8 *vs* 4.8 – 5.5), and a lower a value from *P. porosus* SZ1 than those reported by Liao *et al*. (2011) (14.3 – 16.5 *vs* 17.4 – 17.8) and Zheng *et al*. (2004) (14.3 – 16.5 *vs* 16.0 – 23.0) (Table 2). Morphologically, no apparent difference was found except for a smaller number of ventromedian advulvar body pores from study isolates than those reported by Zheng *et al*. (2004) and Zhao *et al*. (2005) (one

Table 2. Morphometrics of females of studied isolates of *Paratrichodorus porosus* mounted in formalin–glycerin
All measurements in μm and in the format: mean ± s.d. (Range)

Character	*P. porosus* SZ1	*P. porosus* SZ2
Host	*Acacia mangium*	*Gleichenia linearis*
n	10	10
L	568.5 ± 42.3 (520.8 – 601.5)	589.8 ± 75.2 (532.6 – 700.2)
a	15.7 ± 1.2 (14.3 – 16.5)	16.6 ± 2.3 (16.0 – 25.0)
b	4.5 ± 0.2 (4.3 – 4.7)	4.5 ± 0.2 (4.4 – 4.8)
V	53.2 ± 1.5 (52.0 – 54.9)	54.8 ± 3.2 (52.6 – 59.5)
Onchiostyle	51.1 ± 6.5 (45.8 – 58.3)	51.0 ± 4.8 (45.8 – 57.4)
Secretory-Excretory pore from anterior end	98.3±5.0 (93.0 – 103.0)	97.8 ± 9.0 (90.0 – 110.0)
Vagina length	9.1 ± 0.2 (9.0 – 9.3)	9.8 ± 0.5 (9.3 – 10.5)
Size of vaginal pieces	1.3 ± 0.1 (1.1 – 1.4)	1.2 ± 0.1 (1.1 – 1.3)
Anterior genital branch	152.9 ± 6.6 (140.2 – 166.7)	171.9 ± 20.0 (152.6 – 200.0)
Posterior genital branch	145.3 ± 7.5 (136.7 – 150.0)	166.4 ± 13.7 (150.8 – 183.3)
Body diam. (greatest body diam.)	36.1 ± 0.7 (35.4 – 36.7)	36.3 ± 8.4 (28.0 – 48.0)
Pharynx	126.0 ± 6.6 (120.0 – 133.0)	130.1 ± 11.0 (121.8 – 146.2)

pair *vs* two pairs), than that reported by Liao *et al*. (2011) (one pair *vs* three pairs), indicating intraspecies variability in the morphological characters among *P. porosus* isolates from different locations.

Molecular Phylogenetic Relationships

A 1131-bp 18S rDNA and a 732-bp 28S D2/D3 expansion segment were amplified and sequenced. Sequences of the rDNA were compared using blastN search from a diverse collection of *Paratrichodorus* species presented in genBank. Two *P. porosus* isolates (genBank accession No. KJ641548 and KJ641550 for SZ1 and KJ641549 and KJ641551 for SZ2) in this study are identical either for 18S or 28S.

The alignment for the partial 18S rDNA included 71 sequences. Forty-six *Paratrichodorus,* nine *Trichodorus* and 16 *Nanidorus* nominal and putative isolates were included in this analysis. Two studied isolates of *P. porosus* is for 99 % identical with all other 19 Chinese isolates, one Portugal (DQ345524) and one American isolate (JN123368) of *P. porosus,* but 95 % and 91 % with one Brazil (AJ438059) and another Portugal isolate (AJ438060), respectively. Intraspecific sequence variations for *P. porosus* was 0 % – 6.1 % (0 – 68 nt). Without Brazil (AJ438059) and Portugal isolate (AJ438060), the intraspecific sequence variations was 0 % – 0.3 % (0 – 3 nt). The identities of both *P. porosus* isolates are 97 % – 98 %, 98 %, 97 %, 96 %, 95 % – 97 %, 90 % – 93 % with *P. teres, P. allius*, *P. pachydermus, P. divergens, P. hispanus, P. anemones,* 93 %, 93 %, 93 % with *N. minor, N. renifer, N. nanus,* and 93 % – 95 % with *Trichodorus* spp. (*T. beirensis, T. cylindricus, T. pakistanensis, T. primitivus, T. similis, T. sparsus* and *T. variopapillatus*), respectively. Based on these data, the Brazil isolate (AJ438059, 95 % identity) and the Portugal isolate (AJ438060, 91 % identity) are very likely different species rather than *P. po-*

Fig. 2. The 10001st Bayesian tree inferred from *Paratrichodorus porosus* 18S under GTR+I+G model (-lnL=4871.895; freqA=0.2775; freqC=0.2107; freqG=0.2603; freqT=0.2515; R(a)=1.9822; R(b)=3.5626; R(c)=2.2002; R(d)=0.8367; R(e)=7.7939; R(f)=1; Pinva=0.4142; Shape=0.6124). Posterior probability values exceeding 50% are given on appropriate clades.

rosus. It's worthy to note that two isolates of *P. teres* (AM269896 and AM087125) are in a monophyletic clade with many other *P. porosus* isolates and are significantly different from two *P. teres* isolates (AJ439575 and FJ040484). This result revealed misidentification and further morphological examination is needed.

Phylogenetic analysis of the partial 18S and 28S D2/D3 were performed to examine the relationships of two study *P. porosus* isolates among other *Paratrichodorus* species sequenced using the same loci. The dendrogram inferred from SSU (Fig. 2) using *Tylolaimophorus minor* (Thorne, 1939) Goodey, 1963 and *Diphtherophora obesus* Thorne, 1939 as outgroups suggested that: *i*) all the selected trichodorids are in a monophyletic clade in relation to *Tylolaimophorus minor* with 100 % posterior probability (pp); *ii*) both study isolates of *P. porosus* is in a well-supported (pp=100 %) monophyletic clade with five Zhejiang isolates (GU645955, GU645868, GU645957, GU645865, GU645 949), 11 Yunnan isolates (GU645954, GU645862, GU645 952, GU645861, GU645860, GU645859, GU645950, GU645863, GU645864, GU645951, GU645953), three Fujian isolates (GU645867, GU645866, GU645956), one American isolate (JN123368) and one Portugal isolate (DQ345524) of *P. porosus,* one Greece (AM269896) and one American (AM087125) of *P. teres* isolate. One Brazil isolate (AJ438059) and one Portugal isolate (AJ438060) of

P. porosus are in a separate clade close to *N. renifer* and *N. monor* which revealed they are not *P. porosus*. *iii*) Brazil isolate (AJ438059) and Portugal isolate (AJ438060) of *P. porosus* are in a monophyletic clade in relation to one Greece isolate (AM269897) and seven isolates of *N. minor* including four Yunnan isolates (GQ995706, GQ995707, GQ995705, GQ995708) and three Hainan isolates (GQ995710, GQ995703, GQ995704) with 100 % posterior probability. This result supports they are not *P. porosus.*

The alignment for the D2/D3 of 28S rDNA included 43 sequences. Twenty-five *Paratrichodorus,* eight *Nanidorus*, eight *Trichodorus* nominal and two putative isolates were included in this analysis. Both studied *P. porosus* isolates all share identities of 99 % with all 14 Chinese isolates of *P. porosus*, but 98 % with two American ones (JN123405 and JN123406). Intraspecific sequence variations for *P. porosus* was 0 – 1.8 % (0 – 13 nt). The identities of both *P. porosus* isolates with *P. pachydermus* are 82 % – 88 %, and 79 % – 83 % with *Trichodorus* spp. (*T. cylindricus, T. nanjingensis, T. pakistanensis, T. primitivus* and *T. similis*), 79 % with *N. minor,* 78 % with *N. renifer.*

The tree inferred from D2/D3 of LSU (Fig. 3) using *Tripyla filicaudata* de Man, 1880 and *Tripylina tamaki* Zhao, 2009 as outgroups suggested that: *i*) all the selected trichodorids are in a monophyletic clade in relation to *Tripyla filicaudata* with 100 % posterior probability; *ii*) two studied isolates of *P. porosus* are in a well-supported (pp=100 %) monophyletic clade with all other Chinese isolates including six Zhejiang isolates (GU645843, GU645930, GU645933, EU827614, GU645840, GU645929), four Yunnan isolates (GU723295, GU645838, GU645931, GU645839), three Fujian isolates (GU645842, GU645934, GU645841), one Guangdong isolate (GU645932), and two American isolates (JN123406, JN123405) of *P. porosus*; *iii*) all *P. porosus* isolates, including study isolates SZ1 and SZ2, are closer to five Czech isolates (JN123399, JN123404, JN123402,

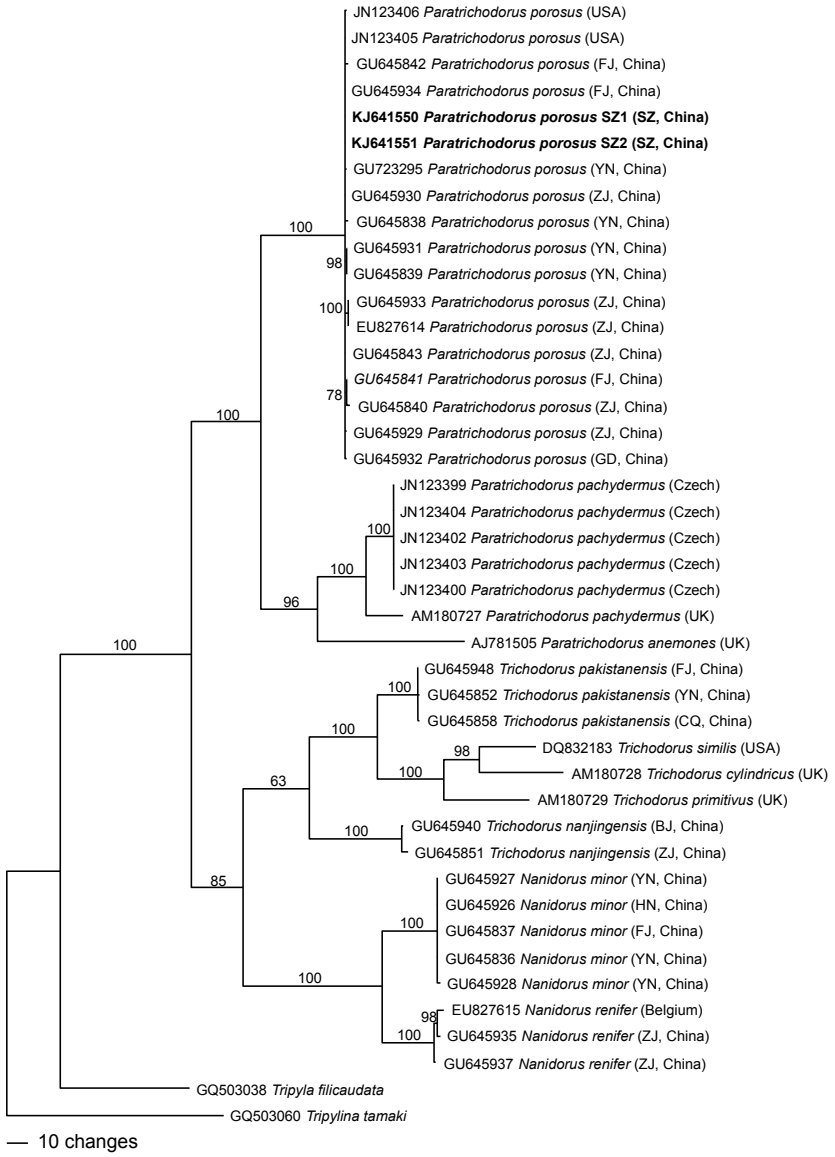

Fig. 3. The 10001st Bayesian tree inferred from *Paratrichodorus porosus* 28S D2/D3 under GTR+I+G model (-lnL=5086.9248; freqA=0.2229; freqC=0.2226; freqG=0.2994; freqT=0.2551; R(a)=0.9556; R(b)=2.4679; R(c)=1.7546; R(d)=0.1858; R(e)=5.0248; R(f)=1; Pinva=0.2515; Shape=0.875). Posterior probability values exceeding 50% are given on appropriate clades.

JN123403, JN123400), one UK isolate (AM180727) of *P. pachydermus* and one UK isolate (AJ781505) of *P. anemones* than five Chinese isolates (GU645927, GU645926, GU645837, GU645836, GU645928) of *N. minor* and two Chinese isolates (GU645935, GU645937) and one Belgium isolate (EU827615) of *N. renifer*.

Discussion

A combined approach that uses morphological and molecular characters is often needed for discrimination of *Paratrichodorus* species at high confidence level. In this study, no apparent differences were found in morphological and morphometric characters between two isolates of *P. porosus* (SZ1 and SZ2) from different localities and vegetation. The analysis of DNA sequences of 18S and 28S D2/D3 confirmed that these two isolates are identical, thus they were considered as the same species. Furthermore, morphological and morphometric characters of both isolates lie within the range of previous reported by Decraemer (1995), although the difference in the number of ventromedian advulvar body pores was observed among several Chinese isolates of *P. porosus*, *i.e.*, one pair for both study isolates, *P. porosus* SZ1 from *A. mangium* and *P. porosus* SZ2 from *G. linearis* in Shenzhen, two pairs for *P. porosus* from *Camellia japonica* and *Prunus serrula* in Zhejiang and *Pyrus* spp. in Yunnan, and three pairs from *Magnoliaceae glance* in Guangdong. Also, analysis of sequences of 18S and 28S D2/D3 indicated that the identities of two studied isolates with other isolates of *P. porosus* except for one Brazil isolate (AJ438059) and one Portugal isolate (AJ438060) are 99 % – 100 %, and molecular phylogenetic analysis revealed that these two isolates are in a monophyletic clade with all other isolates of *P. porosus* except for two isolates (AJ438059 and AJ438060) with 100 % posterior probability. Thus, based upon morphological and molecular characterization, these two isolates from *A. mangium* and *G. linearis*, respectively, are the same species and were identified as *P. porosus* (Allen, 1957) Siddiqi, 1974. To our knowledge, this is the first report of this species on *A. mangium* and *G. linearis*.

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