Eur J Plant Pathol (2010) 128:243–250
DOI 10.1007/s10658-010-9651-8

Molecular characterization of *Xiphinema brevicollum* (Nematoda: Longidoridae) from the Czech Republic

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Accepted: 11 June 2010 / Published online: 4 July 2010

Abstract Populations of *Xiphinema brevicollum* occurring in the Czech Republic were described morphologically and molecularly. Published species-specific primer set BL18 and BV3 was used to amplify three populations of *X. brevicollum* from the Czech Republic. These primers were tested against 9 species of *Xiphinema* and 11 species of *Longidorus.* Amplification was also observed for *X. inaequale*, *X. italiae* and *X. lambertii*. Three additional markers, mitochondrial cytochrome c oxidase subunit 1, ribosomal D2/D3 expansion segment of the 28S gene and 18S gene, were amplified and sequenced for *X. brevicollum*, *X. inaequale* and *X. lambertii* belonging to *X. americanum*-group. Comparison of *cox1* sequences of *X. brevicollum* from the Czech Republic with *X. taylori* from the Slovak Republic (accession number AM086702) suggested that these populations represent the same species.

Keywords *Xiphinema inaequale* · *Xiphinema italiae* · *Xiphinema lambertii* · Nematode · Mitochondrial DNA · Ribosomal DNA

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Introduction

Xiphinema are migratory ectoparasitic nematodes that feed on an extensive range of host plants. Many species are considered putative vectors of nepoviruses (Taylor and Brown 1997). The genus comprises 234 valid species (Coomans et al. 2001). Many species of the *Xiphinema americanum*-group are difficult to differentiate based on morphological features. This is for example well illustrated by *X. brevicollum* (originally described as *X. brevicolle* Lordello and Da Costa 1961) and its large number of junior synonyms (Coomans et al. 2001). One of these junior synonyms in the Coomans et al's list, *X. diffusum*, was re-established as a valid species based on molecular analyses by Oliveira et al. (2005). Since the review by Coomans et al. (2001) *X. petersmithi* was considered a junior synonym of *X. vuittenezi* (Barsi 2005) and additional fourteen new species have been described.

The taxonomic status of *X. brevicollum* occurring in Europe is controversial (Lamberti et al. 1991; Luc et al. 1998; Luc and Baujard 2001; Lamberti et al. 2002). *X. brevicollum* was recorded in European countries (Lamberti and Bleve-Zacheo 1979; Barsi 1989; Lišková 1995); but later on the species occurring in Europe was referred to as a new species *X. taylori* (Lamberti et al. 1991; Lišková et al. 1995; Barsi 1994). According to Lamberti et al. (1991), *X. taylori* has a longer body than *X. brevicollum* , a more anterior vulva position, a shorter odontostyle, a more rounded and expanded lip region and a more symmetrical tail. However, Luc et al. (1998) could not find convincing morphological and morphometric differences to differentiate both species because of overlapping values based upon the study of type specimens or topotypes. Additional features such as length of uteri/ovejector did not provide clear separation of *X. brevicollum* species group. Consequently the authors considered *X. taylori* amongst other species of the *X. brevicollum* group as a junior synonym of *X. brevicollum*. Some nematologists accepted *X. taylori* as a junior synonym while others continued to consider them as two different species. Recently, Repasi et al (2008) reported *X. brevicollum* from Hungary and stressed the use of molecular means to identify the species. The availability of molecular techniques provides new additional tools to differentiate species. Therefore, in this study, our objective was to check and possibly confirm the taxonomic identification of *X. brevicollum* occurring in the Czech Republic by molecular means. To confirm the species status of *X. brevicollum* in the Czech Republic, inclusion of samples from the type locality should have been the best approach but unfortunately samples were not available from the type locality. In the Czech Republic *X. brevicollum* was first recorded from two sites Břeclav and Žadovice by Erbenová (1975) under the rhizosphere of apple. Later on it was recorded from another two sites Hrušky I and Hrušky II under the rhizosphere of grapevine (Kumari et al. 2005). In this study it was recorded from additional two sites.

Materials and methods

Morphometric and morphological analysis

Xiphinema brevicollum specimens from Břeclav (grapevine) and Ratiškovice (peach) were extracted from soil by sieving and decanting methods (Brown and Boag 1988). Specimens for morphological study were heat killed and fixed in TAF, and processed and mounted in anhydrous glycerin. Measurements and photomicrographs were made with the aid of imaging software (Olympus DP-soft) using an Olympus BX-51 light microscope, equipped with a digital camera C4040 and differential interference contrast (DIC, Nomarski). Some specimens were stored in 1M NaCl for molecular analysis. Soil samples for *X. inaequale* and *X. lambertii* were collected and processed by the first author in India and brought to the Czech Republic in TAF for morphological study and in 1M NaCl for molecular study.

DNA extraction, amplification and sequencing

For molecular analysis three populations (Břeclav, Hrušky and Ratiškovcie) of *X. brevicollum* from the Czech Republic were used. Because the status of *X. brevicollum* is controversial, temporary mounts of two specimens of *X. brevicollum* per population (three populations) were made in a drop of 1M NaCl containing glass beads and after taking measurements and photomicrographs the slides were dismantled and total genomic DNA from these specimens was prepared by using a rapid technique described by Stanton et al. (1998). Morphometrics and photomicrographs of these six individuals of *X. brevicollum* used for sequencing are available from the first author on request. Specimens of other nematode species used in this study were not documented prior to DNA extraction.

The species-specific amplification of the partial 18S+ITS1 region of *X. brevicollum* was carried out by using the reverse species-specific primer BV3 and the universal forward primer BL18 (Table 1). This species-specific primer set (BL18+BV3) for *X. brevicollum* was also tested on 9 nematode species belonging to the genus *Xiphinema* (*X. dentatum, X. diversicaudatum, X. inaequale, X. insigne, X. italiae, X. lambertii, X. pachtaicum, X. simile, X. vuittenezi*) and 11 species of the genus *Longidorus* (*L. distinctus, L. elongatus, L. intermedius, L. iuglandis, L. juglandicola, L. juvenilis, L. leptocephalus, L. picenus, L. pisi, L. poessneckensis, L. uroshis).* PCR reactions were performed in a 25 µl volume with the following mastermix: one PCR bead (GE Healthcare, Buckinghamshire, UK), 20.5 µl double distilled sterile water,

Table 1 Primers used to amplify 18S+ITS1 rDNA, *cox1* mtDNA, D2/D3 expansion segments of 28S rDNA and 18S rDNA

Region	Primer name	Direction	Primer sequence 5′–3′	Reference
18S+ITS1	BL18	forward	CGA ATA TAG TAC TTC GAT CAG	Oliveira et al. (2005)
18S+ITS1	BV3	reverse	CCC GTC GMT ACT ACC GAT T	Oliveira et al. (2005)
cox1	COIF	forward	GAT TTT TTG GKC ATC CWG ARG	He et al. (2005)
cox1	COIR	reverse	CWA CAT AAT AAG TAT CAT G	He et al. (2005)
D2/D3	D2A	forward	ACA AGT ACC GTG AGG GAA AGT TG	De Ley et al. (1999)
D2/D3	D3B	reverse	TCG GAA GGA ACC AGC TAC TA	De Ley et al. (1999)
18S	988F	forward	CTC AAA GAT TAA GCC ATG C	Holterman et al. (2006)
18S	1912R	reverse	TTT ACG GTC AGA ACT AGG G	Holterman et al. (2006)
18S	1813F	forward	CTG CGT GAG AGG TGA AAT	Holterman et al. (2006)
18S	2646R	reverse	GCT ACC TTG TTA CGA CTT TT	Holterman et al. (2006)

2.0 μl each primer (10 pmol/μl) and to this 0.5 μl of DNA was added as a template for PCR. A negative control (sterilized water) was included in all PCR experiments. All PCR reactions were performed on a DNA Engine PTC-1148 thermal cycler (Bio-Rad) with heated lid. The cycling conditions were: first denaturation for 3 min at 94°C, 40 cycles with 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final elongation step was run at 72°C for 10 min. An aliquot of each PCR reaction was separated on 1.5 % agarose gel in Tris-Acetate-EDTA (TAE), stained with syber-safe and visualized with UV illumination (312 nm). The remaining DNA of positive samples was purified using High Pure Product Purification kit (Roche Diagnostics GmbH, Manheim, Germany) and directly sequenced in both directions (Macrogen, Korea). Sequencher™ 4.8 (Genes Codes. Corp., Ann Arbor, MI, USA) software was used to assemble and view each sequence and check for base-calling errors.

The cycling profiles for three additional molecular markers *cox1*, D2/D3 expansion segments of 28S and 18S gene were the same as described by Kumari et al. (2009), PCR mix was as described above; primers and references are listed in Table 1. The 18S gene was amplified in two overlapping fragments and primer combination was 988F+1912R for the first fragment and 1813F+2646R for the second fragment. Aliquots of PCR were analyzed by electrophoresis and the remaining products were purified, sequenced and assembled as described above. Sequences have been submitted at the National Centre for Biotechnology Information (NCBI) database and the corresponding accession numbers are reported in Table 2. Pairwise distance was calculated by MEGA (Kumar et al. 2004).

Table 2 NCBI accession numbers for 18S+ITS1 rDNA, *cox1*-mtDNA, D2/D3 expansion segments of 28S-rDNA and 18S-rDNA sequences

Druh	Locality	Morphological reference	NCBI accession numbers			
			18S+ITS1	*cox1*	D2/D3	18S
X. brevicollum	Břeclav[a]	This study	HM163202	HM163206	HM163209	HM163212
X. brevicollum	Hrušky [a]	Kumari et al. (2005)	[b]	[b]	[b]	[b]
X. brevicollum	Ratiškovcie[a]	This study	[b]	[b]	[b]	[b]
X. inaequale	India	This study	HM163203	HM163207	HM163210	HM163213
X. italiae	Slovakia	Lišková et al. (1993)	HM163204	[c]	[c]	[c]
X. lambertii	India	This study	HM163205	HM163208	HM163211	HM163214

[a] Czech Republic; [b] sequences identical to locality Břeclav; [c] Kumari and Lišková 2009

Table 3 Morphometrics of *Xiphinema brevicollum*, *X. inaequale* and *X. lambertii*. Measurements in μm (in form): mean±standard deviation (range)

Country	Czech Republic	Czech Republic		India	India
Locality	Břeclav	Ratiškovice		Baijnath, HP	Baijnath, HP
Specimens	*X. brevicollum*	*X. brevicollum*		*X. inaequale*	*X. lambertii*
	females	females	male	females	females
n	11	5	4	20	20
L	2115±128 (1857–2286)	2250±46 (2220–2331)	2059±97 (1965–2191)	1775±110 (1527–1915)	1519±63 (1410–1657)
a	37.8±1.12 (36.3–39.2)	40.7±0.64 (39.9–41.6)	45.7±3.81 (41.3–49.1)	40.8±3.05 (36.2–46.6)	37.7±3.42 (32.5–47.1)
b	6.8±0.51 (5.9–7.7)	6.7±0.19 (6.4–6.9)	6.1±0.53 (5.7–6.7)	6.3±0.68 (5.2–8.0)	6.5±0.47 (5.8–7.8)
c	84.2±5.73 (74.8–95.3)	80.2±6.29 (72.8–86.0)	69.5±5.40 (63.4–76.5)	51.1±4.97 (45.4–68.4)	41.6±2.53 (38.2–48.2)
c	0.78±0.07 (0.66–0.88)	0.85±0.06 (0.76–0.90)	1.03±0.10 (0.93–1.14)	1.57±0.14 (1.10–1.75)	1.83±0.17 (1.46–2.11)
V/spicule	50±1.00 (48–51)	49±0.80 (48–50)	48±6.22 (41–55)	52.9±1.01 (50.9–54.8)	50.7±1.01 (49.2–52.6)
Odontostyle	88±2.09 (85–92)	90±2.88 (87–94)	93±4.86 (88–99)	92±3.43 (83–98)	58±1.49 (54–60)
Odontophore	57±2.66 (54–62)	58±1.79 (57–61)	55±3.10 (51–58)	48±2.44 (43–52)	43±1.42 (40–45)
Total stylet length	145±3.02 (140–149)	145±10.06 (128–155)	148±2.94 (145–151)	140±4.8 (129–148)	101±1.90 (97–104)
Greatest flange width	10±1.12 (9–12)	9±0.45 (9–10)	7±0.96 (6–8)	8±1.05 (5–9)	7±0.79 (6–9)
Oral aperture to guide ring	76±1.95 (74–81)	79±2.07 (75–80)	78±2.22 (76–81)	76±4.11 (65–81)	49±1.79 (45–52)
Pharyngeal bulb length	86±5.51 (79–96)	87±7.05 (80–99)	87±6.43 (82–94)	67±5.52 (58–78)	62±3.25 (57–68)
Pharyngeal bulb diam.	21±1.62 (17–23)	24±2.77 (21–28)	19±1.53 (18–21)	17±2.39 (13–23)	17±1.29 (15–19)
Tail length	25±1.78 (21–28)	28±2.68 (26–32)	30±2.22 (27–32)	35±3.12 (28–41)	37±2.26 (32–40)
Length of hyaline tip	12±2.00 (8–15)	10±1.67 (8–12)	8±2.22 (5–10)	14±1.63 (11–17)	9±1.27 (7–12)
Number of supplements	–	–	8±0.00 (8–8)	–	–
Body diam. at lip region	12±0.87 (11–14)	13±0.45 (13–14)	13±0.50 (12–13)	9±0.55 (8–10)	8±0.50 (8–9)
at guiding ring	38±2.42 (34–41)	35±0.84 (34–36)	31±0.82 (30–32)	28±1.52 (25–31)	23±2.19 (21–26)
at base of pharynx	50±2.66 (48–55)	49±1.92 (47–52)	39±3.51 (36–43)	38±3.56 (33–45)	35±2.19 (32–40)
at mid body/at vulva	56±3.79 (50–62)	55±0.84 (54–56)	45±4.11 (40–50)	44±4.98 (36–53)	41±3.08 (36–45)
at anus	33±2.58 (29–38)	33±2.45 (30–36)	29±2.16 (27–32)	22±2.04 (18–26)	20±1.93 (17–24)
at beginning of hyaline tip	21±3.63 (16–28)	16±1.82 (14–19)	14±2.06 (12–16)	12±1.53 (8–14)	8±0.75 (7–10)

Results

Morphometric descriptions of females and males of *X. brevicollum* from the new localities Břeclav and Ratiškovice from the Czech Republic along with those of females of *X. inaequale* and *X. lambertii* from India are given in Table 3. The morphological references for each population used in this study are reported in Table 2. So far *X. brevicollum* was recorded from six sites in the Czech Republic, but for molecular study specimens were available only from three sites. The species-specific primer set, BL18 and BV3 (amplifying partial 18S+ITS1 region) yielded the expected PCR product of approximately 462 bp for all three populations of *X. brevicollum* (Fig. 1). By using the same primer set, amplified products were also observed for *X. lambertii*, *X. inaequale* and *X. italiae* (Fig. 1). Bands of equal high intensity were observed for *X. brevicollum* and *X. lambertii* whereas *X. inaequale* and *X. italiae* showed faint bands (Fig. 1). The length of the amplicons of *X. lambertii* and *X. inaequale* were almost similar in size with those of *X. brevicollum*, whereas the amplicon of *X. italiae* was longer. No amplification was observed for the eleven *Longidorus* species tested.

To verify that the amplified products of 18S+ITS1 region by the primer set BL18+BV3 were of *X. brevicollum*, *X. inaquale*, *X. italiae* and *X. lambertii* the products were sequenced in both directions and deposited at the NCBI database (Table 2). Only good quality partial sequences of 18S+ITS1 region amplicon of *X. brevicollum* (166 bp) and *X. lambertii* (364 bp) were deposited whereas the full length amplicon sequences of *X. inaequale* (445 bp) and *X. italiae* (558 bp) were deposited. The accession numbers of additional three molecular markers *cox1*, D2/D3 expansion segments of 28S and 18S gene of *X. brevicollum*, *X. inaequale* and *X. lambertii* are also given in Table 2. The nucleotide sequences of four loci (*cox1*, D2/D3, 18S and18S+ITS1 region) were sequenced from the same DNA extracted from a single individual nematode. The length of *cox1* ranged from 414 to 415 bp, D2/D3 from 771 to 773 bp and 18S from 1660 to 1716 bp for *X. brevicollum*, *X. inaequale* and *X. lambertii*. Difference in 18S nucleotides is mostly because of the unequal ends. No intra-specific nucleotide difference was found between the three populations of *X. brevicollum*.

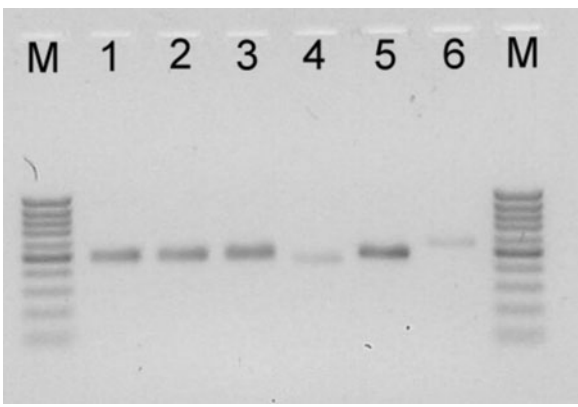

Fig. 1 Electrophoresis of the amplified products from single specimens with BL18 and BV3 primers. Lane M: 100 bp DNA ladder (Fermentas); lane 1, 2, 3: *Xiphinema brevicollum* from the three localities; lane 4: *X. inaequale*; lane 5: *X. lambertii*; lane 6: *X. italiae*

Pairwise distances calculated by MEGA 3.1 (Kumar et al. 2004) among *X. brevicollum*, *X. inaequale*, *X. lambertii* and *X. italiae* (accession numbers of *X. italiae* from the Genbank FJ713151; FJ711353; FJ713154) for *cox1*, D2/D3 and 18S are given in Table 4. Pairwise distance was calculated from the alignments produced by clustalW (as implemented in MEGA) after trimming flanking regions. Alignments of *cox1* and 18S were without any gaps but the alignment of D2/D3 produced many gaps because of the sequence of *X. italiae* which is different from the other three species. Except for trimming the flanking regions, no further refinement of the alignment was done to calculate pairwise distance. The pairwise distances among the three species *X. brevicollum*, *X. inaequale* and *X. lambertii* belonging to *X. americanum*-group varied between 17.5–20.3% for *cox1*, 0.8–2.6% for D2/D3 and 0.1% for 18S (Table 4).

Discussion

Morphometrically two populations of *X. brevicollum* (Table 3) from new localities were similar to the previous populations described from the Czech Republic (Erbenová 1975; Kumari et al. 2005). Morphometrics were also compared with the original description of *X. brevicollum* (Loredello and Da Costa 1961) and re-description of topotypes (Luc et al. 1998). Further the data were compared with *X. taylori*

Table 4 Pairwise distance matrix of *cox1*, D2/D3 expansion segments of 28S and 18S nucleotide sequences

cox1-mtDNA	*X. brevicollum*	*X. lambertii*	*X. inaequale*	*X. italiae*
X. brevicollum				
X. lambertii	0.175			
X. inaequale	0.203	0.188		
X. italiae	0.225	0.235	0.260	
D2/D3-28S rDNA	*X. brevicollum*	*X. lambertii*	*X. inaequale*	*X. italiae*
X. brevicollum				
X. lambertii	0.008			
X. inaequale	0.026	0.026		
X. italiae	0.205	0.205	0.205	
18S-rDNA	*X. brevicollum*	*X. lambertii*	*X. inaequale*	*X. italiae*
X. brevicollum				
X. lambertii	0.001			
X. inaequale	0.001	0.001		
X. italiae	0.041	0.041	0.041	

(Lamberti et al. 1991). Minor overlapping differences were observed. These differences can be explained by the occurrence of intraspecific variability. Morphometrics of *X. lambertii* (Table 3) closely agree with those reported in the original description of the species (Bajaj and Jairajpuri 1976). Morphometrics of *X. inaequale* were compared with the original description of the species (Khan and Ahmad 1975) and some differences were found such as shorter odontostyle length (83–98 vs 98–105 μm); shorter odontophore 43–52 vs 56–61 μm. Photomicrographs of *X. lambertii* and *X. inaequale* are available on request from the corresponding author.

Molecular features are considered to be particularly useful where morphological characters lead to an ambiguous interpretation or when dealing with cryptic species which are clearly different based on their genes, but are difficult to distinguish based on morphological characteristics (Tandingan De Ley et al. 2007; Vovlas et al. 2008). Since the identification of *X. brevicollum* is controversial and morphological analysis (e.g. shape of lip region, tail shape) and morphometric data alone do not appear reliable, molecular means were used here to assess the taxonomic status of *X. brevicollum* occurring in the Czech Republic. Species-specific primers designed by Oliveira et al. (2005) for *X. brevicollum* amplified the expected fragment for all three populations from the Czech Republic, but the primer set (BL18+BV3) also amplified the corresponding DNA region in *X. inaequale*, *X. italiae* and *X. lambertii*. These finding suggested that the primers designed for *X. brevicollum* are not species-specific. Because all these four species (*X. brevicollum, X. inaequale, X. italiae* and *X. lambertii*) were positive by the primer set BL18+BV3, therefore three additional markers (*cox1*, D2/D3 and 18S) of these species were sequenced to supplement their morphological identification. Moreover, sequences of these markers will be useful in DNA-barcode studies of genus *Xiphinema*. These three loci from the same isolate of *X. italiae* were already sequenced by Kumari and Lišková (2009).

Table 5 Basic Local Alignment Search Tool (BLAST) results for *Xiphinema brevicollum* nucleotides

Region	BLAST to accession numbers	Identities
ITS1	AY359858 *X. diffusum*	68/69
	FM211426 *X. brevicollum*	67/69
	FM211425 *X. brevicollum*	67/69
	FM211424 *X. brevicollum*	67/69
cox1	AM086703 *X. taylori*	404/405
	AM086702 *X. taylori*	404/405
	AM086701 *X. diffusum*	352/407
	AM086700 *X. diffusum*	349/403
D2/D3	AY601602 *X. taylori*	771/773
	AY601603 *X. taylori*	768/775
	AY601604 *X. brevicollum*	765/773
	FM211649 *X. brevicollum*	764/773
18S	AM086676 *X. taylori*	1660/1660
	AM086675 *X. taylori*	1660/1660
	AM086670 *X. incognitum*	1660/1660
	AM086669 *X. diffusum*	1660/1660

Mitochondrial *cox1* region is widely used to differentiate between closely related species (Derycke et al. 2006, 2007). The pairwise distance of *cox1* between the three species belonging to *X. americanum*-group was 17.5% between *X. brevicollum* and *X. lambertii*; 20.3% between *X. brevicollum* and *X. inaequale* and 18.8% between *X. inaequale* and *X. lambertii*. Mitochondrial DNA sequence divergence between closely related species is typically in the range of 10 to 20% (Blouin et al. 1998). But the differentiation of species solely on genetic distance is also not reliable and the integration of molecular and morphological analyses is required to identify nematodes, therefore morphological references to all species and populations used in this study are given in Table 2.

The sequences obtained were compared by BLAST (Basic Local Alignment Search Tool) in NCBI. Sequences of *X. lambertii* and *X. inaequale* are not available in GenBank but BLAST result showed that our sequences are different compared to the sequences available in GenBank. BLAST results of first four top hits for *X. brevicollum* are shown in Table 5. The first top hit of ITS1 was *X. diffusum* and further three hits to *X. brevicollum*. The top hits of *cox1* were to *X. taylori* and *X. diffusum*. The top hits of D2/D3 were to *X. taylori* and *X. brevicollum*. The 18S top four hits were to *X. taylori*, *X. incognitum* and *X. diffusum* with 100% identity. On the basis of BLAST results of ITS1, D2/D3 and 18S, it was not possible to make any conclusion on the species status of *X. brevicollum* occurring in the Czech Republic, but comparison of *cox1* sequence of *X. brevicollum* from the Czech Republic with published sequences (accession no. AM086702 and AM086703) (Lazarova et al. 2006) of *X. taylori* from the Slovak Republic revealed that the observed diversity between these two species is indeed low (difference only one substitution). Almost identical sequence of *cox1* possibly suggests that *X. brevicollum* and *X. taylori* occurring in the Czech and Slovak Republic represent a single species. The sequence of *X. brevicollum* from the Czech Republic of *cox1* region is different than the published sequence of *X. brevicollum* from Brazil accession number AM086707 (Lazarova et al. 2006). The pairwise distance between *X. brevicollum* from the Czech Republic and Brazil is 20%. But the differentiation of species based solely on genetic distance of a single molecular marker is also not reliable. No major morphological difference was observed between the four populations of *X. brevicollum* from the Czech Republic and published data from literature on *X. brevicollum* and *X. taylori*. Moreover, specimens of *X. taylori* from Slovakia and specimens of *X. brevicollum* from the type locality were not included in this study for molecular anaylsis; therefore no definitive statement was made on the species status of *X. brevicollum* occurring in the Czech Republic. Further research, in which more molecular markers and different geographical populations of *X. brevicollum* and *X. taylori* are included, is needed to make a definitive statement on the species status of *X. brevicollum* occurring in the Czech Republic.

Acknowledgements The authors are thankful to M. Vítámvásová for preparing permanent slides and technical assistance. The work was supported by the Ministry of Agriculture of the Czech Republic, Project number MZe-0002700604.

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