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Cytochrome c oxidase subunit 1 analysis of *Xiphinema diversicaudatum, X. pachtaicum, X. simile* and *X. vuittenezi* (Nematoda, Dorylaimida)

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Abstract Genetic analyses using sequences of partial cytochrome c oxidase subunit 1 (*cox1*) gene of mitochondrial DNA were conducted to determine the extent of genetic variation within and among *Xiphinema diversicaudatum, X. pachtaicum*, *X. simile* and *X. vuittenezi* populations. Pairwise distance among the four species was 22.5 to 31.2%. Four different sequence variants of *cox1* were determined among six populations of *X. diversicaudatum* and three variants among three populations of *X. simile*. Nucleotide variation was detected at 18 of 414 bp (1.9 to 2.7%) in *X. diversicaudatum* and 4 of 435 bp (0.2 to 0.9%) in *X. simile*. All changes were at silent sites. No nucleotide variation was detected within three populations of *X. pachtaicum* and within three populations of *X. vuittenezi*.

Keywords Czech Republic · Mitochondrial DNA · Dagger nematode

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Introduction

Plant-parasitic nematodes are important agricultural pests and are widely being studied at the molecular level in order to develop molecular diagnostic markers. In the last decade, molecular approaches have been largely used to characterize and explore the population genetic structures in nematodes. Genetic variation is widespread in nematode populations (Derycke et al. 2005, 2007, 2008; Otranto et al. 2005), and the accurate analysis of genetic variation in nematodes has important implications for studying population biology, epidemiology, and genetic structure of nematodes. Mitochondrial DNA has been used to study population genetic in some plant parasitic nematodes of economical importance and diverse patterns of population genetic structure have been observed in some taxa such as *Meloidogyne* (Hugall et al. 1997).

However, except for one study (Lazarova et al. 2006), there is no information on the genetic variation among different populations of taxa of the virus vector families utilizing mitochondrial DNA, e.g. for any *Xiphinema* species. Thus, more comparative studies on genetic structure of different populations of *Xiphinema* species are needed. To study population variability of four *Xiphinema* species, we focused our attention on mitochondrial DNA, because mitochondrial genome is a rich source of markers for population genetic and systematic studies (Hu and Gasser 2006).

The objective of this study was to analyze the genetic variation of different populations of *X. diversicaudatum*, *X. pachtaicum*, *X. simile* and *X. vuittenezi* occurring in the Czech Republic by using cytochrome *c* oxidase subunit 1 (*cox1*) gene of mitochondrial DNA. Till now six species (*X. brevicollum*, *X. dentatum, X. diversicaudatum, X. pachtaicum, X. simile* and *X. vuittenezi*) of the genus *Xiphinema* have been recorded in the Czech Republic (Kumari et al. 2005; Kumari 2006, 2009). *X. dentatum* was not included in the present study because it was found only at one site and *X. brevicollum* is a part of another study. For comparison two populations of *X. diversicaudatum* from the neighbouring country Slovakia and one population from Austria were also included.

Material and methods

Total DNA lysis

Specimens of the four *Xiphinema* species were stored in 1 M NaCl. Total genomic DNA was prepared by a rapid technique (Stanton et al. 1998). Single specimens were added into 0.5 ml Eppendorf microtubes containing 20 µl of 0.25 M NaOH under a binocular microscope, incubated overnight at room temperature and thereafter heated to 99°C for 3 min. Afterwards 10 µl of 0.25 M HCl, and 5 µl each of 0.5 M Tris-HCl (pH 8) and 2% Triton X-100 were added and the mixture was incubated for another 3 min at 99°C. Finally, the DNA suspension was cooled and the DNA was either used directly for PCR or stored at −20°C until template was needed for PCR reactions.

Table 1 *Xiphinema* populations used for sequencing and their accession numbers

Species	Locality	Host	No. of specimen sequenced	Accession number
X. diversicaudatum	Bílé Podolí (CZ)	sour cherry	8 (4♀+4♂)	GU222421
	Hrušky (CZ)	grapevine	8 (4♀+4♂)	GU222422
	Lhenice (CZ)	sweet cherry	8 (4♀+4♂)	[a]
	Marchegg (Austria)	–	8 (4♀+4♂)	GU222423
	Kľúčovec (Slovakia)	–	3 ♀	[b]
X. pachtaicum	Kurdějov (CZ)	grapevine	2♀	GU222424
	Mohyla-míru (CZ)	apple	2♀	[c]
	Sokolnice (CZ)	grapevine	2♀	[c]
X. simile	Čejkovice (CZ)	sour cherry	8♀	GU222425
	Dětkovice (CZ)	sweet cherry	8♀	GU222426
	Velké Pavlovice (CZ)	apricot	8♀	GU222427
X. vuittenezi	Brno (CZ)	pear	2 ♀	[d]
	Pavlov (CZ)	grapevine	2 ♀	[d]
	Slaný (CZ)	apple	2 ♀	[d]

CZ Czech Republic

[a] Identical to Hrušky

[b] Identical to Marchegg

[c] Identical to Kurdějov

[d] Identical to accession number EF614265

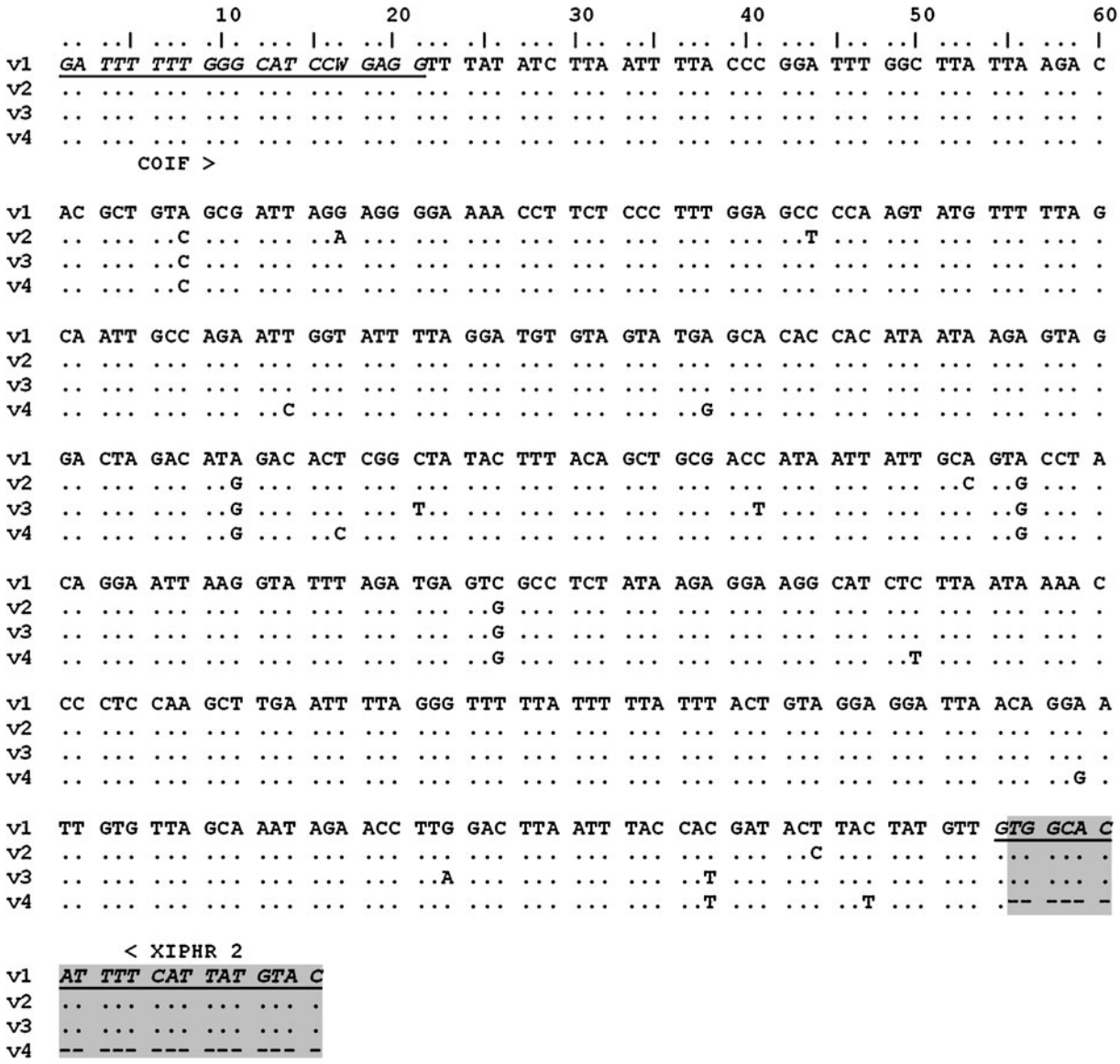

Fig. 1 Alignment of the four variants of *X. diversicaudatum*. **v1** population from Bílé Podolí ; **v2** Hrušky and Lhenice; **v3** Marchegg (Austria) and Kľúčovec (Slovakia); **v4** accession number EF538749 (Somotor, Slovakia). Grey high-lighted part was not taken into account during analysis. Italic and underlined are the sequences of the oligonucleotide primers used in the PCR

Table 2 Pairwise comparison of sequence differences among the four sequence variants of *Xiphinema diversicaudatum* and three variants of *X. simile*

X. diversicaudatum	1	2	3	*X. simile*	1	2
1 Bílé Podolí				1 Čejkovice		
2 Hrušky/Lhenice	1.9			2 Dětkovice	0.2	
3 Austria/Slovakia	1.9	1.9		3 Velké Pavlovice	0.9	0.7
4 EF538749	2.7	2.7	2.2			

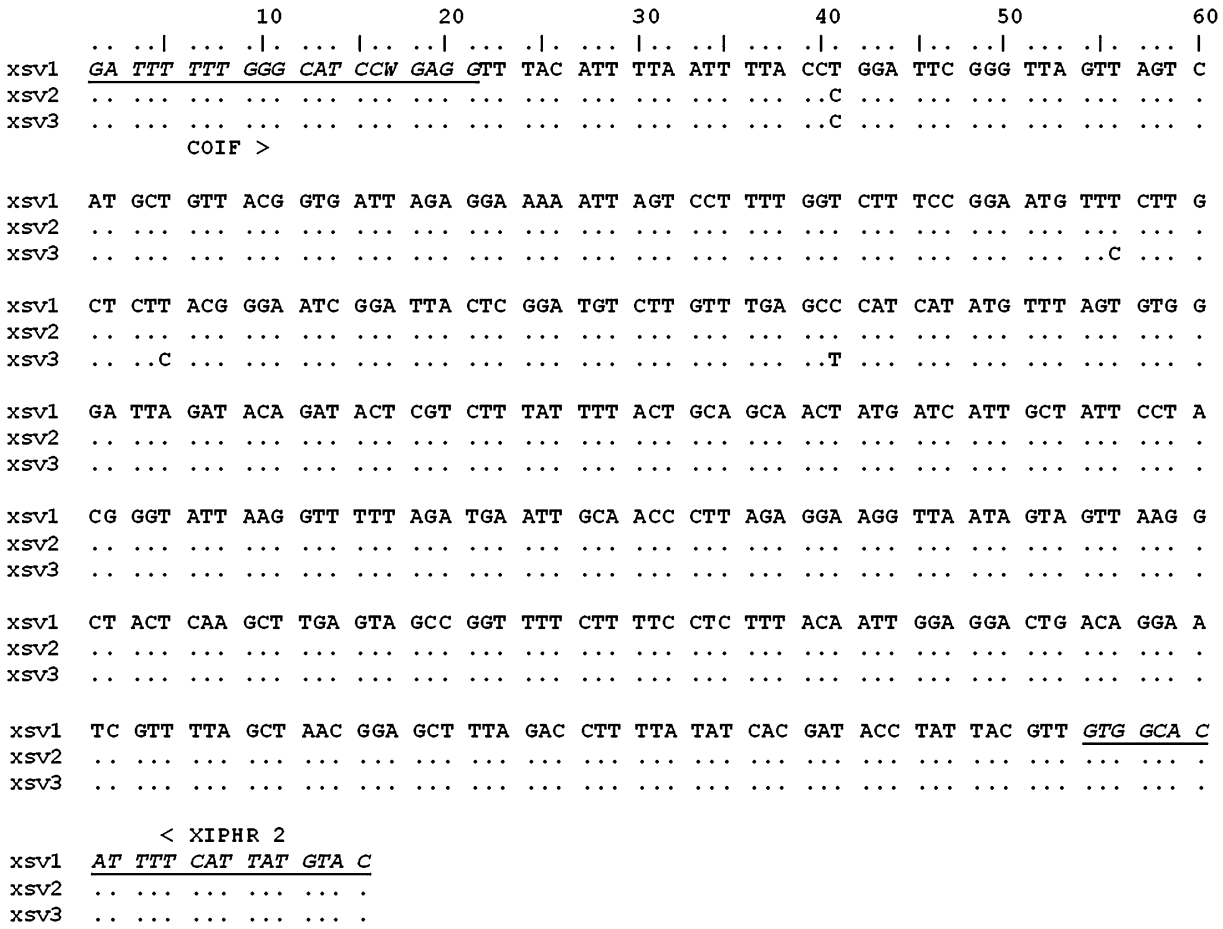

Fig. 2 Alignment of the three variants of *X. simile*. **xsv1** population from Čejkovice; **xsv2** Dětkovice; **xsv3** Velké Pavlovice. Italic and underlined are the sequences of the oligonucleotide primers used in the PCR

Amplification and sequencing

The *cox1* gene was amplified using forward primer COIF (5′-GAT TTT TTG GKC ATC CWG ARG-3′) and reverse primer COIR (5′-CWA CAT AAT AAG TAT CAT G-3′) (He et al. 2005) or XIPHR2 (5′-GTA CAT AAT GAA AAT GTG CCA C-3′) (Lazarova et al. 2006). Five populations of *X. diversicaudatum* and three populations of *X. simile* were amplified by using primers COIF and XIPHR2, while three populations of *X. pachtaicum* and *X. vuittenezi* were amplified using COIF and COIR primers. One population of *X. diversicaudatum* was used from Genbank (accession number EF538749) from our previous study (Kumari et al. 2009). The PCR was performed in a 25 µl total volume containing 1 PCR bead (GE Healthcare, Buckinghamshire, UK), 20.5 µl double distilled sterile water, 2.0 µl each primer (10 pmol/µl) (synthesized by Generi Biotech, Hradec Králové, Czech Republic), and 0.5 µl of DNA lysis. 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). The cycling profile was as described by He et al. (2005): 95°C for 10 min, 5 cycles at 94°C for 30 s, 45°c for 40 s, and 72°C for 1 min, and further 35 cycles at 94°C for 30 s, 37°C for 30 s, and 72°C for 1 min, followed by an extension at 72°C for 10 min. Aliquots of PCR were analysed by electrophoresis and the remaining products were purified using High Pure Product Purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and sequenced in both directions using each primer pair one forward and one reverse (Macrogen, Korea). Initially two specimens per population were sequenced

for *X. diversicaudatum* (five populations), *X. pachtaicum* (three populations), *X. simile* (three populations) and *X. vuittenezi* (three populations). For populations of *X. diversicaudatum* and *X. simile* which showed variation in nucleotides, the number of nematodes for sequencing was increased to eight per population, except for one population from Kľúčovec where the number of specimens sequenced was only three (see Table 1). Total numbers of specimens sequenced were 35 (*X. diversicaudatum*), 6 (*X. pachtaicum*), 24 (*X. simile*) and 6 (*X. vuittenezi*). Sequencher™ 4.8 (Genes Codes, Corp., Ann Arbor, MI, USA) was used to assemble and view each of the sequences and check for base-calling errors. Pairwise distance was calculated by Mega 3.1 (Kumar et al. 2004) after trimming unequal ends. The sequences were deposited at National Centre for Biotechnology Information (NCBI) database and their accession numbers are listed in Table 1.

Results

The length of partial *cox1* gene sequenced in this study was 435 bp, 416 bp, 435 bp and 416 bp for *X. diversicaudatum*, *X. pachtaicum*, *X. simile* and *X. vuittenezi* respectively. Difference in length is due to the different primers used (see "Material and methods"). Identical sequences were obtained for all individuals studied from the same population. Inter-population genetic variation among *X. pachtaicum* and *X. vuittenezi* were not found between three populations examined for each species. Minor inter-population variation found among three populations of *X. simile*, whereas relatively high inter-population variability was found among six populations of *X. diversicaudatum*, including one population from the GenBank (accession number EF538749).

Four different sequence variants of *cox1* were determined from six population of *X. diversicaudatum*. The alignment of these variants revealed nucleotide variation at 18 sites (15 transitions and 3 transversions) (Fig. 1). The 18 variable nucleotide sites among all four *cox1* sequence variants represented transitions T↔C (n=9) and A↔G (n=6) and transversions C↔A (n=2) and G↔C (n=1) (Fig. 1). The majority (n=17; 94.4%) of the nucleotide variability was at the third codon position, whereas in only one instance was variability (5.6%) at the first codon position. Pairwise comparisons among the four *cox1* sequence variants (excluding the unequal flanking sequence) revealed sequence variation ranging from 1.9 to 2.7% (Table 2). The A+T content was 54.3% at the first, 58.3% at second codon positions,

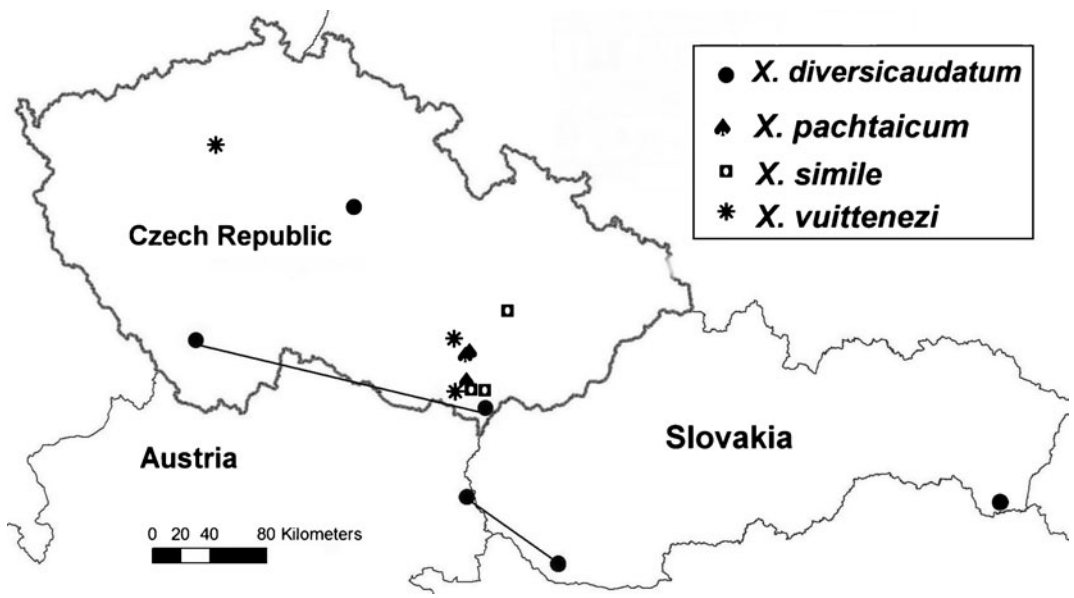

Fig. 3 Geographical location of populations of *X. diversicaudatum*, *X. pachtaicum*, *X. simile* and *X. vuittenezi*. Lines indicate the two populations of *X. diversicaudatum* which represent same variants

and the third codon position had a considerably greater A+T bias (73.0%) compared with the other two positions.

Three different sequence variants of *cox1* were determined from three populations of *X. simile*. The alignment of these variants revealed nucleotide variation at 4 positions. All four variations were transitions (C↔T) (Fig. 2) and at the third codon position. Pairwise comparisons among the three *cox1* sequence variants revealed sequence variation ranging from 0.2 to 0.9% (Table 2). The A+T content was 49.0% at the first, 58.7% at the second codon positions, and the third codon position had a considerably greater A+T bias (76.4%) compared with the other two positions.

In order to ensure open reading frame and exclude any pseudogenes, all *cox1* nucleotide sequences determined were conceptually translated into amino acid sequences. All nucleotide changes were silent in *X. diversicaudatum* as well as in *X. simile*.

Discussion

Except for one study (Lazarova et al. 2006), nothing is known about the *cox1* gene variability in *Xiphninema* species. Therefore, to characterize the population genetic structure of *X. diversicaudatum, X, simile, X, pachtaicum* and *X. vuittenezi*, the nucleotide variability of *cox1* was investigated.

The *cox1* sequence data revealed a comparatively high level of inter-population sequence variation in *X. diversicaudatum* (average 2.2%) and minor inter-population variation in *X. simile* (average 0.6%). Average nucleotide diversity within *X. diversicaudatum* is some 4-fold higher than in *X. simile*. These intra-specific sequence variations lie within the average sequence variation observed among nematodes of the same species (Blouin et al. 1998). Identical sequences were obtained between three populations of *X. vuittenezi* and *X. pachtaicum*. Identical sequences were also observed among three populations of the parthenogenetic *X. brevicollum* (Kumari et al unpublished). Three geographical variants of *X. simile* were separated by much smaller distance compared to *X. diversicaudatum* variants (Fig. 3).

Patterns of mtDNA variation have been informative at lower taxonomic levels of nematodes, revealing low intraspecific diversity and large interspecific divergences (Blouin et al. 1998). In our study, pairwise distance among *X. diversicaudatum, X. pachtaicum, X. simile* and *X. vuittenezi* (22.5–31.2%) was also greater than intra-species sequence variation (0.2–0.9 and 1.9–2.7%).

To our knowledge, only Lazarova et al. (2006) has utilized *cox1* gene sequence of mitochondrial DNA to study *Xiphinema* species. Clearly this is an important field of study that deserves more attention. In this study, patterns of *cox1* variation have been informative for *Xiphinema* species, revealing higher diversity within *X. diversicaudatum* and low (*X. simile*) or no divergences (*X. pachtaicum* and *X. vuittenezi*) among parthenogenetic species. Populations were studied from a small geographical area (Fig. 3), therefore future studies including populations from different geographical areas may show more divergence among these species.

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