Variability of D2/D3 Segment Sequences of Several Populations and Pathotypes of Potato Cyst Nematodes (*Globodera rostochiensis*, *Globodera pallida*)

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Abstract

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Potato cyst nematodes (*Globodera rostochiensis*, *Globodera pallida*) remain a key pest in the main potato growing regions of the Czech Republic. Due to difficult direct management and presence of diverse pathotypes attacking different potato cultivars the rapid and reliable diagnostics is of crucial importance. Currently, efforts are aimed at a description of different pathotypes based on DNA analysis. The main objective of this study was to evaluate the homogeneity of sequences of D2/D3 segments of the 28S rDNA gene obtained from 3 populations of *G. rostochiensis* and 5 populations of *G. pallida* and estimate their value for diagnostic purposes. PCR amplification yielded a single fragment of the length of 700 bp approximately in all populations. The alignment score of the vast majority of all pair comparisons of *G. rostochiensis* and *G. pallida* populations varied from 98 to 99. In total 14 point deletions and 3 substitutions were observed. The variability of D2/D3 segments of potato cyst nematodes is rather low and this DNA region can be used for diagnostics on a species level because more differences were found after comparing with *G. tabacum* and *G. millefolii* sequences obtained from Gene Bank; however the applicability of D2/D3 sequences to routine diagnostics of potato cyst nematodes could be complicated by its similarity to corresponding sequences of the nematode *G. artemisiae*.

Keywords: *Globodera rostochiensis*; *G. pallida*; pathotype; D2/D3 segment

The presence of quarantine pests is often a limiting factor of commercial crop production. Considering potato production, two species of the potato cyst nematode (*G. rostochiensis*, *G. pallida*) pose a significant challenge in plant protection. Despite of the availability of resistant and tolerant cultivars, potato cyst nematode remains a major pest requiring the adoption of severe quarantine measures. In the Czech Republic it is especially essential to keep the potato seed areas localised in a mountainous region of the Bohemian-Moravian border free of this pest. Like in the case of other plant parasitic nematodes direct management is considerably difficult. Furthermore, with the potato cyst nematode the situation is more complicated by the presence of different pathotypes.

Pathotypes are defined as a nematode population characterised by their ability to multiply on

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certain tubers of *Solanum* spp. clones and hybrids (Quader 2009). Some pathotypes show the almost total inability to multiply on specific cultivars of potato (single-gene resistance); for example, the most commonly grown resistant potato cultivars (based on H1 gene derived from clones of *S. tuberosum* subsp. *andigena*) are resistant to pathotype Ro1 of *G. rostochiensis* only (EPPO 2004). Five pathotypes are recognised in *G. rostochiensis* (Ro1-Ro5 international notation) and three in *G. pallida* (Pa1-Pa3) in Europe (Kort *et al.* 1977). For effective use of resistant cultivars in field conditions the precise diagnostics of pathotypes remains a key factor. Considering plant parasitic nematodes ITS sequences were used e.g. for the discrimination of *Ditylenchus dipsaci* populations (Marek *et al.* 2005; Kerkoud *et al.* 2007).

D2/D3 region sequences were already used in research on the evolution relationship of different taxonomic units. Courtright *et al*. (2000) successfully exploited these regions of ribosomal DNA for the interspecific analysis of several populations of the nematode *Scottnema lindsaya* occurring in separate Antarctic valleys. De Ley *et al*. (1999) used the D2/D3 sequences for distinguishing the nematodes *Acrobeloides bodenheimeri* and *Acrobeloides camberenensis*; these species differ in mirror-image anatomy only, but 45 differences in their D2/D3 regions were found. A comparison of the D2/D3 sequences of 12 isolates of 8 different *Meloidogyne* species was performed by Tenente *et al*. (2004). In this case it was revealed that in *Meloidogyne* species D2/D3 sequences are more suitable for distinguishing the separate species groups rather than for species specific diagnostics.

Analyses of the D2/D3 sequences were also used in research on interrelationships within higher taxa. An extensive study of nematodes belonging to the suborder Criconematina based on D2/D3 sequences was performed by Subbotin *et al*. (2005). Results principally confirmed the taxonomic system based on morphological properties and documented the existence of cryptic, morphologically undistinguishable however sexually isolated species. An analogous study was performed for the whole order Tylenchida (Subbotin *et al.* 2006). The correctness of the present taxonomic system was also confirmed in this case although some differences in lower taxonomical units were found. According to Subbotin *et al*. (2006) the molecular analysis of ribosomal DNA can provide a base for additional morphology studies.

D2/D3 sequences also became a common part of descriptions of new plant parasitic nematode species. E.g. Handoo *et al*. (2005a) used these regions for the *Longidorus americanum* description and D3 solely for the characterization of the new species Meloidogyne thailandica (Handoo *et al.* 2005b); sequences of both regions were published by Castillo *et al*. (2003) when describing the new gall-forming nematode species *Meloidogyne baetica*.

The primary goal of this study was to evaluate the homogeneity of sequences of D2/D3 segments of the 28S rRNA gene obtained from 3 populations of *G. rostochiensis* and 5 populations of *G. pallida* and estimate its value for diagnostic purposes.

MATERIALS AND METHODS

Nematode populations, DNA extraction

Potato cyst nematode populations used in this study are summarised in Table 1. Potato nematode cysts were extracted from soil with Fenwick's can, in some cases cysts already extracted were obtained.

Table 1. Populations of the nematodes used in this study, accession numbers refer to D2/D3 region sequences obtained in this study

No.	Species	Pathotype	Origin	Source	Designation	Accession number
1.		Ro1	Šluknov (Czech Republic)	V. Gaar	Ro1	GU338015
2.	*G. rostochiensis*	Ro3	Obersteinbach (Germany)	M. Zouhar	Ro3	GU338016
3.		Ro5	Harmerz Germany (SPA)	V. Gaar	Ro5	GU338017
4.		Pa1	UK	J. Pickup	Pa1	GU338018
5.		Pa2	Aveest (Netherlands)	V. Gaar	Pa2A	GU338019
6.	*G. pallida*	Pa2	Kalle (France)	V. Gaar	Pa2K	GU338020
7.		Pa3	Delmsen (Germany)	V. Gaar	Pa3	GU338021
8.		unknown	Kašperské Hory (Czech Republic)	V. Gaar	PaKa	GU338022

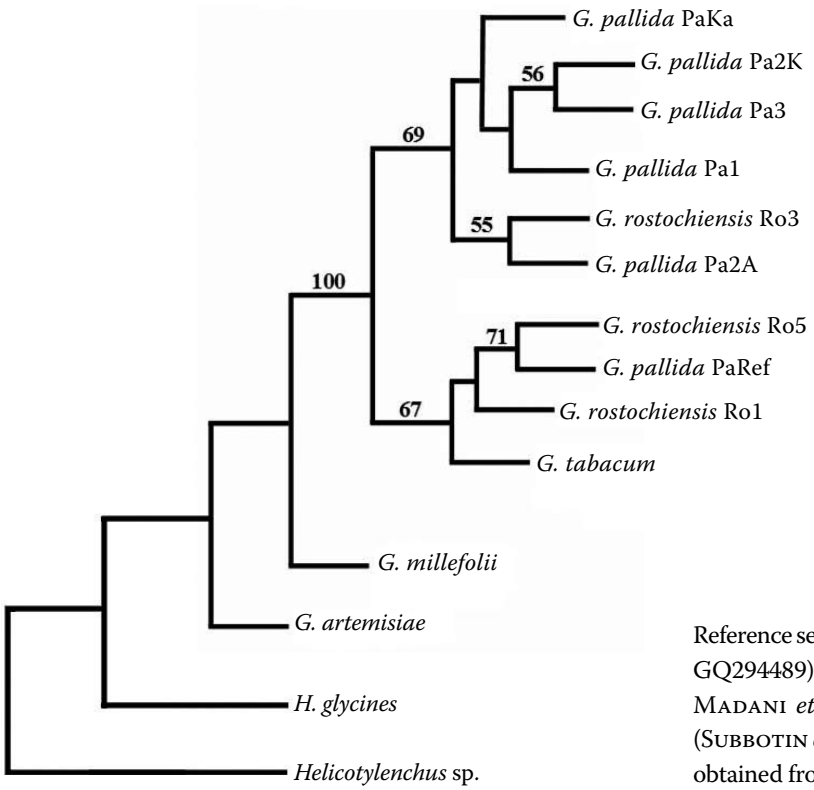
DNA was extracted from single cysts with the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manual; cysts were crushed in a ball mill prior to extraction, the foam which was formed in microtubes was sent down with the help of liquid nitrogen. The volume of elution solution was decreased to 50 µl and samples were incubated for 15 min at room temperature to increase the elution efficiency.

PCR amplification and purification of PCR product, sequencing and sequence alignment

Universal primers (Ellis *et al.* 1986; Courtright *et al.* 2000) D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') were used for D2/D3 segment amplification.

PCR was performed in 0.2 ml microtubes with the final volume of 25 µl of reaction mixture containing 10× PCR buffer for DNA polymerase (Fermentas), **TOWN, STATE**); 3.0mM of $MgCl_2$ (Fermentas); 300mM of dNTP (Fermentas); 0.4µM of primers (mix); 2.5 U of Taq polymerase (Fermentas), 1.00 µl of DNA and 17.7 µl of ddH_2O. Conditions for the amplification of the D2/D3 expansion region were as follows: denaturation 2 min, then 35 cycles consisting of 94°C 1 min, 53°C 30 s, 72°C 1 min and additional 4 min at 72°C. In the negative control DNA template was replaced by water. PCR products were analysed by electrophoresis in 1% gel in 1× TBE, stained with ethidium bromide and visualized under UV light.

Positive variants were amplified once again using LA polymerase (Fermentas) to minimize possible errors during amplification. PCR products were purified by a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Purified DNA fragments were sequenced directly in both directions (Genomac International, Prague, Czech Republic). Sequence alignment and calculation of alignment score were performed in Clustal W software available on-line at default settings. The D2/D3 sequence of *G. pallida* designated as PaRef (NCBI number GQ294489; Madani *et al.* unpublished) obtained from NCBI database was added for alignment. D2/D3 sequences of another three *Globodera* species obtained in the same manner were also used for comparison. The following species were concerned:



Reference sequences of *G. pallida* (PaRef; NCBI number GQ294489), *G. tabacum* (NCBI number GQ294492; Madani *et al.* 2009, unpublished) and *G. millefolii* (Subbotin *et al.* 2006; NCBI number DQ328700) were obtained from NCBI database

Figure 1. Comparison of the consensus sequences of five *G. pallida* and three *G. rostochiensis* populations. Point substitutions and deletions are marked in light grey. Differences caused by inconsistent results of sequencing (3 PCR products were sequenced from each population) are marked in black

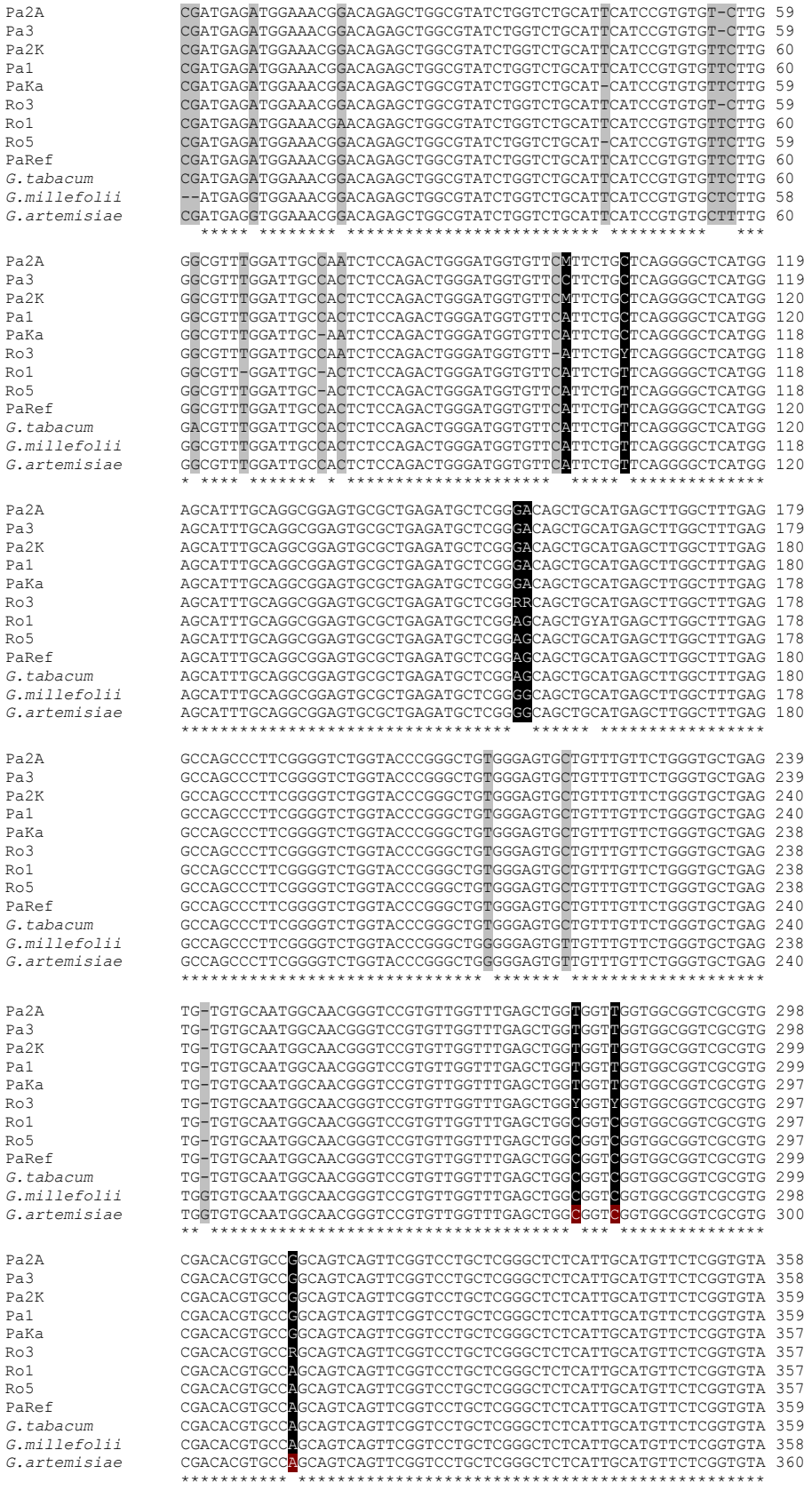


Figure 2. Consensus tree from the maximum likelihood analysis generated by Hidden Markov Model from the alignment of 12 sequences of the D2/D3 expansion region of the 28S-rRNA gene from nematodes of the genus *Globodera*. Bootstrap values higher than 50% are given on appropriate clades

Figure 2 to be continued

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Pa2A            AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 418
Pa3             AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 418
Pa2K            AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 419
Pa1             AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 419
PaKa            AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 417
Ro3             AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 417
Ro1             AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 417
Ro5             AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 417
PaRef           AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 419
G.tabacum       AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 419
G.millefolii    AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 418
G.artemisiae    AAAGCCGGTCATCTGTCCGACCCGTCTTGAAACACGGACCAAGGAGTTTAGCGTGTGCGC 420
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Pa2A            GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 478
Pa3             GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 478
Pa2K            GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 479
Pa1             GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 479
PaKa            GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 477
Ro3             GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 477
Ro1             GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 477
Ro5             GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 477
PaRef           GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 479
G.tabacum       GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 479
G.millefolii    GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 478
G.artemisiae    GAGTCATTGGGAGTTGAAAACCCAAGGGCGTAATGAAAGTGAAGGTCTCCTTGCGGAGCT 480
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Pa2A            GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 538
Pa3             GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 538
Pa2K            GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 539
Pa1             GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCAATCGCATG 539
PaKa            GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 537
Ro3             GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 537
Ro1             GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 537
Ro5             GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 537
PaRef           GATGTGTGATCTCGTGCACCCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 539
G.tabacum       GATGTGTGATCTCGTGCACTCCGGTGTGCGGGCGCAACATAGTCCCGTCCCGATCGCATG 539
G.millefolii    GATGTGTGATCCCGTGCACCACGGTGTGCGGGCGCAACATAGTCCCGTTCCTATCGCATG 538
G.artemisiae    GATGTGTGATCCCGTGCACCACGGTGTGCGGGCGCAGCATAGTCCCGTTCCTATCGCNTG 540
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Pa2A            CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 598
Pa3             CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 598
Pa2K            CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 599
Pa1             CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 599
PaKa            CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 597
Ro3             CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 597
Ro1             CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 597
Ro5             CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 597
PaRef           CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 599
G.tabacum       CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 599
G.millefolii    CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 598
G.artemisiae    CGATGGGGCGGAGACAGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATTCCTGAG 600
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Pa2A            CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGA-GCGATTCTGACGTGCAAATCGA 657
Pa3             CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 658
Pa2K            CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 659
Pa1             CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 659
PaKa            CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 657
Ro3             CAGGATGAAGCCAGAGGAAACTCTGGTGGA-GTCCGA-GCGATTCTGACGTGCAAATCGA 655
Ro1             CAGGATGA-GCCAGAGGAAACCTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 656
Ro5             CAGGATGAAGCCAGAGGAAC-TCTGGTGGA-GTCCGA-GCGATTCTGACGTGCAAATCGA 654
PaRef           CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 659
G.tabacum       CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 659
G.millefolii    CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 658
G.artemisiae    CAGGATGAAGCCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGA 660
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Pa2A            TCG 660
Pa3             TCG 661
Pa2K            TCG 662
Pa1             TCG 662
PaKa            TCG 660
Ro3             TCG 658
Ro1             TCG 659
Ro5             TCG 657
PaRef           TCG 662
G.tabacum       TCG 662
G.millefolii    TCG 661
G.artemisiae    TCG 663
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Table 2. Alignment score of all pair comparisons of D2/D3 region sequences

Name	Len (nt)	Name	Len (nt)	Score	Name	Len (nt)	Name	Len (nt)	Score
Ro1	659	Ro3	658	98	Pa2K	662	Pa3	661	99
Ro1	659	Ro5	657	99	Pa2K	662	PaKa	660	99
Ro1	659	Pa1	662	98	Pa2K	662	PaRef	662	98
Ro1	659	Pa2A	660	98	Pa2K	662	*G. tabacum*	667	98
Ro1	659	Pa2K	662	98	Pa2K	662	*G. millefolii*	661	97
Ro1	659	Pa3	661	98	Pa3	661	PaKa	660	99
Ro1	659	PaKa	660	98	Pa3	661	PaRef	662	98
Ro1	659	PaRef	662	99	Pa3	661	*G. tabacum*	667	98
Ro1	659	*G. tabacum*	667	99	Pa3	661	*G. millefolii*	661	97
Ro1	659	*G. millefolii*	661	97	PaKa	660	PaRef	662	98
Ro3	658	Ro5	657	98	PaKa	660	*G. tabacum*	667	98
Ro3	658	Pa1	662	98	PaKa	660	*G. millefolii*	661	97
Ro3	658	Pa2A	660	98	PaRef	662	*G. tabacum*	667	99
Ro3	658	Pa2K	662	98	PaRef	662	*G. millefolii*	661	98
Ro3	658	Pa3	661	98	*H. glycines*	656	Ro1	659	89
Ro3	658	PaKa	660	98	*H. glycines*	656	Ro3	658	87
Ro3	658	PaRef	662	98	*H. glycines*	656	Ro5	657	88
Ro3	658	*G. tabacum*	667	98	*H. glycines*	656	Pa1	662	88
Ro3	658	*G. millefolii*	661	97	*H. glycines*	656	Pa2A	660	88
Ro5	657	Pa1	662	98	*H. glycines*	656	Pa2K	662	89
Ro5	657	Pa2A	660	98	*H. glycines*	656	Pa3	661	88
Ro5	657	Pa2K	662	98	*H. glycines*	656	PaKa	660	88
Ro5	657	Pa3	661	98	*H. glycines*	656	PaRef	662	89
Ro5	657	PaKa	660	98	*H. glycines*	656	*G. tabacum*	667	90
Ro5	657	PaRef	662	100	*H. glycines*	656	*G. millefolii*	661	89
Ro5	657	*G. tabacum*	667	99	*H. glycines*	656	*G. artemisiae*	663	89
Ro5	657	*G. millefolii*	661	98	*H. glycines*	656	*Helicotylenchus*	665	83
Pa1	662	Pa2A	660	99	*Helicotylenchus*	665	*G. artemisiae*	663	85
Pa1	662	Pa2K	662	99	*Helicotylenchus*	665	*G. millefolii*	661	85
Pa1	662	Pa3	661	99	*Helicotylenchus*	665	*G. tabacum*	667	84
Pa1	662	PaKa	660	99	*Helicotylenchus*	665	PaRef	662	85
Pa1	662	PaRef	662	98	*Helicotylenchus*	665	PaKa	660	84
Pa1	662	*G. tabacum*	667	98	*Helicotylenchus*	665	Pa3	661	84
Pa1	662	*G. millefolii*	661	97	*Helicotylenchus*	665	Pa2K	662	84
Pa2A	660	Pa2K	662	99	*Helicotylenchus*	665	Pa2A	660	84
Pa2A	660	Pa3	661	99	*Helicotylenchus*	665	Pa1	662	84
Pa2A	660	PaKa	660	99	*Helicotylenchus*	665	Ro5	657	84
Pa2A	660	PaRef	662	98	*Helicotylenchus*	665	Ro3	658	84
Pa2A	660	*G. tabacum*	667	98	*Helicotylenchus*	665	Ro1	659	84
Pa2A	660	*G. millefolii*	661	97					

G. artemisiae (NCBI number EU855121; Nowaczyk *et al.* unpublished), *G. tabacum* (NCBI number GQ294492; Madani *et al.* 2009 – unpublished) and *G. millefolii* (Subbotin *et al.* 2006; NCBI number DQ328700).

Secondary rRNA structures were determined by the CLC Main Workbench 4 software. A phylogram was constructed using the Phylip software package, version 3.69 (Felsenstein 2005) and the maximum likelihood algorithm assuming a molecular clock (10 000 rearrangements) implying Hidden Markov Model (Felsenstein & Churchill 1996) was used. The reliability of the resulting phylogram was estimated using 1000 replicates in bootstrap test.

RESULTS

PCR amplification yielded a single fragment of about 700 bp in length in all populations. After

sequence alignment it was ascertained that the length of amplified sequences varied from 684 bp in Ro5 population to 691 bp in Pa2 and Pa1 populations. The nucleotide composition for each PCR product was as follows: 18.73% A, 22.48% C, 34.86% G, and 23.93% T. Thus, the percentage of purine nucleotides in the fragment was slightly higher than the pyrimidine nucleotides.

Alignment scores ranged from 97 to 100 in all pair alignments. The highest level of variability was detected in the following pair comparisons: Ro1 vs. Ro3, Ro1 vs. Pa2A and Ro1 vs. PaKa. In contrast, no differences were observed when Ro5 was compared with the reference sequence of *G. pallida* obtained from Gene Bank (pathotype unspecified, NCBI number GQ294489; Mandani *et al*. unpublished). The alignment score of the vast majority of all other pair comparisons varied from 98 to 99. For the complete sequence alignment see Figure 1, for alignment scores see Table 2.

Sequences of both *Globodera* species were further compared using the BLAST algorithm. In both cases a high level of similarity was observed when compared with the sequences of *G. artemisiae* (Gene Bank number EU855121; Nowaczyk *et al*. unpublished), *G. tabacum* (NCBI number GQ294492; Madani *et al*. 2009, unpublished) and *G. millefolii* (NCBI number DQ328700; Subbotin *et al.* 2006). The alignment score revealed more differences in the case of comparison with *G. millefolii* and *G. artemisiae* (score 97 was the most frequent), smaller differences were found when *G. tabacum* was compared (score 98 in most cases).

After comparison with the D2/D3 sequences of other important plant parasitic species of the family Heteroderidae present in NCBI database a certain level of similarity was found in the case of *Heterodera glycines* (DQ328692.1; Subbotin *et al.* 2006). Surprisingly, a similarly high level of resemblance with the *Helicotylenchus* sp. sequence (DQ077794; De Ley *et al.* 2005) was observed.

The phylogram revealed two basic clades separating the researched *Globodera* populations according to different species. However, *G. rostochiensis* population Ro3 was grouped with *G. pallida* populations and reference population of *G. pallida* PaRef was included in *G. rostochiensis* clade. *G. tabacum* was positioned close to *G. rostochiensis* Ro1 and Ro5 populations, *G. millefolii* and *G. artemisiae* were more distant (Figure 2).

The secondary structure of the D2 segment of *G. rostochiensis* and *G. pallida* consists of three long

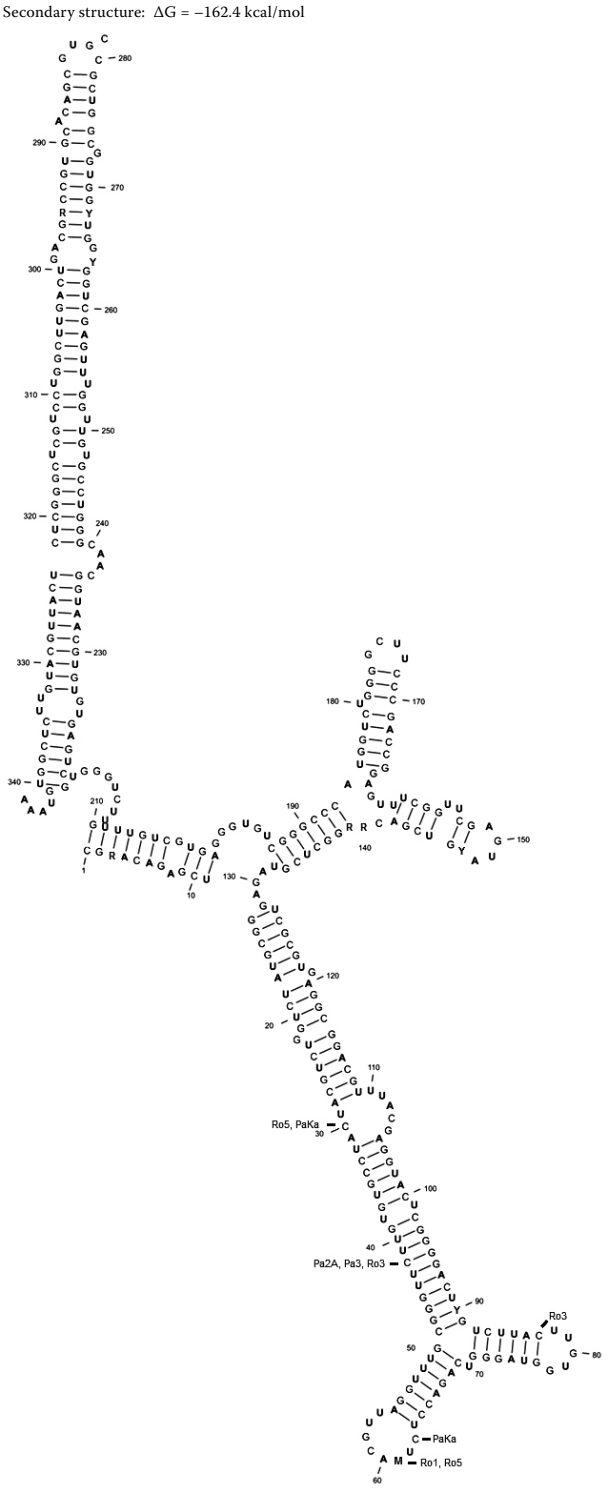

Figure 3. The general secondary structure of the D2 segment of 28S rRNA gene of *G. pallida* and *G. rostochiensis*; deletions are marked by bold dashes including the codes of corresponding populations

hairpins, the first of which is connected with the other two by a shorted helix; the second and the third hairpin are forked (Figure 3). Point mutations and deletions detected in the sequences did not cause any

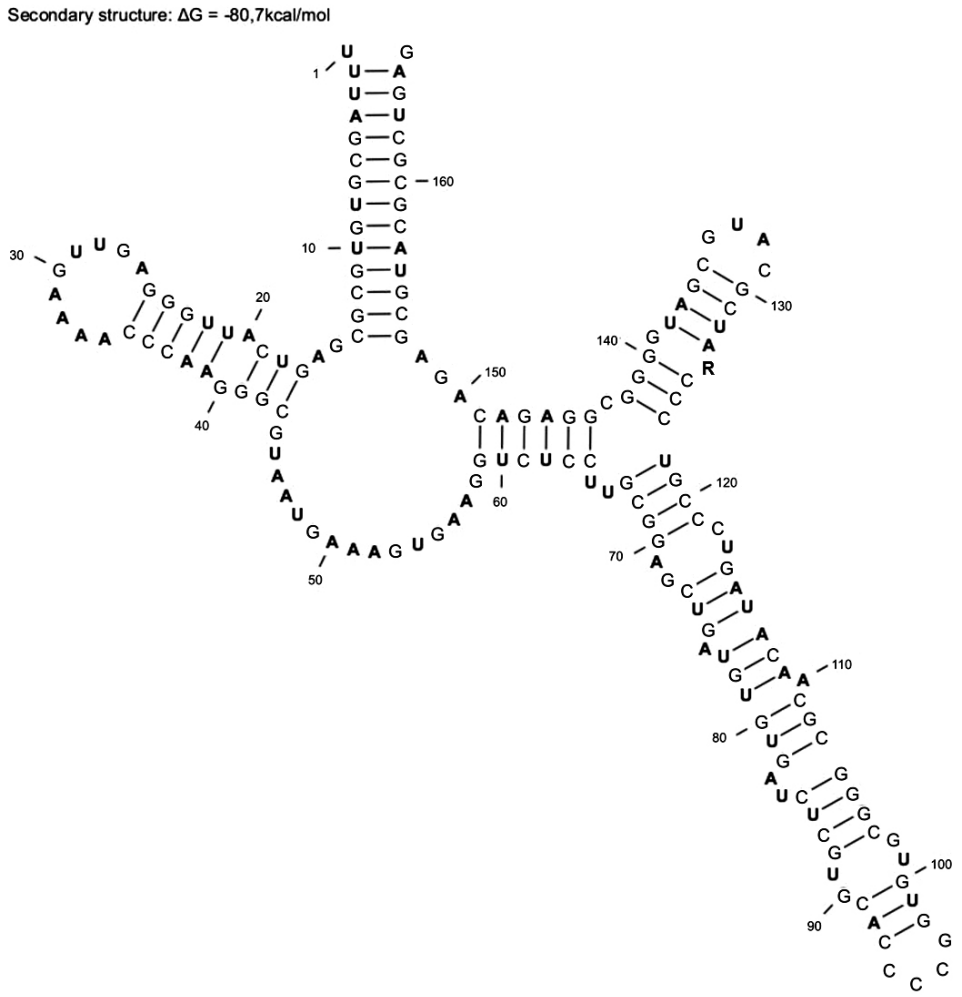

Figure 4. The general secondary structure of the D3 segment of 28S rRNA gene of *G. pallida* and *G. rostochiensis*

substantial differences in secondary structures of the two species. A rather dissimilar structure was found in the D3 segment. In both species the structure of this segment consisted of one long forked hairpin connected together by a longer single strand section with the second simple hairpin (Figure 4). Neither in this case were any substantial differences detected among the particular *G. pallida* and *G. rostochiensis* populations and between both species.

DISCUSSION

The method of DNA isolation used in this study was extremely successful. Under no circumstances is it problematic to use it for DNA isolation from a single potato cyst nematode cyst.

The expansion segments are a useful source of phylogenetic information regarding relatively recent evolutionary events (Subbotin *et al.* 2005). The structure of the D2/D3 segment can be used for solving taxonomic problems on different levels (Hillis & Dixon 1991). However, in the case of both potato cyst nematode species, the variability of their pathotypes and rDNA D2/D3 expansion segment region is rather low. Diversity detected in this study among the particular potato cyst nematode pathotypes is basically the same as when two *G. pallida* populations of the same pathotype (*G. pallida* Pa2A and *G. pallida* PaK) were compared.

Nevertheless, it appeared that D2/D3 expansion segment sequences could be used in some phylogenetic studies at least; however, the correct designation of *G. rostochiensis* and *G. pallida* populations is of crucial importance. Anyway it seems that in the case of potato cyst nematode the variability of D2D3 segments is not suitable for routine diagnostics but rather for basic research tracking recent evolutionary events.

The phylogram tree created from herein presented sequences clearly showed differences between both potato cyst nematode species, but the situation is complicated by the presence of *G. pallida* reference population PaRef in the *G. rostochiensis* group and *G. rostochiensis* Ro3 in the *G. pallida* cluster. The taxonomic status of these two populations needs further verification; their designation was done according to original sources or publications. Probably sequencing of another DNA segment or performing of biological tests would be desirable in this case.

According to the authors of this study the applicability of D2/D3 sequences to routine diagnostics of potato cyst nematodes could be complicated by their similarity to corresponding sequences of the nematode *G. artemisiae* (Nowaczyk *et al.* unpublished). However, the same problem arises when PCR diagnostic targeting Internal Transcribed Spacers (ITS) of rDNA with primers localised in 18S and 28S genes are used. According to Blok *et al.* (1998), who performed the PCR-RFLP analysis of ITS of several *G. pallida* populations, the digestion of the amplified fragment with a number of restriction enzymes showed differences among the populations, thus intraspecific variation was proved, but it was not clear if differences occurred among the particular populations only or whether there also existed variability within the particular populations. Single D3 sequences were obtained from *G. pallida* and *G. rostochiensis* by Subbotin *et al*. (2000); according to the same author they are unlikely to be useful for the identification of *Globodera* species parasitising plants of the Solanaceae family as it is highly conserved. This is consistent with our findings; however it seems that D2 segment sequences which are first presented in this study possess a somewhat greater potential as most differences among *Globodera* populations were detected in this region.

Another approach to discrimination of potato cyst nematode populations utilises mitochondrial DNA (Armstrong *et al.* 2007); in this case certain differences between American and European populations of *Globodera pallida* were found. Similar analysis of the population studied in this work would also be desirable.

By contrast, higher variability of the D2/D3 sequences of *G. tabacum* and *G. millefolii* (Subbotin *et al.* 2000, 2006) with corresponding sequences of *G. pallida* and *G. rostochiensis* suggests that in these species this region possesses a certain diagnostic potential. Anyway, the authors conclude that in future it will be necessary to design a reliable molecular diagnostic method for discriminating the species *G. pallida* and *G. rostochiensis* from *G. artemisiae* and *G. tabacum* because the morphological methods are not effective enough and a novel objective diagnostic protocol is needed (Gaar, personal commun.). Joint occurrence of this species with quarantine potato cyst nematodes could cause faulty diagnostics at least in Central Europe.

According to the authors of this study the applicability of D2/D3 sequences to routine diagnostics of potato cyst nematodes could be complicated by their similarity to corresponding sequences of the nematode *G. artemisiae* (Nowaczyk *et al.* unpublished). However, the same problem arises when PCR diagnostic targeting Internal Transcribed Spacers of rDNA with primers localised in 18S and 28S genes are used (Blok *et al.* 1998).

In the secondary RNA structure we can claim that the statement of largely conservative configuration was proved (Hillis & Dixon 1991). The secondary structure of D2/D3 segments of both species matches the general eukaryote model (Michot *et al.* 1984).

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