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Sequence analysis of the ribosomal internal transcribed spacer DNA of the crayfish parasite *Psorospermium haeckeli*

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ABSTRACT: Two morphotypes of the crayfish parasite *Psorospermium haeckeli* were isolated from 2 crayfish species of different geographical origin. The oval-shaped sporocysts were obtained from the epidermal and connective tissue beneath the carapace of the noble crayfish *Astacus astacus* from Sweden and Finland. Elongated spores were isolated from the abdominal muscle tissue of the red swamp crayfish *Procambarus clarkii* from USA. To compare genetic divergence of 2 morphotypes of the parasite, the ribosomal internal transcribed spacer (ITS) DNA (ITS 1 and ITS 2) and the 5.8S rRNA gene were cloned and sequenced. The analysed region is variable in length, with the ribosomal ITS sequence of the European morphotype longer than the North American one. Sequence diversity is found mainly in ITS 1 and ITS 2 regions, and there is 66% and 58% similarity between the 2 morphotypes, respectively. Thus, analysis of the ribosomal ITS DNA suggests that *P. haeckeli* forms obtained from Europe and North America are genetically diverse, which supports the previously reported morphological characteristics.

KEY WORDS: *Psorospermium haeckeli* · *Astacus astacus* · *Procambarus clarkii* · Crayfish · Parasite · Ribosomal RNA · Internal transcribed spacer

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INTRODUCTION

The crayfish parasite *Psorospermium haeckeli* is a unicellular organism characterised by an immobile sporocyst that is 100 to 200 µm in length, has an oval-elongated shape and a thick-layered shell, and is stuffed with globular contents. This parasite was first documented in Europe from native noble crayfish *Astacus astacus* by Haeckel (1857). It has been found in 17 species of freshwater crayfish worldwide (Vogt 1999). The pathogenicity of this parasite to the crayfish host is not clear. However, in aquaculture and during moulting, *P. haeckeli* has been associated with crayfish mortalities (Vey 1978, Söderhäll 1988). Several studies on host defence mechanism have shown that *P. haeckeli* can induce the activation of haemocytes and the prophenoloxidase-activating system of freshwater crayfish (Kobayashi & Söderhäll 1990, Söderhäll et al. 1990, Cerenius et al. 1991, Thörnqvist & Söderhäll 1993).

Although several life stage forms of *Psorospermium haeckeli* have been described in crayfish hosts (Henttonen et al. 1997) and its histology has been investigated (Rug & Vogt 1995, Vogt & Rug 1995), the life cycle and taxonomic status of this parasite are still mysterious. Recently, Vogt & Rug (1999) proposed a diphasic life cycle of *P. haeckeli* which comprises a histozoic and a free-living phase, and it was speculated that a new crayfish host is probably infected by the

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amoeboid form of the parasite. Analysis of the nuclear-encoded small subunit (SSU) rRNA gene of *P. haeckeli* showed that it belongs to a phylogenetic clade near the animal-fungal dichotomy (Ragan et al. 1996, Baker et al. 1999, Herr et al. 1999, Benny & O'Donnell 2000, Figueras et al. 2000, Ustinova et al. 2000), and it was suggested that this novel taxon be named Ichthyosporea according to its class status (Cavalier-Smith 1998). Like *P. haeckeli*, the members of this clade, except for *Amoebidium parasiticum*, are unicellular parasites of animals, and no complete life cycle of any species is known (Ragan et al. 1996, Herr et al. 1999).

At least 4 morphotypes of *Psorospermium haeckeli* have been described, as determined by size, morphology and histology. It seems likely that the occurrence of different morphotypes may be linked to crayfish species of different geographical origin. Of these, 2 morphotypes are found in Europe (Rug & Vogt 1995), the third morphotype is present in North America (Henttonen et al. 1992, 1994) and the fourth one is found in Australia (Herbert 1987). The different morphotypes have been suggested to constitute different species (Vogt & Rug 1995, Vogt et al. 1996). However, whether different morphotypes are different strains or species is still not resolved. The DNA sequences of non-coding regions of the large pre-rRNA coding segment, i.e., internal transcribed spacer (ITS) 1 and 2, are less conserved than the regions-encoding genes and have been used to distinguish between strains or species of organisms (Hillis & Dixon 1991). We analysed the nucleotide sequence of the ribosomal ITS DNA (ITS 1, 5.8S rRNA and ITS 2) from 2 morphotypes of *P. haeckeli*, isolated from *Astacus astacus* obtained in Sweden and Finland, and *Procambarus clarkii* from USA. The aim of this study was to compare genetic divergence between 2 morphotypes of the crayfish parasite *P. haeckeli* based on ribosomal ITS sequences.

MATERIALS AND METHODS

Isolation of *Psorospermium haeckeli*. Crayfish, infected naturally by *P. haeckeli*, were used as a source of this organism. The noble crayfish *Astacus astacus* was obtained from the Aquatic Research Unit, Kuopio University, Finland and from Lake Ubbemålasjön, Sweden. The red swamp crayfish *Procambarus clarkii* was from the University of Southwestern Louisiana Experimental Farm, USA. The epidermal and connective tissue beneath the carapace of *A. astacus* or the abdominal muscle tissue of *P. clarkii* (300 to 400 mg wet wt) was dissected and cut into small pieces in a Petri dish in 2 ml of 0.15 M NaCl. The preparation was filtered through cheesecloth and washed 3 times with 2 ml of 0.15 M NaCl. The suspension was centrifuged at 2000 × *g* for 2 min and the pellet was washed twice with 2 ml of 0.15 M NaCl. Then the suspension was laid on the top of a Percoll cushion (50% Percoll [Pharmacia] in 0.15 M NaCl) and centrifuged at 2800 × *g* for 10 min. The resulting pellet contained *P. haeckeli* relatively free of crayfish tissue. Sometimes, if the last suspension contained too much host tissue, it was mixed with an equal volume of sterile water and centrifuged at 13 000 × *g* for 10 min to obtain parasites free of host tissue. Then the preparation was checked under a light microscope and used for genomic DNA extraction.

DNA isolation. Genomic DNA was extracted from the parasite suspension using either (1) phenol:chloroform extraction and ethanol precipitation as described by Sambrook et al. (1989) or (2) Puregene kit, following the supplied protocol for DNA isolation from body fluid (Gentra Systems, Minneapolis, MN, USA). The DNA was stored at –20°C until required.

Amplification of ribosomal ITS DNA. The complete ITS 1, 5.8S rRNA and ITS 2 of 2 different morphotypes of *Psorospermium haeckeli* were amplified by using an upstream primer at the 3′ end of small subunit (SSU) rRNA and a downstream primer at the 5′ end of large subunit (LSU) rRNA. The forward primer Pso-1 (5′ ATGAAATACTGCCTAGTCGTC 3′) was designed from the sequence of *P. haeckeli* SSU rRNA (Ragan et al. 1996; GenBank accession no. U33180). The reverse primer ITS 4 (5′ TCCTCCGCTTATTGATATGC 3′) was designed from a highly conserved region of LSU rRNA sequence (White et al. 1990). The PCR reactions were carried out in 50 µl volumes containing 100 ng of genomic DNA, 200 µmol of each deoxyribonucleoside-5′-triphosphate (Amersham Pharmacia Biotech, Uppsala, Sweden), 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer and 2 units of Taq DNA polymerase (Gibco BRL, UK). Amplifications were performed in a Perkin-Elmer GeneAmp PCR system 2400 programmed for 1 cycle of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 30 min. From each parasite preparation (i.e., Swedish *Astacus astacus*, Finnish *A. astacus* and American *Procambarus clarkii*), 3 independent PCR reactions were carried out to ensure that the PCR products were free of errors during amplification with Taq DNA polymerase.

DNA cloning and sequencing. The PCR products were analysed on 1% agarose gel. Lambda DNA/*Hin*d III markers (Promega, Madison, WI, USA) and 100 bp ladder DNA (Amersham Pharmacia Biotech) were used as molecular weight markers. The amplified products were excised from agarose gel and purified by GFX PCR DNA and Gel Band Purification Kit

(Amersham Pharmacia Biotech, Piscataway, NJ, USA). Purified products were cloned into either pBluescript KS-vector (Strategene, La Jolla, CA, USA) or pCR®2.1-TOPO® vector (Invitrogen, Breda, The Netherlands) according to manufacturers' instructions. From each independent PCR reaction as above, at least 3 clones were picked and sequenced in both directions. This means that each ITS sequence shown is the consensus of at least 9 determinations. The sequencing was performed using Big Dye terminator reactions and an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Forest City, CA, USA).

Sequence data analysis. The sequences were aligned using the multiple alignment program ClustalW, and the pairwise similarity was calculated.

RESULTS

Morphotypes of *Psorospermium haeckeli*

Two different morphotypes of mature sporocysts of *Psorospermium haeckeli* were obtained from 2 crayfish species. *P. haeckeli* from the noble crayfish *Astacus astacus* (from both Sweden and Finland) were oval-shaped, measuring 80 to 110 × 55 to 70 µm (Fig. 1a). The parasites were often found in the epidermal and connective tissue beneath the carapace of *A. astacus* and sometimes in the abdominal muscle and gill. In the red swamp crayfish (*Procambarus clarkii*), *P. haeckeli* had elongated spores of 120 to 200 × 35 to 70 µm predominantly in the abdominal muscle tissue (Fig. 1b). Although *P. haeckeli* in the crayfish tissue was not quantified, the amount of parasites was estimated to range from a few individuals to thousands in both *A. astacus* and *P. clarkii*.

Analysis of ribosomal ITS DNA

The ribosomal ITS regions of the 2 morphotypes of *Psorospermium haeckeli* were amplified and sequenced (Fig. 2). The ITS sequence of *P. haeckeli* from *Astacus astacus* isolates (Sweden and Finland) was 1500 bp in total length, while the *Procambarus clarkii* isolate was 1300 bp in length. The position of the 3′ end of the SSU rRNA gene was determined by comparison with the complete sequence of *P. haeckeli* SSU rRNA (Ragan et al. 1996; GenBank accession no. U33180) and the position of the 5′ end of the LSU rRNA gene was assigned by comparing the current sequences with the sequences of *Cyanea capillata* (U65481), *Distichopora* sp. (U65483), *Typhula ishikariensis* (AF-193364) and *Gerronema marchantiae* (U66432). The 5.8S rRNA sequences from *P. haeckeli* were estimated by comparison with the sequences of *Pseudoperkinsus tapetis* (AF268423), *Bilinopsis* sp. (U65480), *Cyanea capillata* (U65481) and *Dendronephthya aurea* (AF-320107). The partial sequences of SSU and LSU rRNA from *A. astacus* (Sweden and Finland) isolates and the *P. clarkii* isolate were 100% identical (Fig. 2) and the sequence similarity of 5.8S rRNA among *P. haeckeli* isolates in this study was 99 to 100%.

The size and G+C contents of each ITS 1, 5.8S rRNA and ITS 2 sequence were calculated (Table 1), and the pairwise sequence similarity was constructed (Table 2). The lengths of both ITS 1 and ITS 2 from *Astacus astacus* (Sweden and Finland) isolates were identical, with 493 and 729 nucleotides, respectively, and the sequence similarity of ITS 1 and ITS 2 between these isolates was >99%. The lengths of both ITS 1 and ITS 2 from the *Procambarus clarkii* isolate were shorter than those of *A. astacus* isolates (Table 1). The ITS 1 and ITS 2 of *P. clarkii* and *A. astacus* isolates were distinct; the

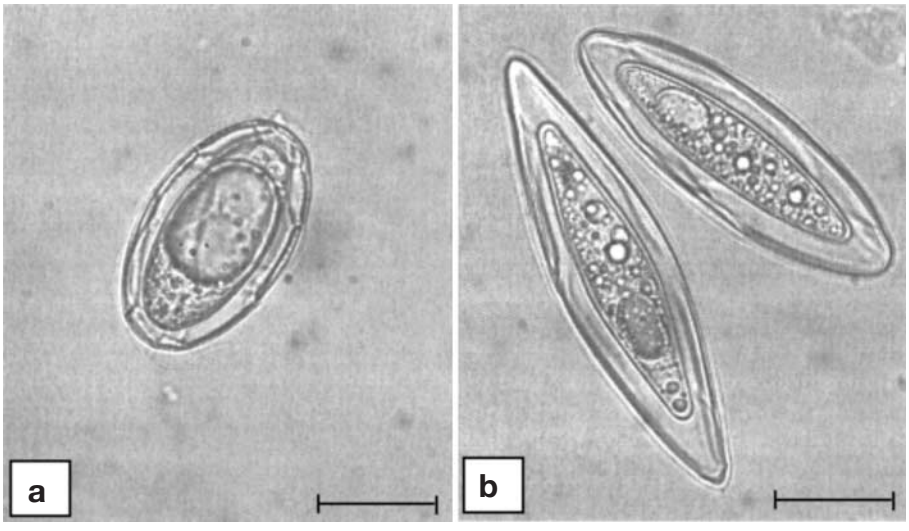

Fig. 1. *Psorospermium haeckeli*. Mature sporocysts (a) from the epidermal and connective tissue under the carapace of *Astacus astacus* and (b) from the abdominal muscle tissue of *Procambarus clarkii*. Scale bar = 50 µm

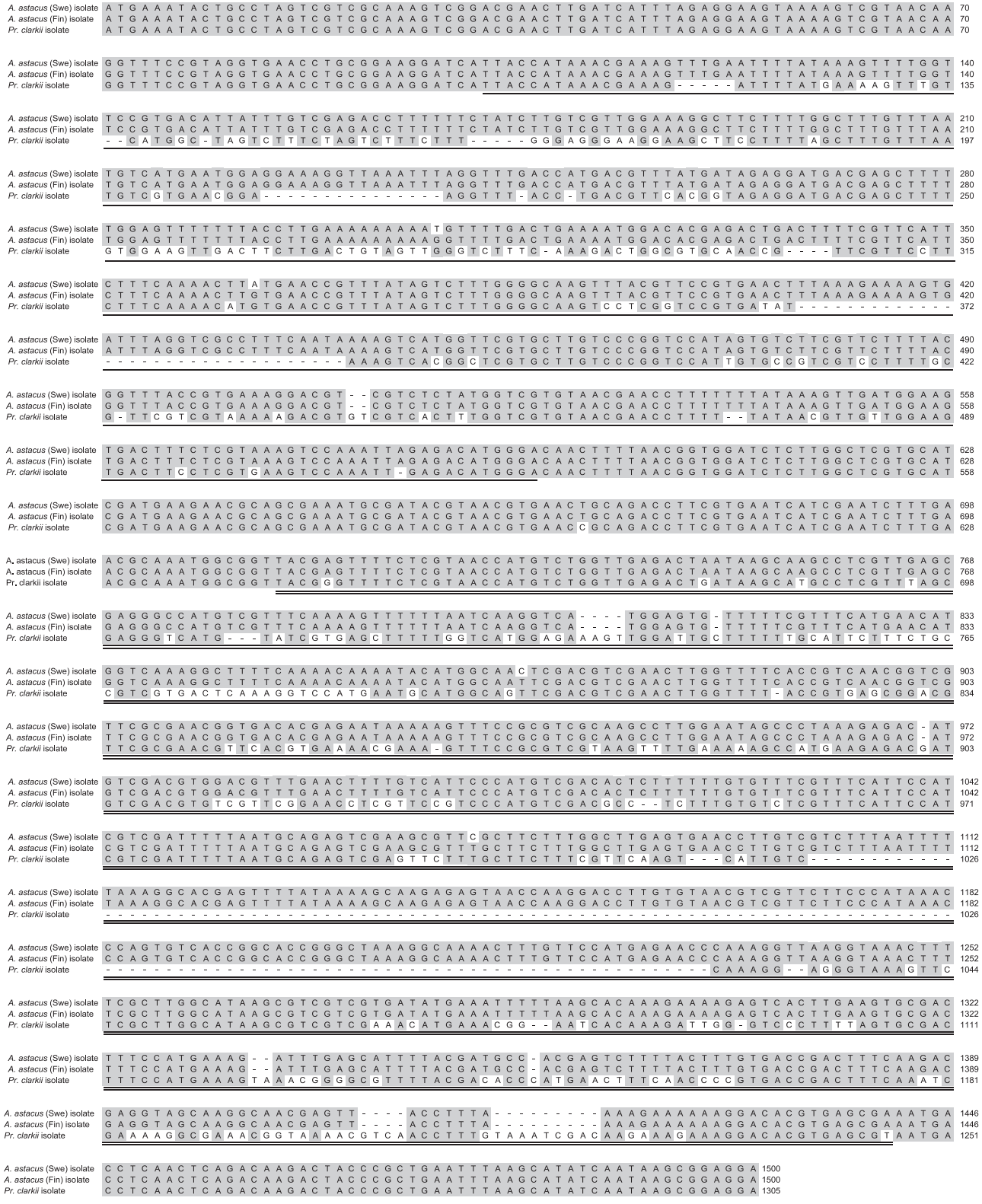

Fig. 2. *Psorospermium haeckeli* isolates. Alignment of the consensus nucleotide sequences of amplified ribosomal internal transcribed spacer (ITS) DNA. The numbers show the order of nucleotide sequence of each parasite isolate. The shaded boxes enclose conserved regions. Gaps generated by alignment are shown by dashes. The single line indicates the ITS 1 region and the double line represents the ITS 2 region

Table 1. *Psorospermium haeckeli* isolates. Length (bp) and G+C contents (%) of the internal transcribed spacing (ITS) 1, 5.8S rRNA and ITS 2 DNA. Fin: Finland; Swe: Sweden

Psorospermium haeckeli	ITS 1		5.8 S rRNA		ITS 2		GenBank
	Length	G+C (%)	Length	G+C (%)	Length	G+C (%)	accession no.
Astacus astacus isolate (Swe)	493	36.51	118	47.46	729	42.66	AF353179
Astacus astacus isolate (Fin)	493	36.92	118	47.46	729	42.39	AF353180
Procambarus clarkii isolate	423	44.44	118	48.31	604	46.36	AF353181

Table 2. *Psorospermium haeckeli* isolates. Sequence similarity of the internal transcribed spacer (ITS) 1, 5.8S rRNA and ITS 2 DNA. Fin: Finland; Swe: Sweden

Psorospermium haeckeli	ITS 1			5.8S rRNA			ITS 2		
	1	2	3	1	2	3	1	2	3
1. *Astacus astacus* isolate (Swe)	1.000			1.000			1.000		
2. *Astacus astacus* isolate (Fin)	0.996	1.000		1.000	1.000		0.997	1.000	
3. *Procambarus clarkii* isolate	0.665	0.669	1.000	0.992	0.992	1.000	0.582	0.585	1.000

ITS 1 and ITS 2 regions showed an average similarity of 66.7% and 58.4%, respectively.

DISCUSSION

At least 4 morphotypes of *Psorospermium haeckeli* have been discovered and they are present in several crayfish species of different geographical origin. Two morphotypes are present in Europe: Type 1 has an oval-shaped sporocyst, and the more elongated or rod shape is considered to be Type 2. The size of the morphotypes is slightly different, although the internal architecture and histological composition are identical (Rug & Vogt 1995). The third morphotype is present in North America. It has an elongated shape and is longer than the European morphotypes (Henttonen et al. 1992, 1994). The fourth is found in Australia, has an oval or curved shape and is slightly smaller than the European types (Herbert 1987, Henttonen 1996). Therefore, the 2 morphotypes of *P. haeckeli* examined in this study belong to the European morphotype 1 and to the North American morphotype, based on shape, size and geographical origin.

The primers designed for amplification of the ribosomal ITS DNA of *Psorospermium haeckeli* are specific. We always obtained a single amplification product from PCR reactions, indicating that there was no amplification of the crayfish host DNA. Moreover, no amplification product was observed when genomic DNA from apparently non-infected crayfish (*Astacus astacus* and *Procambarus clarkii*) was used as a control DNA template (data not shown). Thus, these primers could be used for analysis of the parasite ribosomal ITS sequence.

The ribosomal ITS sequence analysis obtained in this study clearly indicates a genetic divergence between the 2 morphotypes of *Psorospermium haeckeli* originating from Europe and North America. The sequence differences between the 2 types occurs largely in the ITS 1 and ITS 2 regions. The partial sequence of SSU and LSU rRNA and the complete sequence of 5.8S rRNA of European (type 1) and North American morphotypes are very conserved. This is not surprising since nuclear rRNA sequences evolve relatively slowly, whereas ITS regions evolve more rapidly and may vary among species within a genus or among populations (White et al. 1990, Hillis & Dixon 1991). This result also suggests that the 4 morphotypes of *P. haeckeli*, which are found in different geographical areas, may have genetic divergence.

The SSU rRNA sequence of the European morphotype 1 was determined by Ragan et al. (1996). Although the SSU rRNA from other morphotypes has not been sequenced, it is suggested to be similar since the coding regions of 5.8S rRNA from both morphotypes in this study are highly conserved. Several putative life cycle stages of the parasite have been described, but a complete life cycle and the mode of infection have not been established (Henttonen et al. 1997, Vogt 1999, Vogt & Rug 1999). Thus, oligonucleotide probes complementary to the specific sequence of the ribosomal ITS may be useful for investigation and confirmation of other life stages of different *Psorospermium haeckeli* morphotypes and to elucidate how the parasite is transmitted from one host to another.

The issue of whether these different morphotypes of *Psorospermium haeckeli* are separate species is unresolved. Even though the *P. haeckeli* European morphotype 1 and North American morphotype have clearly

distinct ribosomal ITS sequences, it would be premature at this stage to conclude that the 2 morphotypes belong to 2 separate species. Obviously, considerable genetic variation may exist between different strains, but more data on possible sequence variations between and within the different morphotypes combined with detailed morphological characteristics of the life cycle stages are required to split *P. haeckeli* into separate species.

In conclusion, the genetic evidence obtained in this study strongly indicates that the different forms of *Psorospermium haeckeli* isolated from Europe (Type 1) and North America are not due to morphological variations of the same organism, but that they are genetically different.

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