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Random Amplified Polymorphic DNA Fingerprinting as a Marker for *Paramecium jenningsi* Strains*

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The aim of the present study is to establish a common RAPD marker for *P. jenningsi* using a series of Ro primers and to investigate if strains originating from distant and isolated localities (Japan, China, India, Saudi Arabia) have isolated gene pools and represent distinct species. An analysis of dendrograms constructed on the basis of RAPD-PCR fingerprints with four primers (Ro 460-04, 460-06, 460-07, and 460-10) from the first part of this project (SKOTARCZAK *et al*. 2004), assigns the strains to two groups consisting of the continental strains (India, Saudi Arabia, China) and Japanese strains that have been considered as a separate sibling species within *P. jenningsi*. The genetic similarity of the Indian and Arabian strains was ascertained, whereas the Chinese strain formed an independent branch in this sibling species. The primers Ro (460-01, 460-02, 460-03, 460-05, 460-08) also distinguish between two groups of strains, although they divide the Japanese strains into two subgroups that are not reproductively isolated. This probably indicates genetic variation within this sibling species. However, it comprises one common gene pool (successful inter-strain crosses) and is reproductively isolated from the other sibling species. The results presented in these papers confirm that the construction of ten band patterns having marker attributes is possible on the basis of DNA amplification from 9 strains of *P. jenningsi* with the RAPD-PCR fingerprinting method using five primers from the Ro series. The patterns can be assigned to three marker-groups: a general species group, a group differentiating between sibling species, and accessory strain markers.

Key words: *Paramecium jenningsi*, RAPD-PCR fingerprinting, marker bands.

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Paramecium jenningsi was described from India (Bangalore) in 1958 (Diller & Earl). Later, other strains of the species were also described from different localities, mainly situated in tropical regions (PRZYBOŚ *et al*. 1999). Several studies carried out on different *P. jennningsi* strains including genetic, karyological, and cytological studies (PRZYBOŚ 1975, 1978, 1980, 1986; JURAND & PRZYBOŚ 1984) as well as biochemical investigations (ALLEN *et al*. 1983) have shown that the strains represent only one species. The existence of only one species of *P. jenningsi* was generally accepted (cf PRZYBOŚ 1986) according to SONNEBORN's earlier (1958, 1970) opinion.

However, the discovery of new habitats of *P. jenningsi* in Japan, China, and Saudi Arabia expanded the range of the species, and prompted the problem of the species structure, i.e. ascertainment if strains originating from remote and isolated habitats represent one species or more. The studies carried out by PRZYBOŚ *et al.* (2003) revealed the presence of separate two species within *P. jenningsi.* One species is confined to six strains from Japan, the other species includes strains from India, Saudi Arabia, and China. The mentioned studies were conducted by the classical genetic method (strain crosses) and a molecular technique, the method of RAPD-PCR fingerprinting with primer Ro460-04. The existence of two separate species of *P. jenningsi* was later confirmed by further stud-

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ies applying other primers from the Ro series (SKOTARCZAK *et al*. 2004).

The two species of *P. jenningsi* are real biological species isolated genetically (they do not cross with each other) and geographically (group of continental strains from Saudi Arabia, India, and China and the Japanese strains). However, in spite of these differences the two *P. jenningsi* species are in practice impossible to differentiate using traditional methods (cytological examination or morphometrically). It seems necessary to elaborate a quick and effective method of identification of the two sibling species as well as the taxonomic species of *P. jenningsi.*

The aim of the present paper was to apply the RAPD-PCR fingerprinting method with several primers from the Ro series for amplifying band patterns containing characteristic marker bands for the taxonomic species *P. jenningsi*, its two sibling species, and for the particular strains.

Material and Methods

Material

Strains used in the study are listed in Table 1.

Methods

DNA was isolated as in PRZYBOŚ *et al.* (2003) from two cultures each of *P. jenningsi* using the QIAamp DNA Mini Kit (Qiagen, Germany). Random Amplified Polymorphic DNA fingerprinting was performed according to STOECK and SCHMIDT (1998) using Fermentas polymerase (Lithuania). The following primers were used: Ro 460-01 (5' TGCGCATCG 3'), Ro 460-02 (5' GCAGGATCG 3'), Ro 460-03 (5' CTGCGATACC 3'), Ro 460-05 (sequence: 5' CTAGCTCTGG 3') and Ro 460-08 (5' CGATGAGCCC 3'), Ro 460-09 (sequence: 5' CGCTGTTACC 3') (Roth, Karsruhe, Germany).

Reaction products were separated in 1.5% agarose gels for 3.5 hours at 85 volts accompanied by a molecular mass marker (Smartladder, Bioline, Germany). Repeatability was ascertained by performing every RAPD reaction several times for each strain and culture. RAPD-PCR fingerprints were visualized in UV light with ethidium bromide and stored in computer memory using the Biocapt program (Vilbert Lourmat, France). Analysis of phylogenetic similarity was carried out by comparing the molecular mass of the RAPD-PCR amplified DNA fragments by means of the program Bio1D (Vilbert Lourmat, France). This program follows NEI and LI's (1979) similarity coefficients defined by the formula:

Table 1

Paramecium jenningsi strains

Origin and collector's name	References
Saudi Arabia, neighbourhood of Riyadh; K.A.S. Al-Rasheid, July 1999	FOKIN *et al*. 2001
India, Bangalore; P.B. Padmavathi, 1955*#	DILLER & EARL 1958; PRZYBOŚ 1975, 1978, 1986
China, Shanghai; M. Fujishima, November 1999	FOKIN *et al*. 2001
Japan, Honshu island, Yamaguchi prefecture, Ube city; M. Fujishima, October 2000	
Japan, Honshu island, Yamaguchi prefecture, Nagato city; M. Fujishima, September 2000	
Japan, Honshu island, Yamaguchi prefecture, Shinnamyou; M. Fujishima, October 2000	
Japan, Honshu island, Yamaguchi prefecture, Hagi city; *S. Fokin, September 1997	PRZYBOŚ *et al*. 1999
Japan, Honshu island, Yamaguchi prefecture, Yamaguchi city; S. Fokin, November 1999	FOKIN *et al*. 2001
Japan, Okinawa island, Okinawa prefecture, Hujigawa; M. Fujishima 2000	

* the strains were studied by RAPD-PCR previously (PRZYBOŚ *et al*. 1999),

the strain from India was studied genetically and karyologically (PRZYBOŚ 1975, 1978, 1986).

$$a = 2nxy/(nx + ny),$$

where nx and ny are the number of bands in RAPD profiles x and y, and nxy the number of bands of equal molecular mass shared by both profiles. Phylogenetic trees were constructed on the basis of a similarity matrix of the values of this formula.

Results

Results of RAPD-PCR fingerprinting

RAPD-PCR products, amplified by primers Ro 460-01, Ro 460-02, 460-03, 460-05, 460-08, 460-09 from nine strains (two cultures each) of *P. jenningsi* originating from different geographic localities are presented in Figures 1-5. Each strain was characterized by a series of RAPD-PCR products of different molecular mass (observed in UV light as bands in agarose gels). RAPD profiles for each geographic locality and primer were constructed on the basis of photographs of gels.

A list of the approximate molecular weight of RAPD-PCR products reveals 100% concordance in all 9 strains (two cultures per strain).

Analysis of RAPD-PCR fingerprints revealed a 551 bp band, present in all strains, when the primer Ro 460-01 was used (Fig. 1). Furthermore, an 1148 bp band was present in every strain except for the Saudi Arabian. All Japanese strains shared an 815 bp band. A single marker (855 bp) was obtained from three non-Japanese strains (India, Saudi Arabia, China). Additionally, the latter groups shared a 491 bp band with the Japanese strains from Hagi, Yamaguchi and Ube. A large band of 1456 bp was shared by the Japanese strains Hagi and Ube, similar to an additional band (1367 bp) present in Shinnamyou and Okinawa.

RAPD fingerprints from the Ro 460-02 primer disclosed the presence of 3 ubiquitous bands (Fig. 2): a 1762 bp marker for all continental strains (India, Saudi Arabia, China) and 3 Japanese strains (Magi, Yamaguchi and Okinawa), another band (1541 bp) for the continental strains, Hagi and Yamaguchi, and a third band (1115 bp) shared by all non-Japanese strains and 3 island strains (Ube, Nagato and Shinnamyou). Specific bands were obtained for the Indian (787 bp), Chinese (1376 and 1055 bp), and Japanese Hagi (469 bp) strains. Large-sized, specific bands were discovered in the Japanese strains Hagi and Nagato (2289 bp), and Yamaguchi and Okinawa (1928 bp).

RAPD-PCR fingerprint analysis for the Ro 460-03 primer (Fig. 3) demonstrated common bands of 1069 bp and 829 bp for all strains. The Japanese and the Indian sample shared a band (1393 bp). Additionally, the Chinese and all Japanese strains except for the Ube sample had a characteristic band (665 bp). A specific band (900 bp) was found in the Japanese samples with the exception of Hagi and Yamaguchi. A 248 bp band was shared by all continental (India, Saudi Arabia, China) and 2 Japanese (Ube and Nagato) strains.

Analysis of RAPD-PCR fingerprints revealed a 1031 bp band, present in all strains, when the primer Ro 460-05 was applied (Fig. 4). All continental strains and two of the Japanese strains (Hagi and Yamaguchi) shared a 1589 bp band. Similarly, non-Japanese strains and the Hagi strain from Japan had an 1186 bp band. However, another band (748 bp) was present in the continental strains and Japanese Hagi and Ube. All Japanese strains, except for Hagi and Yamaguchi, had a 1457 bp band. Strain specific bands were obtained for six strains: Arabian, Chinese and four Japanese strains (Hagi, Ube, Shinnamyou, Okinawa). The Saudi Arabian strain had a molecular mass of 673 bp, the Chinese a 495 bp band, the Hagi strain a 394 bp band, 1702 bp for Ube, 1862 bp for Shinnamyou and 700 bp, 636 bp, and 417 bp for the Okinawa strain.

Analysis of RAPD-PCR fingerprints revealed 1333 bp and 722 bp bands, present in all strains, when the primer Ro 460-08 was used (Fig. 5). Moreover, all strains except for Okinawa shared

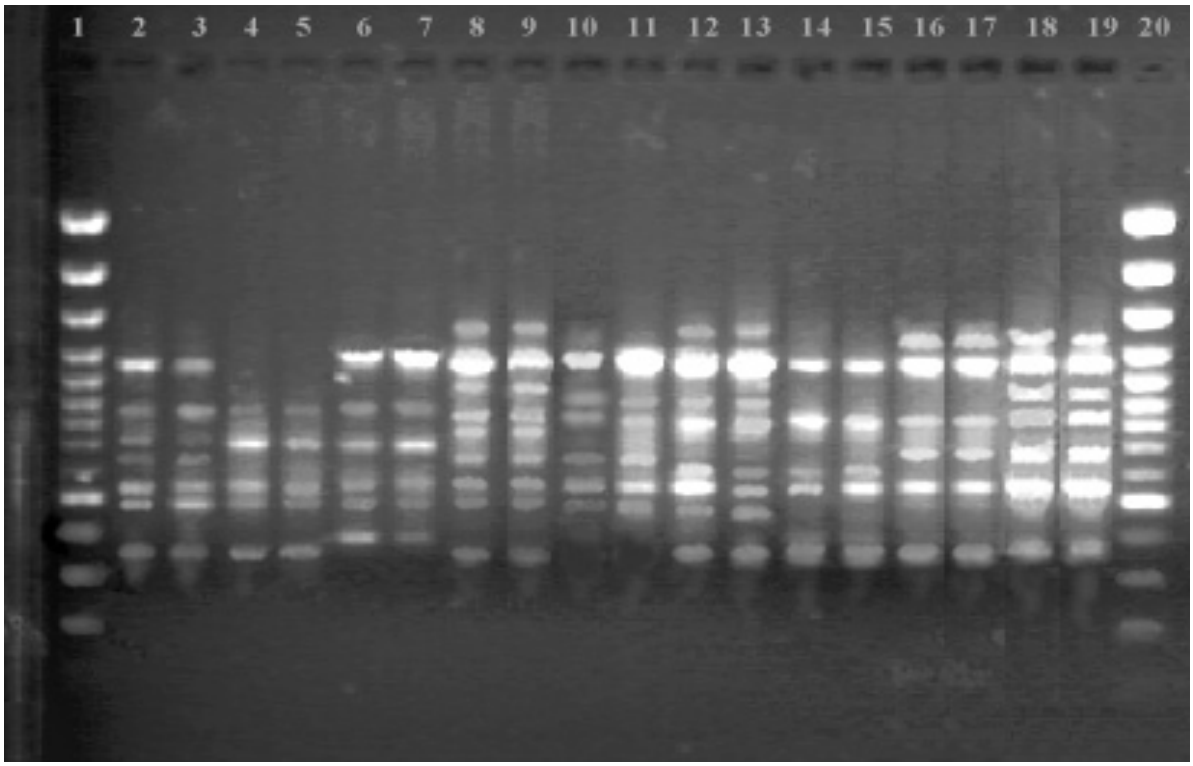

Fig. 1. RAPD reaction products for primer Ro 460–01 separated in an agarose gel. Lanes: 1 and 20 – weight marker, 2, 3 – Indian strain (Bangalore), 4, 5 – Saudi Arabian strain, 6, 7 – Chinese strain (Shanghai), 8, 9 – Japanese strain (Hagi), 10, 11 – Japanese strain (Yamaguchi), 12, 13 – Japanese strain (Ube), 14, 15 – Japanese strain (Nagato), 16, 17 – Japanese strain (Shinnamyou), 18, 19 – Japanese strain (Okinawa, Hujigawa).

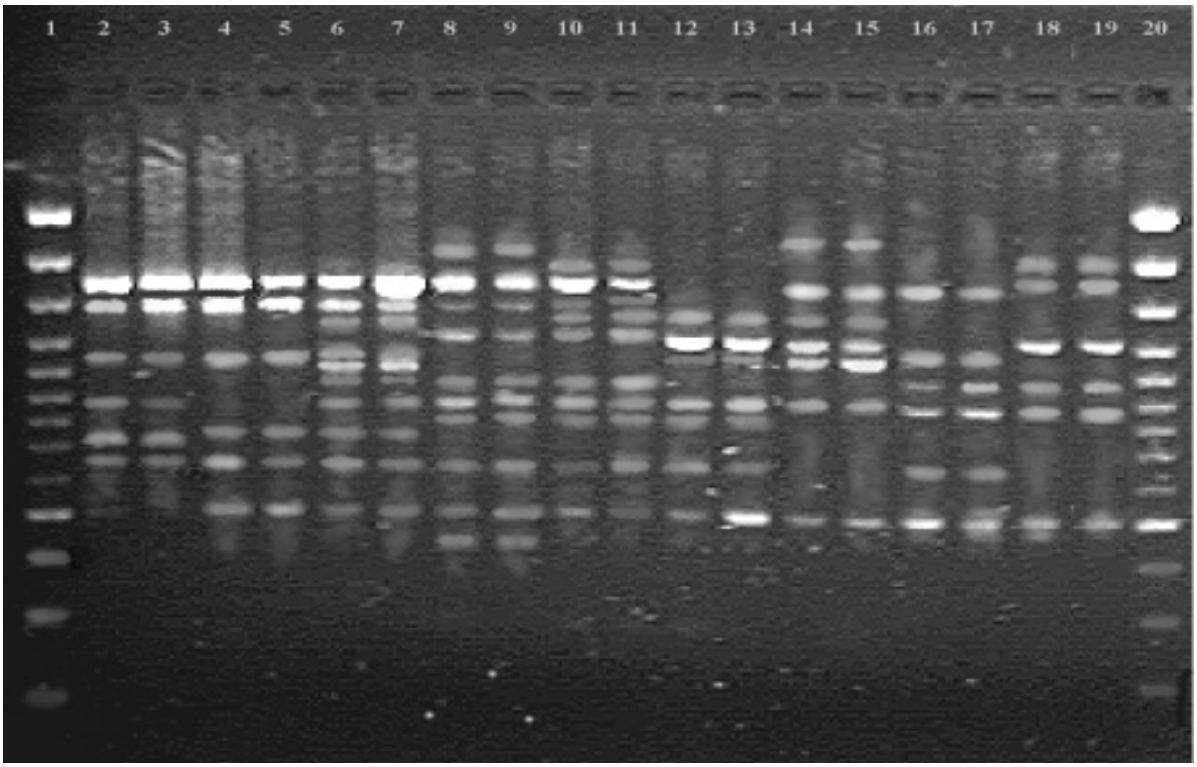

Fig. 2. RAPD reaction products for primer Ro 460–02 separated in an agarose gel. Lanes: 1 and 20 – weight marker, 2, 3 – Indian strain (Bangalore), 4, 5 – Saudi Arabian strain, 6, 7 – Chinese strain (Shanghai), 8, 9 – Japanese strain (Hagi), 10, 11 – Japanese strain (Yamaguchi), 12, 13 – Japanese strain (Ube), 14, 15 – Japanese strain (Nagato), 16, 17 – Japanese strain (Shinnamyou), 18, 19 – Japanese strain (Okinawa, Hujigawa).

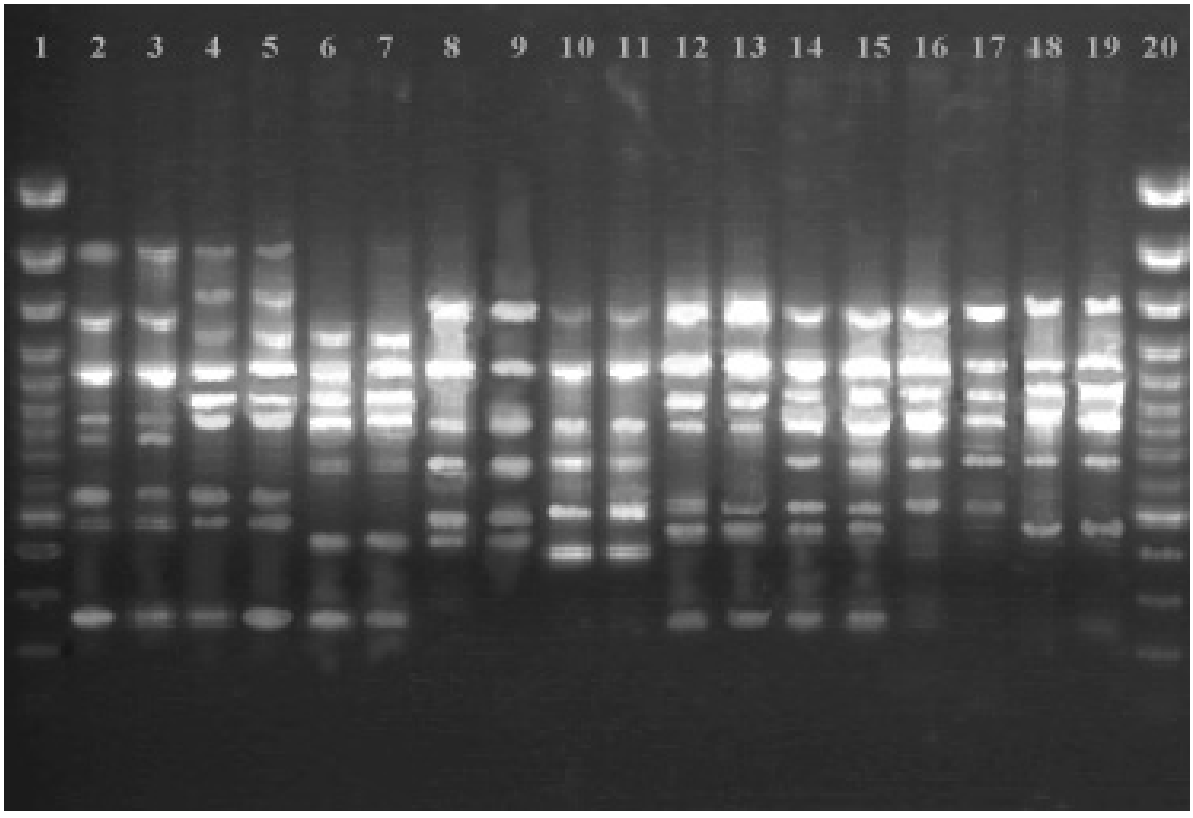

Fig. 3. RAPD reaction products for primer Ro 460–03 separated in an agarose gel. Lanes: 1 and 20 – weight marker, 2, 3 – Indian strain (Bangalore), 4, 5 – Saudi Arabian strain, 6, 7 – Chinese strain (Shanghai), 8, 9 – Japanese strain (Hagi), 10, 11 – Japanese strain (Yamaguchi), 12, 13 – Japanese strain (Ube), 14, 15 – Japanese strain (Nagato), 16, 17 – Japanese strain (Shinnamyou), 18, 19 – Japanese strain (Okinawa, Hujigawa).

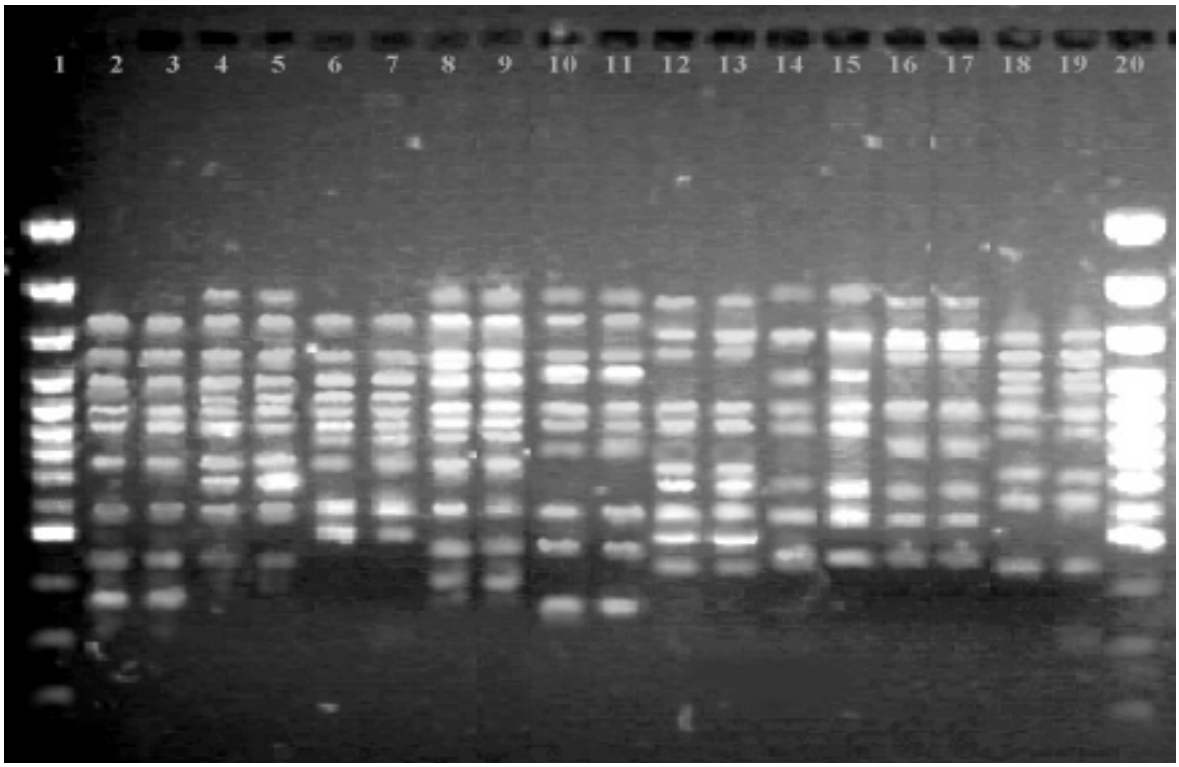

Fig. 4. RAPD reaction products for primer Ro 460–05 separated in an agarose gel. Lanes: 1 and 20 – weight marker, 2, 3 – Indian strain (Bangalore), 4, 5 – Saudi Arabian strain, 6, 7 – Chinese strain (Shanghai), 8, 9 – Japanese strain (Hagi), 10, 11 – Japanese strain (Yamaguchi), 12, 13 – Japanese strain (Ube), 14, 15 – Japanese strain (Nagato), 16, 17 – Japanese strain (Shinnamyou), 18, 19 – Japanese strain (Okinawa, Hujigawa).

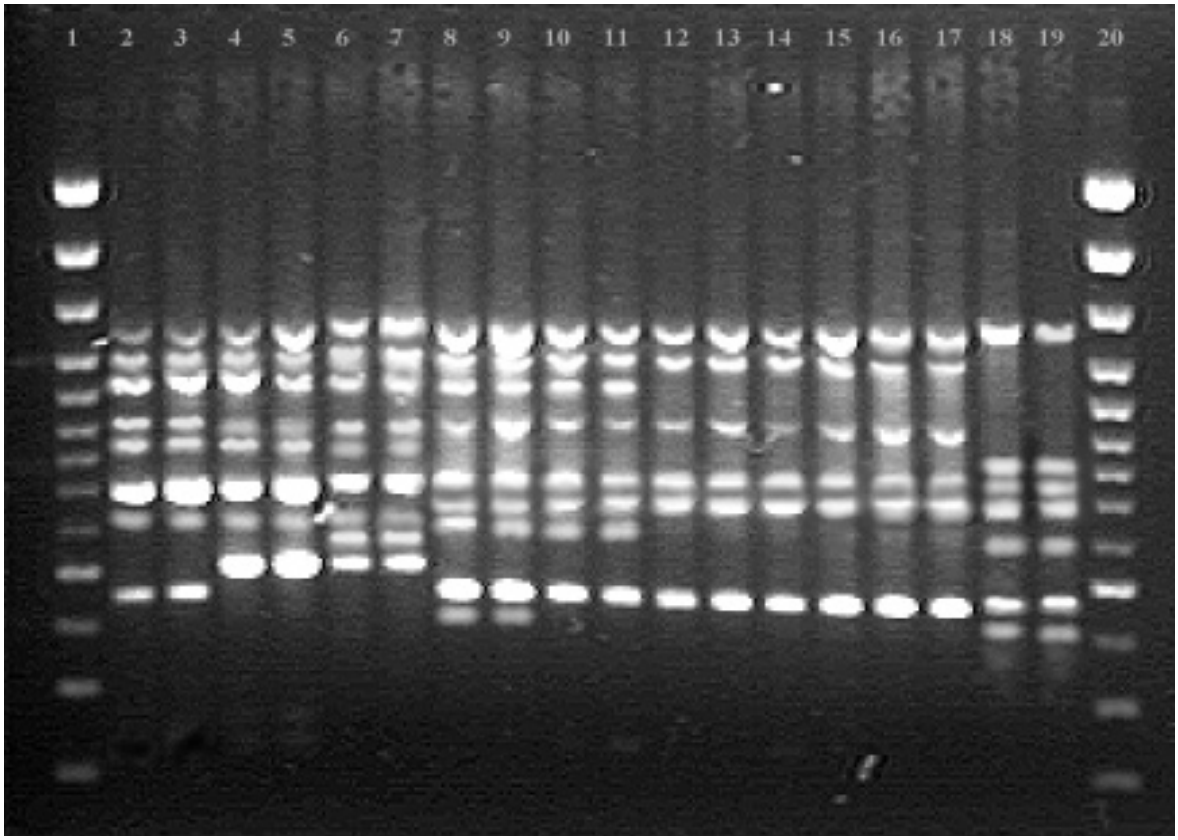

Fig. 5. RAPD reaction products for primer Ro 460–08 separated in an agarose gel. Lanes: 1 and 20 – weight marker, 2, 3 – Indian strain (Bangalore), 4, 5 – Saudi Arabian strain, 6, 7 – Chinese strain (Shanghai), 8, 9 – Japanese strain (Hagi), 10, 11 – Japanese strain (Yamaguchi), 12, 13 – Japanese strain (Ube), 14, 15 – Japanese strain (Nagato), 16, 17 – Japanese strain (Shinnamyou), 18, 19 – Japanese strain (Okinawa, Hujigawa).

1189 bp and 920 bp bands. Japanese strains had a 679 bp band in common. Additionally, the Japanese and Indian strains shared a band of 460 bp. The continental strains had a marker of 833 bp. These and the Japanese strains Hagi and Yamaguchi shared 1099 bp and 625 bp bands. Specific bands were produced for two Japanese strains, Hagi and Okinawa. The band for the Hagi strain had a molecular mass of 431 bp; bands for the Okinawa strain had 800 bp and 408 bp.

Analysis of RAPD-PCR fingerprints revealed an 838 bp band, present in all strains, when the primer Ro 460-09 was applied. It can be used as a molecular marker for *P. jenningsi*. Three products between 838 bp and 589 bp were obtained for the Indian strain; the same was true for the Arabian, Chinese and Japanese Hagi strains. The Japanese strains Yamaguchi and Ube shared two products (1240 bp and 838 bp), while the Nagato strain had only one with a molecular mass of 838 bp. Three products were obtained for the Shinnamyou strain (from 838 bp to 412 bp) and two for the Okinawa strain with a molecular mass of 838 bp and 589 bp . RAPD-PCR fingerprinting with primer Ro 460-09 did not reveal a marker specific to all Japanese strains, or for the non-Japanese localities.

Results of genetic similarity analysis

The dendrogram obtained from the results of RAPD fingerprinting using the Ro primers (Fig. 6) divides the 9 strains of *P. jenningsi* into 2 groups: the first large group includes all continental strains and 4 Japanese strains (Hagi, Yamaguchi, Ube and Nagato), the second involves the Japanese strains from Shinnamyou and Okinawa (similarity between groups equaled 42%). Two subgroups could be distinguished in the first group: continental and Japanese strains (similarity of 57%). The Japanese strains from the second group (Shinnamyou and Okinawa) had a similarity of 92%.

Discussion

The aim of the present study was to establish a common RAPD marker for *P. jenningsi* using a series of Ro primers. Amplification with these primers resulted in several products for each strain, allowing the construction of a schematic band pattern. Some of amplified products are typical for all strains, another for sibling species or for one strain only.

The Ro 460-01 primer produced band patterns for each of the 9 different strains and can be useful as a strain-specific marker for geographic differentiation. Additionally, one band was shared by all strains (551 bp); therefore it is considered as a marker for the entire species. Two bands were obtained for "continental" and Japanese strains considered as separate sibling species (855 bp and 815 bp). All of these traits deem the primer Ro 460-01 useful in RAPD analysis for species and strain identification in *P. jenningsi*.

The RAPD-PCR primer Ro 460-02 does not seem to give a specific band for the species, or a

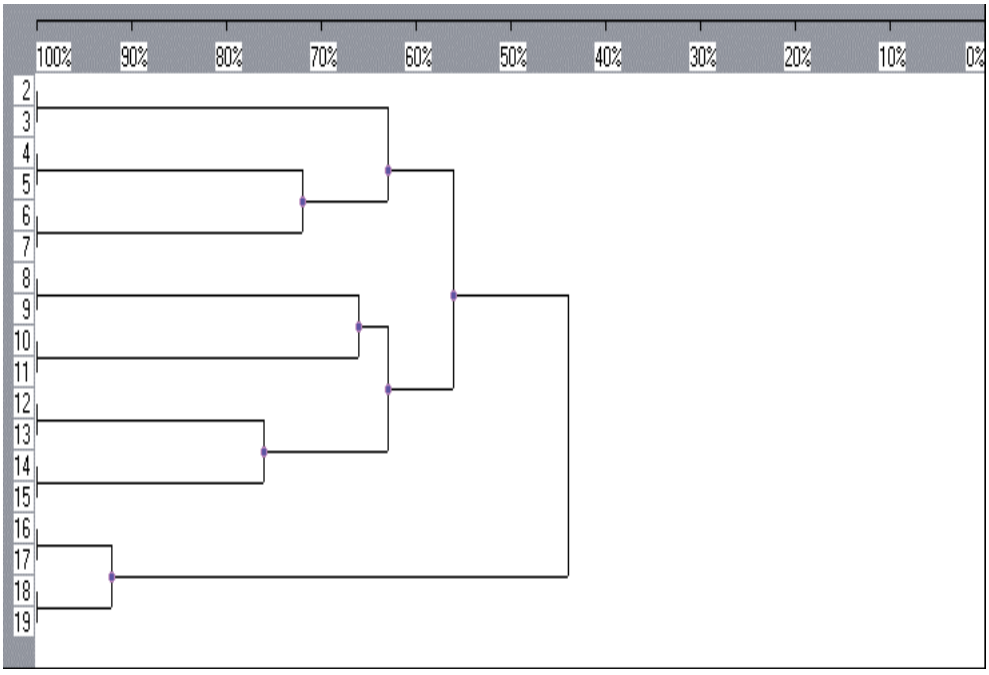

Fig. 6. Dendrogram representing phylogenetic relationships among strains of *P. jenningsi* with RAPD fingerprint data from the Ro 460-01, Ro 460-02, Ro 460-03, Ro 460-05, Ro 460-08 primers (2, 3 – Indian strain, 4, 5 – Saudi Arabian strain, 6, 7 – Chinese strain, 8, 9 – Japanese strain from Hagi, 10, 11 – Japanese strain from Yamaguchi, 12, 13 – Japanese strain from Ube, 14, 15 – Japanese strain from Nagato, 16, 17 – Japanese strain from Shinnamyou, 18, 19 – Japanese strain from Okinawa).

typical band for the Japanese and non-Japanese groups. Instead, markers were obtained for the continental strains and two groups of Japanese strains. This primer is complementary to at least several DNA sequences in each strain, allowing the construction of schematic band patterns showing variability between strains and is therefore considered useful in strain identification, for example in confirming questionable results.

Amplification with the RAPD-PCR primer Ro 460-03 resulted in two bands shared by all members of *P. jenningsi* and two types of schematic band patterns allowing for strain differentiation into a continental group (including 2 Japanese strains) and an assemblage consisting of the remaining Japanese strains.

Amplification with RAPD-PCR primer Ro 460-05 showed the presence of one band common to all the studied strains, therefore it can be used as a molecular marker for *P. jenningsi*. Strain specific bands suitable for use as strain markers were obtained for six strains: Arabian, Chinese and four Japanese strains (Hagi, Ube, Shinnamyou, Okinawa). RAPD profiles for primer Ro 460-05 permit the identification of *P. jenningsi* and to distinguish between two groups of strains. One of these is the Indian, Saudi Arabian, Chinese and part of the Japanese strains; the other comprises the remaining Japanese strains. Additionally, it can be used for inter-strain differentiation.

The band profiles obtained from RAPD-PCR fingerprinting with primer Ro 460-08 can be accepted as good general species markers because of the presence of two bands for *P. jenningsi*. Additionally, it is very useful as an inter-strain marker and for differentiating Japanese and continental strains. It can be used much like the profile for Ro 460-03. In this pair of primers, variation in band pattern for particular strains is considerably larger for Ro 460-03. These profiles are therefore better suited for strain identification than those from Ro 460-08. However, because of the distinct bands in Japanese and continental strains (679 bp and 833 bp) from primer Ro 460-08, it seems more appropriately applied to research requiring a general distinction between Japanese and non-Japanese strains.

The results presented in this and former papers (SKOTARCZAK *et al*. 2004) confirm that the construction of ten band patterns having marker attributes is possible on the basis of DNA amplification from 9 strains of *P. jenningsi* with the RAPD-PCR fingerprinting method using eleven primers from the Ro series. The patterns can be assigned to three marker-groups; a general species group, a group differentiating between sibling species, and accessory strain markers.

General species markers can be used in quick and precise identification of *P. jenningsi* in samples obtained from the natural environment. Band patterns obtained earlier from RAPR-PCR fingerprints include primers Ro 460-04 and Ro 460-10, and now Ro 460-01 and Ro 460-05, reveal single bands for the species. Additionally, excellent markers can be procured by amplification with primers Ro 460-03 and Ro 460-08, which produce several bands for *P. jenningsi*.

Markers differentiating between groups of strains obtained from RAPD-PCR fingerprints include primers Ro 460-04, Ro 460-01 and Ro 460-08. In these profiles specific bands were observed exclusively for continental strains and Japanese strains, making them helpful in the geographic differentiation of strains.

Accessory strain markers have patterns that do not allow for species or broad geographic determination and preclude their use in basic species identification. However, because of their distinct inter-strain variation, they can be used as accessory markers in confirmation studies.

This paper is the first to apply the aforementioned primers in *P. jenningsi* and consequently, because of the lack of published research concerning their use, a comparative analysis of results is impossible.

Many investigators point out the problems associated with repeatability of RAPD-PCR fingerprinting (SKROCH & NIENHUIS 1995; WOODBURN *et al.* 1995, after FOISSNER *et al*. 2001). Because of the variation in intensity of bands in parallel samples, interpretation of results is problematic and demands extensive experience, being prone to subjective judgment (FOISSNER *et al*. 2001). STOECK and SCHMIDT (1998) proved that the intensity of bands could change in subsequent experiments in relation to template DNA. Relatively weak bands may not show up at all in successive runs. This is why reliable results required the analysis of several isolates of each strain of *P. jenningsi* in multiple replicates.

Phylogenetic analysis of the 9 strains of *P. jenningsi* based on RAPD-PCR fingerprints permit genetic similarity assessment and the construction of dendrograms reflecting phylogenetic relationships in this species. Four primers (Ro 460-04, Ro 460-06, Ro 460-07, Ro 460-10) distinguished between two groups of strains: the first combining strains from India, Saudi Arabia and China; the second was reserved to the Japanese strains. For four constructed dendrograms we found an identical, hierarchical arrangement of subgroups and lineages within the first group when the abovementioned primers were applied (unpublished data). The genetic similarity of the Indian and Ara-

bian strains was confirmed, whereas the Chinese strain forms an independent branch in this species (SKOTARCZAK *et al*. 2004).

These results are consistent with former phylogenetic analyses on the basis of RAPD-PCR fingerprinting with primer Ro 460-04 (PRZYBOŚ *et al.* 2003), where two syngens (cryptic species) were also found, consisting of the continental and Japanese strains. The Indian and Saudi Arabian strains were most similar and the Chinese strain was isolated within the continental species (SKOTARCZAK *et al*. 2004).

However, phylogenetic relationships within the second sibling species differ. In dendrograms constructed on the basis of each primer (Ro 460-04, Ro 460-06, Ro 460-07, Ro 460-10) a different arrangement of subgroups and lineages was observed. Also, considerable variation in values of genetic similarity was detected. This necessitates a detailed analysis among the Japanese strains of *P. jenningsi* in order to clarify their phylogenetic relationships and further studies with 6 other primers can be expected to verify these results.

The dendrogram constructed on the basis of results from 5 Ro primers (460-10, 02, 03, 05, 08) revealed the existence of two groups of strains: the first with continental strains from India, Saudi Arabia, Chaina, and second with Japanese strains, excluding the strains from Shinnamyou and Okinawa, which formed a second independent branch.

In spite of this uncertainty, we acknowledge the presence of two sibling species in *P. jenningsi.* Taking into account the fact that the values of the similarity indexes are not absolute but rather relative – they change depending on the applied primer (CHAPCO *et al.* 1992; PRZYBOŚ *et al*. 1999) – they cannot accurately describe the phylogenetic structure of this species. LANDRY and LAPOINTE (1996) concluded that at least 12 primers were needed for appropriate phylogenetic reconstruction. Still, the arrangement of groups and subgroups established on the basis of the similarity matrices is relatively constant and probably reflects actual phylogenetic relationships in *P. jenningsi*.

Our results are congruent with inter-strain crossing experiments in which reproductive isolation was found when continental and Japanese strains were tested (PRZYBOŚ *et al.* 2003). This is further evidence for the existence of two sibling species, whose reproductive isolation is genetically induced.

The primers Ro (460-01, Ro 460-02, Ro 460-03, Ro 460-05, Ro 460-08) also distinguish between two groups of strains, although they divide the Japanese strains into two subgroups that are not reproductively isolated. This probably indicates genetic variation within this species. However, it comprises one common gene pool (successful inter-strain crosses) and is reproductively isolated from the other species.

The genetic structure revealed by RAPD-PCR fingerprinting is closely linked to the life strategy of the species and its evolutionary history.

Genetic variation between continental and Japanese strains of *P. jenningsi*, reflected by the presence of two sibling species, is therefore a result of the geographic dispersal of this species.

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