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Tandem arrangement of tRNA^{Asp}-encoding genes in *Phytophthora* spp.

(Oomycetes; RNA polymerase III; internal control region; tRNA structure; fungus; repetitive DNA)

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SUMMARY

We have cloned a region of repetitive DNA from the phytopathogenic fungus, *Phytophthora parasitica*. The cloned region consists of 17 highly homologous units arranged in tandem. The consensus sequence is 562 bp long and carries the information for a tRNA^{Asp}. All sequence motifs required for efficient RNA polymerase III transcription are present, and the tRNA derived from the nucleotide sequence is able to form a complete cloverleaf structure with high homology to previously characterized tRNA^{Asp} molecules. The isolated tRNA^{Asp} gene cluster is located at a distance of 20 kb from the *TRP1* gene of *P. parasitica*. It comprises about 0.1% of the total genomic DNA. Similar clusters were detected in four other *Phytophthora* species.

INTRODUCTION

In the translation system of every living organism tRNA plays a crucial role as adapter between mRNA and aa. In eukaryotes the genes encoding the tRNA molecules share special features: they are transcribed by PolIII (Sentenac, 1985) and thus contain an internal split promoter (Galli et al., 1981). Transcription is terminated by one or more short clusters of T residues in the 3′ flanking region (Geiduschek and Tocchini-Valentini, 1988). Unlike their prokaryotic equivalents, eukaryotic tRNA genes do not encode the 3′-CCA terminus of mature tRNA (Clarkson, 1983). With a few exceptions 5′ flanking regions of eukaryotic tRNA genes share little homology and common sequence motifs could not be identified. Nevertheless, several in vitro experiments (reviewed in Geiduschek and Tocchini-Valentini, 1988) have demonstrated an influence of 5′ flanking regions on PolIII transcription.

To meet temporary high demands for tRNA molecules generally more than one gene for any given tRNA species is present in the genome. In eukaryotic organisms these identical genes are widely dispersed throughout the genome, either solitary or in heteroclusters together with other tRNA genes (Clarkson, 1983; Sharp et al., 1985). The rare exceptions from this kind of organisation were exclusively found in higher eukaryotes. In *Xenopus laevis* for instance a heterocluster containing eight tRNA genes is tandemly amplified (Fostel et al., 1984) and in *Bombyx mori* about 20 copies of a silk-gland-specific tRNA^{Ala}-encoding gene form a homocluster (Underwood et al., 1988). In lower eukaryotes tandem arrangement of tRNA genes or gene clusters has not yet been observed.

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Abbreviations: aa, amino acid(s); bp, base pair(s); dNTP, deoxyribonucleoside triphosphate; InGP, indole-3-glycerolphosphate; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); *P.*, *Phytophthora*; PEG, polyethylene glycol; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; PolIII, RNA polymerase III; PRA, phosphoribosyl-anthranilate; pTrp1 and pTrp2, cosmids carrying the repetitive tRNA gene region; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na_3·citrate pH 7.6; TAE, 40 mM Tris·acetate pH 8.0/2 mM EDTA; TE, 10 mM Tris·HCl pH 7.4/1 mM EDTA; tRNA, transfer RNA; *TRP1*, gene encoding InGP synthase and PRA isomerase.

In the present study we provide evidence that a repetitive DNA region isolated from the plant pathogenic fungus *P. parasitica* represents a homocluster of tandemly arranged tRNA^{Asp}-encoding genes. As far as we know, this is the first example for the characterization of tRNA genes from an oomycetous fungus.

RESULTS AND DISCUSSION

(a) Identification of cosmids pTrp1 and pTrp2

A genomic library of *P. parasitica* DSM1829 was constructed in the cosmid vector pHC79 (Hohn and Collins, 1980) and maintained in *E. coli* DH1 (Hanahan, 1983). Plasmid DNA prepared from this library was used to transform the *E. coli* strain W3110*trpC*1117 (Yanofsky et al., 1971), which is deficient in PRA-isomerase. Two different cosmids (pTrp1 and pTrp2) could be identified by their ability to complement the *trpC*1117 mutation. Since W3110*trpC*1117 is not *recA*$^-$, these two plasmids were picked up from their corresponding clones of the DH1 library. These clones were detected by hybridization with the isolated *TRP1* gene (P.K., in preparation) as probe. Restriction analysis of both cosmids (Fig. 1) revealed the presence of a 10-kb repetitive DNA region, about 20 kb away from the *TRP1* gene.

(b) Restriction mapping of the repetitive DNA region

Restriction mapping (Fig. 2) reveals that the 10-kb repetitive DNA region consists of 17 tandemly reiterated units, each about 560 bp long. Although there is no significant deviation in repeat unit length, some units are slightly different on the restriction enzyme level, i.e., some sites for *Hin*dIII or *Eco*RV are missing. A loss of these sites during plasmid propagation in *E. coli* can be ruled out (*i*) by the fact that they are also absent in pTrp2 (data not shown) and (*ii*) by data from Southern (1975) hybridization with genomic DNA (see section **f**). The restriction data further suggest that the repetitive region was not cloned completely, i.e., it may be continued at its left side within the *P. parasitica* genome.

(c) Consensus nt sequence of the repetitive units

The sequence obtained is shown in Fig. 3. The fact that sequencing all the different monomeric units together (Fig. 3 legend) resulted in a clearly interpretable banding pattern with hardly any background (data not shown), indicates that there is a strong similarity between these units and that divergences (Fig. 2B) are presumably restricted to a few single nt exchanges.

A salient feature of the presented sequence is a putative coding region for tRNA^{Asp}. Other tRNA species or 5S rRNA are not encoded.

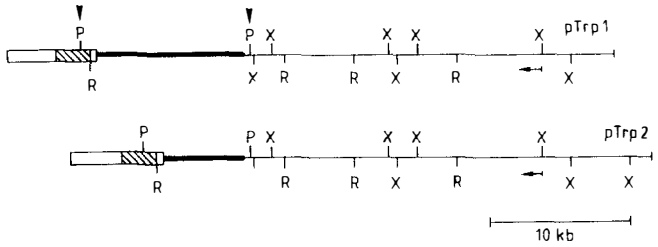

Fig. 1. Restriction maps of cosmids pTrp1 and pTrp2, shown in linearized form. **Symbols**: open boxes, vector DNA; hatched boxes, regions with 100% homology to pUC19 (Yanisch-Perron et al., 1985); thin lines, *P. parasitica* DNA; heavy lines, repetitive part of *P. parasitica*. P, *Pst*I, R, *Eco*RI and X, *Xho*I. Only two (of the frequently occurring) *Pst*I sites are presented, but between these two sites no further *Pst*I recognition sequences are found. In pTrp1, the *Pst*I sites (labeled by arrowheads) enclose a 12.1-kb fragment containing 10 kb of repetitive DNA. This *Pst*I fragment has been characterized in detail (see sections **b** and **c**). The horizontal arrows indicate the coding sequence for the *TRP1* gene, 20 kb distant from the repetitive region. **Methods**. The cosmid library, from which pTrp1 and pTrp2 were isolated, was constructed as follows: protoplasts of *P. parasitica*, generated by the method of Jahnke et al. (1987), were lysed with 1 vol. phenol, containing 2% SDS. The high-M_r DNA in the supernatant was precipitated twice with 1 vol. 98% ethanol and finally resolved in TE buffer. RNA was not removed. Following partial digestion with *Sau*3A, molecules with a length of 30–50 kb were purified from a 10–40% sucrose gradient (Maniatis et al., 1982) and cloned into the *Sal*I site of cosmid pHC79 (Hohn and Collins, 1980). The protruding ends of vector and insert were made compatible by partial filling in with the appropriate nucleotides (Zabarovsky and Allikmets, 1986). To promote the formation of high-M_r vector-insert concatemers the ligation was accomplished in the presence of 15% PEG (Pheiffer and Zimmerman, 1983). Preparation of the packaging extracts from *E. coli* SMR10 (Rosenberg et al., 1985) and the in vitro packaging procedure were performed as described (Rosenberg et al., 1985). Infection of *E. coli* DH1 (Hanahan, 1983) followed the protocols of Maniatis et al. (1982). The recombinant clones obtained were stored separately in 17% glycerin at −70°C.

The tRNA^{Asp} region exhibits high homology to tRNA^{Asp} from *X. laevis* (Haumont et al., 1984) and the tRNA^{Asp}-encoding gene from rat (Rosen et al., 1984) whereas similarities to fungal, plastid and plant tRNA^{Asp}-encoding genes (listed in Sprinzl et al., 1989) are lower. The *P. parasitica* tRNA^{Asp}-encoding gene contains neither an intron nor the information for the 3′-end CCA of mature tRNA. The hypothetical tRNA derived from the coding region is able to form an intact cloverleaf structure (Fig. 4) with all invariable or semi-invariable nt present. Sequence deviations from rat or *Xenopus* tRNA^{Asp}, for instance, only occur at variable positions in the tRNA structure (Fig. 4). An interesting peculiarity is the presence of a T^2-A^{71} bp in the aa acceptor stem. Surprisingly, this bp seems not to occur at that position in any other tRNA^{Asp} so far characterized.

The putative tRNA^{Asp}-encoding gene contains the typical PolIII promoter (Galli et al., 1981), which is split into two internal control regions called A- and B-block (Fig. 3). In the 3′ flanking region a signal for PolIII transcription

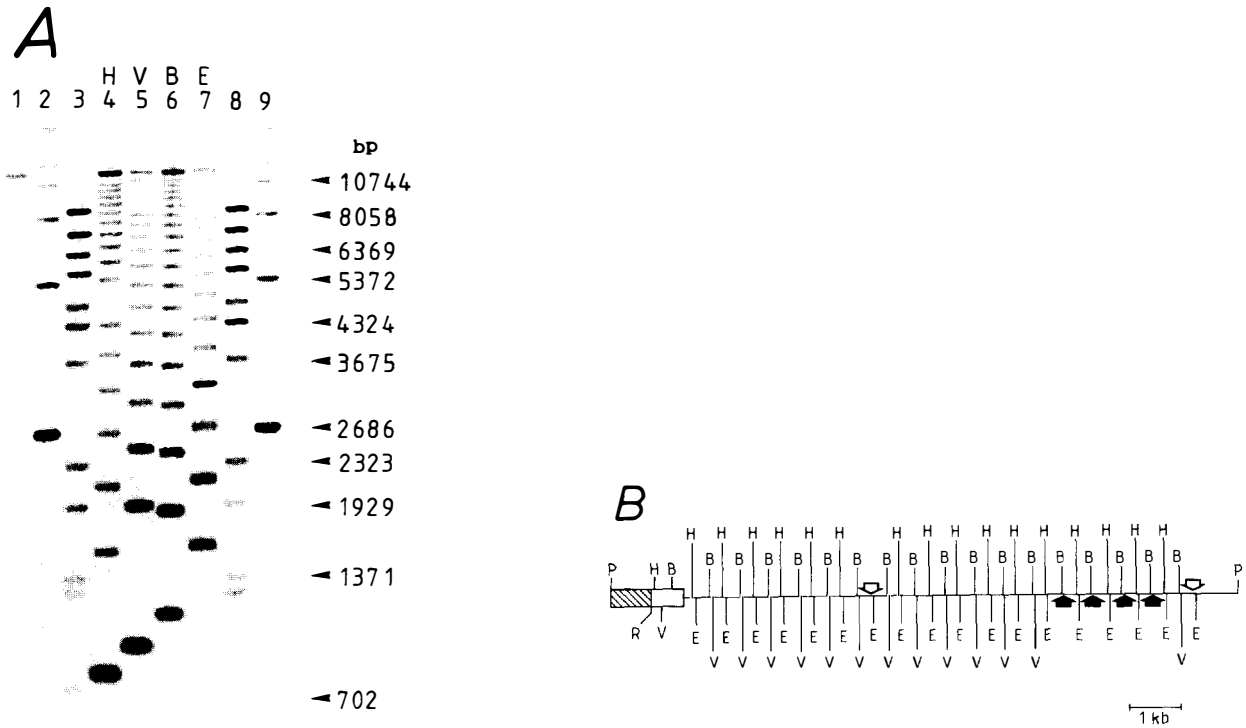

Fig. 2. Direct restriction mapping (Smith and Birnstiel, 1976) of the 12.1-kb *Pst*I fragment from pTrp1 (see Fig. 1).**Methods**. The fragment was purified from 0.6% agarose gel as follows: the excised gel matrix containing the DNA was solubilized by addition of 2.5 times the gel volume (determined by weight) of 7 M NaI/0.1 M Na_2SO_3 (Vogelstein and Gillespie, 1979). Then PEG4000 (Serva) and NaCl were added to a final concentration of 7% and 0.5 M, respectively. Following incubation overnight at 37°C, the DNA was sedimented by centrifugation for 10 min at 13 000 rpm in an eppendorf centrifuge. After washing twice with 70% ethanol, the DNA pellet was dried and resuspended in TE-buffer. (Panel **A**) The 12.1-kb *Pst*I fragment (lane **1**) was partially digested with H (*Hin*dIII), V (*Eco*RV), B (*Bam*HI) and E (*Bst*EII) (lanes **4–7**, respectively). Following electrophoresis in 0.8% agarose gel and Southern blotting with the VacuGene blotting system (Pharmacia/LKB), the DNA was hybridized with biotin-labeled pUC19 DNA. The hybridization procedure followed the protocols of Anderson and Young (1985). The 'stringency wash' was carried out in 2 × SSC/0.1% SDS for 30 min at 65°C. The bands were made visible with the BLUEGENE kit (BRL). Since pUC19 labels only one end of the 12.1-kb fragment, only those partial digestion products become visible that contain this end. Consequently the band pattern reveals the ordered restiction map. Size markers are pUC19 ladder (lanes **2+9**) and phage λ/*Bst*EII DNA (lanes **3+8**). The latter was made visible by mixing the pUC19 probe with biotin-labeled phage λ DNA. Length (in bp) of some size marker fragments is displayed on the right margin. (Part **B**) Restriction map of the 12.1-kb fragment. P, *Pst*I; R, *Eco*RI; B,E,H,V as in panel **A**. Other symbols like in Fig. 1. Open arrows indicate missing *Hin*dIII sites, blackened arrows missing *Eco*RV sites.

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   BamHI
 1 GGATCCGCCA AGACGTTAGT AAAGGCGCCG AAGTGGGGTG GCGGAGCTGT

                                                  AvaII
51 AGGTATGGGA AATCGCCGCT GCACGAGACG GGCGCAAGTG GGGTCCACGA

101 TGGGTTTGTG TGTACATGAC AGTGGTGAGG GTGCTGTAGA GGGTTTGTGG

151 TAGCAGGCTG AGGCGATGTA GCGTGCGTGC GATGCGTGAT TTGGGGAGTA

201 TGTGTGTATT TGTTTTGAAA TTTTGTGTGG CGTGTGAGTT GTCGTACAGA

                                                  BstEII
251 TTCTCGTTAG TATAGTGGTT AGTATACCCG CCTGTCACGC GGGTGACCCG
          A                        Anticodon
                  HindIII
301 GGTTCAATTC CCGGACGGGA AGCTTCTTTT GCTCTTTCAC TCTTTCATGC
      B                      TS       TS       TS

351 CACCCTTCGT TTTGGGCCCC ACGCTGCCTT CACATACACC GCCGCAGCAC
          TS
                          SalI                SalI
401 GTAAACCACG CGCACGTAGC GTGTCGACCG CCTCCGTCGA CGAAGGCCGA

451 GAAGAGGCGA GCGTTTCGTT GGAACTCGAT CCCAGGCCAT CGGTCTCTGT

        EcoRV
501 TGGCAATTGA TATCGTCGCC AGATGCAACG GATGGCGTCT TGACCGGATC

                BamHI
551 GGCGCTCAAG GTggatcc
                 |
               562
```

Fig. 3. Consensus nt sequence of the 562-bp *P. parasitica* repeat. Monomeric repeat units were released from the 10-kb repetitive region of pTrp1 by complete digestion with *Ava*II or *Hin*dIII+*Sal*I and then purified from other restriction products by agarose gel (0.8%) elution with the Gene-Clean Kit (Bio 101). The *Ava*II as well as the *Hin*dIII-*Sal*I fragments were sequenced from both ends, following the protocol of Maxam and Gilbert (1980). Since we did not separate the different monomers from each other, the resulting nt sequence represents a consensus sequence of all units. **Symbols**: upper-case letters, the 562-bp consensus nt sequence; lower-case letters, beginning of the adjacent repeat unit; boxed, putative coding region for $tRNA^{Asp}$; A and B below underlining, internal control regions of the PolIII promoter; TS below underlining, sequences possibly involved in transcription termination; underlined with dots, putative inhibitory purine-pyrimidine tract (see section **c**). The GenBank accession number for this sequence is M60656.

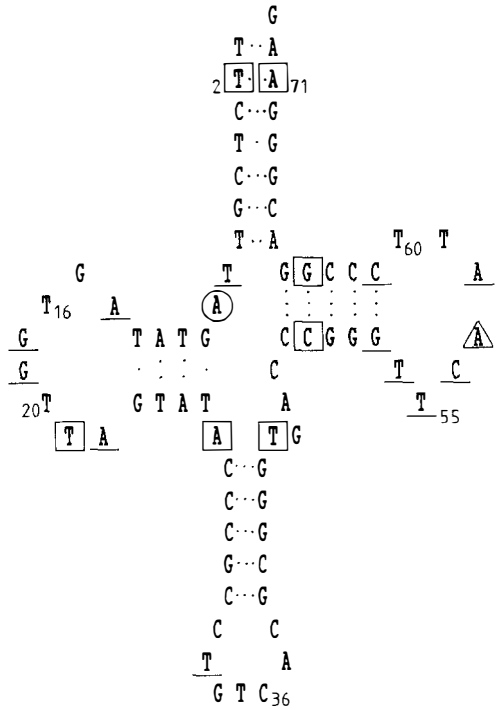

Fig. 4. Sequence and clover-leaf structure of *P. parasitica* $tRNA^{Asp}$. The numbering system is that of yeast $tRNA^{Phe}$. Underlined, nt which are invariant in all tRNAs of all species so far analysed; boxed, different nt both in *X. laevis* and in rat $tRNA^{Asp}$; circled, different nt in *X. laevis*, only; triangle, different nt in rat only.

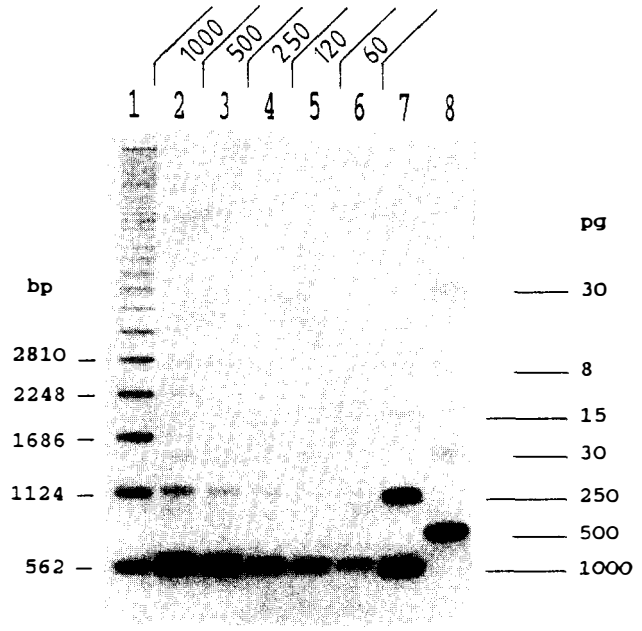

Fig. 5. Quantitation of the 562-bp repeat unit in the *P. parasitica* genome. Different amounts of genomic DNA (values in 60–1000 ng at the top) were digested to completion with *Hin*dIII and separated on 0.8% agarose gel (lanes **2–6**), parallel with known amounts (in pg; see values on the right margin) of purified repeat monomers (*Sal*I fragments; lanes **7+8**). Several portions of repeat DNA were loaded to lanes **7+8** with temporal distance, i.e., each of these lanes contains more than one sample of the same DNA. Size marker (lane **1**; fragment length in bp to the left) is a ladder of the repeat element created by partial *Sal*I digestion of the repetitive DNA region. Following Southern blotting (as described in Fig. 2), the DNAs were hybridized with biotin labeled 12.1-kb *Pst*I fragment, containing the 10-kb repetitive DNA region (see Fig. 2B). Biotin labeling was done by nick-translation; probe concentration was 150 ng/ml. Stringency conditions were 2 × SSC/0.1% SDS at 65 °C. By comparison, the signal intensity of the lowest band in lane **2** (1000 ng genomic DNA) is equal to the 1000 pg band in lane **7**. Likewise, the lowest bands in lanes **3** (500 ng genomic DNA) and **4** (250 ng genomic DNA) are equal to the 500-pg band in lane **8** and the 250-pg band in lane **7**, respectively. Thus, the fraction of the repetitive DNA element in the genomic DNA can be estimated at 0.1%.

termination is present. An interesting feature of the 5′ flanking region is the occurrence of two tracts of alternating purines and pyrimidines (Fig. 3). Similar purine-pyrimidine stretches have previously been identified as transcription inhibitory signals in front of a variant $tRNA^{Met}$-encoding gene of *X. laevis* (Hipskind and Clarkson, 1983).

(d) Quantitation of the tRNA-encoding gene repeat in the genome

The portion of 562-bp repeat in the genomic DNA was estimated by Southern-blot hybridization of known amounts of purified monomeric units and chromosomal DNA. Comparison of the hybridization signals (Fig. 5) shows that the repeated DNA comprises about 0.1% of the total genomic DNA. Up to now no data concerning the size of the *P. parasitica* genome have been published. In fact it seems that only one evaluation of the genome size of a *Phytophthora* species (*P. megasperma* f.sp. *glycinea*) has appeared in the literature (Rutherford and Ward, 1985). The authors report a value of 20.7×10^{-11} mg DNA per diploid nucleus, which corresponds to 4.5×10^7 bp/haploid genome, about three times more than for the *Saccharomyces cerevisiae* genome. If the genome of *P. parasitica* is supposed to have a similar size, the copy number of the 562-bp repetitive element will be about 80 per haploid genome.

(e) Analogues of the 562-bp tRNA gene repeat in other Oomycetes

DNA-DNA hybridization reveals that in at least four other *Phytophthora* species, *P. iranica*, *P. infestans* (Fig. 6) and *P. cactorum*, *P. megasperma* (data not shown) a repetitive DNA element is present with nt sequence, copy number and repeat unit length similar to the 562-bp tRNA-encoding gene repeat of *P. parasitica*. The occurrence of oligomeric repeat molecules in at least one digestion from each *Phytophthora* species indicates that in all these organisms the repeat is arranged in tandem.

On the restriction enzyme level the repeat analogues

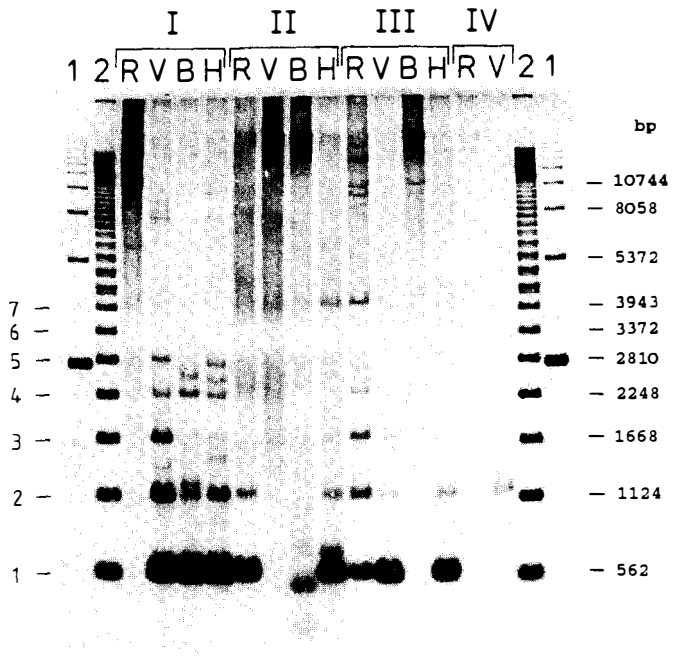

Fig. 6. Repeat analogues of 562 bp in other *Phytophthora* species. Total genomic DNA from I, *P. parasitica*; II, *P. infestans*; III, *P. iranica* and IV, *Pythium arrhenomanes* was digested to completion with R, *Eco*RI; V, *Eco*RV; B, *Bam*HI and H, *Hin*dIII. Following electrophoresis in 0.8% agarose (3 μg DNA/lane) and Southern transfer the DNA was hybridized with a biotin-labeled 12.1-kb *Pst*I fragment. Probe, probe concentration and stringency conditions are described in Fig. 5. Size markers (fragment length in bp on the right margin) are pUC19 ladder (lanes **1**) and 562-bp repeat ladder (lanes **2**; see also Fig. 5); Numbers on the left margin indicate degree of repetition (1 = monomer, 2 = dimer, etc.).

show significant diversification, i.e., they possess a different number of sites for a certain enzyme. Only the *Hin*dIII site occurs in the repeat analogues of all species tested. This may reflect the fact that this enzyme cuts within the putative coding region for tRNA^{Asp} (Fig. 3).

The *Hin*dIII-digested *P. parasitica* DNA shows a strong dimer band (Fig. 6, lanes I/H). Since the hardly visible trimer band suggests nearly complete restriction, the strong dimer signal is thought to result from the absence of *Hin*dIII sites in some repeat units. This finding, which is consistent with the restriction data presented in Fig. 2, indicates that some units within the tRNA^{Asp}-encoding gene cluster may be inactive, i.e., may not produce functional tRNA^{Asp} molecules.

A hybridization signal also occurs within the genomic DNA of *Pythium*, another member of the Oomycetes. According to the intensity of the signal the tRNA^{Asp}-encoding gene is present in only one or a few copies in *Pythium*. The presence of a tRNA^{Asp}-encoding gene cluster in five *Phytophthora* species and of a single- or low-copy tRNA^{Asp}-encoding gene in *Pythium* indicates that the amplification of the tRNA^{Asp} unit occurred after the diversification of *Phytophthora* and *Pythium* lineages but before speciation within *Phytophthora*. Thus, the amplification of tRNA^{Asp}-encoding genes could provide a useful reference point in the phylogeny of the Oomycetes. Hybridization with *Phytophthora* species more distant to *P. parasitica* (e.g., marine species) would be especially interesting in this respect.

(f) Conclusions

(1) Based on the facts that the obtained sequence (*i*) represents the consensus of 17 adjacent units and (*ii*) contains information for only one tRNA species (tRNA^{Asp}) we assume to have cloned a homocluster of tRNA^{Asp} genes.

(2) The *P. parasitica* tRNA^{Asp}-encoding genes described here are rather true structural genes than pseudogenes. Evidences for this are (*i*) the ability of the derived tRNA to form a complete cloverleaf structure, (*ii*) the high homology to previously characterized tRNA^{Asp}-encoding genes and (*iii*) the presence of all sequence motifs required for PolIII transcription. Nevertheless, direct experimental proof for biological function of the presently isolated tRNA^{Asp}-encoding genes will have to be provided in the future.

(3) Using a published genome size of *P. megasperma*, the copy number of the tRNA^{Asp}-encoding gene was estimated at about 80 per haploid genome. Since only 17 copies have been characterized so far the genomic arrangement of the remaining tRNA^{Asp}-encoding genes is still unclear.

(4) Similar tRNA^{Asp} clusters are present in five *Phytophthora* species but only a single- (or low-) copy gene for tRNA^{Asp} was detected in the Oomycete *Pythium arrhenomanes*.

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