GENETIC DIVERSITY ANALYSIS OF *Pseudomonas cichorii* ISOLATES FROM BRAZIL

Suzete A. L. Destéfano[1]
Irene M. G. Almeida[1]
Júlio Rodrigues Neto[1]

ABSTRACT

Thirty-five strains of *Pseudomonas cichorii* isolated from different hosts and geographic regions were investigated. The amplification of the 16S-23S spacer region rDNA yielded a band of approximately 1,000 base pairs for all strains tested. PCR products were digested, individually, with *Afa* I, *Dde* I and *Taq* I and the results showed that *P. cichorii* is a highly heterogeneous species. The DNA profiles were combined and analyzed by a similarity dendrogram, calculated with the algorithm UPGMA. Strains isolated from different hosts and geographical regions showed identical profiles whereas strains isolated from the same hosts and geographical regions showed distinct patterns. The amplification of the 16S rDNA gene confirms that all isolates belong to *Pseudomonas* genus but more studies should be obtained to clarify the low level of similarity of the 16S-23S spacer region rDNA detected among them.

Key words: 16S-23S spacer region rDNA, bacterial diseases.

[1]Instituto Biológico, Caixa Postal 70, CEP 13001-970, Campinas, SP, Brazil. e-mail: suzete@biologico.br

RESUMO

ANÁLISE DA DIVERSIDADE GENÉTICA DE ISOLADOS DE *Pseudomonas cichorii* DO BRASIL

Trinta e cinco linhagens de *Pseudomonas cichorii*, isoladas de diferentes hospedeiros e regiões geográficas, foram investigados. A

amplificação da região espaçadora 16S-23S DNAr resultou num fragmento único de aproximadamente 1.000 pares de bases para todas as linhagens testadas. Os produtos de amplificação foram digeridos com as enzimas de restrição *Afa* I, *Dde* I e *Taq* I e os resultados mostraram que *P. cichorii* é uma espécie altamente heterogênea. Os dados de digestão foram combinados e analisados através da construção de dendrograma de similaridade, obtido com o uso do algorítmo UPGMA. Nesta análise verificou-se que linhagens isoladas de diferentes hospedeiros e regiões geográficas apresentaram perfis idênticos, enquanto que linhagens isoladas de mesmo hospedeiro e região geográfica apresentaram perfis distintos. A amplificação do gene 16S DNA ribossomal sugere que todos os isolados pertencem ao gênero *Pseudomonas*, porém os baixos valores de similaridade da região espaçadora entre determinados isolados indicam que mais estudos deverão ser efetuados para esclarecer os resultados obtidos.

Palavras-chave: região espaçadora 16S-23S DNAr, doenças bacterianas.

INTRODUCTION

Pseudomonas cichorii (Swingle 1925) Stapp 1928 was first identified as a pathogen of *Cichorium intybus* L. and has become an increasingly important problem. This bacterium is one of the most widely distributed in the world and infects a very wide range of plants. More than 45 natural host species have been reported by several authors (Bradbury, 1986; Young *et al.*, 1987; Holcomb & Cox, 1998; Putnam, 1999; Pernezny & Raid, 2001; Obradovic & Arsenijevic, 2002). However, there are no reports comparing the strains of this species at genetic diversity level.

In Brazil, *P. cichorii* was identified causing serious damage in several crops such as common chicory (*Cichorium intybus* L.), cauliflower (*Brassica oleracea* var. *botrytis*), cabbage (*Brassica oleracea* var. *capitata*), lettuce (*Lactuta sativa*), egg-plant (*Solanum melongena*), sugar-beet (*Beta vulgaris* var. *cicla*), muskmelon (*Cucumis melo*), tobacco (*Nicotiana tabacum*), coffee (*Coffea arabica*) and many ornamentals like chrysanthemum (*Chrysanthemum morifolium*), sunflower (*Helianthus annuus*), gerbera (*Gerbera jamesonii*), philodendron (*Philodendron* sp.) and others (Bergey & Buchanan Nota

2001).

In spite of the variability in pathogenicity, all strains belong to the same bacterial species and therefore, the genetic and pathogenic relationships amongst pathotypes remain unknown.

Molecular approaches are being used increasingly as a tool to clarify the taxonomic positions of several groups of microorganisms. Nucleotide sequences of 16S-23S rDNA spacer regions represent genetic marker for assessing the phylogenetic relationships between closely related organisms (Gürtler & Stanisich, 1996) and has been used as a target for PCR-based identification and the typing of many bacteria by fragment length profiling, restriction fragment length profiling or DNA sequencing (Seal *et al.*, 1990; Honeycut *et al.*, 1995; Gürtler & Stanisich, 1996; Maes *et al.*, 1996; Mendoza *et al.*, 1998; Riffard *et al.*, 1998). In addition, the differentiation at infra-specific level of *Pseudomonas syringae* strains was performed due to the high variability amongst 16S-23S spacer regions (Manceau & Horvais, 1997).

In this study, the spacer region between 16S and 23S rDNA were used in order to verify the genetic diversity of populations of *P. cichorii* strains isolated from different geographic regions and hosts in Brazil, and to analyze the relationships between the strains.

MATERIALS AND METHODS

Thirty five strains of *Pseudomonas cichorii* isolated from different hosts and geographic regions, mainly of the State of São Paulo were used in this study (Table 1). Strains of *P. fluorescens* (IBSBF 532, 539), *P. agarici* (IBSBF 1458) and *P. toolasi* (IBSBF 1459) where also included for comparative purposes. All strains are deposited in the IBSBF Culture Collection of the Laboratório de Bacteriologia Vegetal of Instituto Biológico, Campinas, SP, Brazil. The strains were grown on NA medium (Levine, 1954) at 28°C for 24 h.

Genomic DNA was extracted as described by Pitcher *et al.* (1989) and the concentration determined by comparison with lambda bacteriophage (λ) DNA in 0.6% ethidium bromide-stained agarose gel. PCR was performed using ~ 100 ng of genomic DNA in 25 µl reaction solutions containing 2.0 U *Taq* polymerase (Amersham-Pharmacia

Tabela 1. Bacterial strains of *Pseudomonas cichorii* used in this study.

Strain IBSBF*	Host	Origin
0233	*Nicotiana tabacum*	Unknown
0234	*Lactuca sativa*	Suzano - SP
0258	*Solanum melongena*	Miguel Pereira, RJ
0262	*Chrysanthemum morifolium*	Piedade - SP
0402	*Solanum melongena*	Andradas - MG
0403	*Solanum melongena*	Andradas - MG
0508	*Gerbera jamesonii*	Holambra - SP
0521	*Abelmoschus esculentus*	Botucatu - SP
0587	*Coffea arabica*	Machado, MG
0951	*Apium graveolens*	Campinas - SP
1013	*Raphanus sativus*	Cascavel - PR
1021	*Lactuca sativa*	Mogi das Cruzes - SP
1053	*Gerbera jamesonii*	Holambra - SP
1059	*Brassica oleracea* var. *botrytis*	Holambra - SP
1060	*Brassica oleracea* var. *acephala*	Mogi das Cruzes -SP
1061	*Cichorium endivia*	Mogi das Cruzes - SP
1069	*Gerbera jamesonii*	Holambra - SP
1070	*Gerbera jamesonii*	Holambra - SP
1084	*Helianthus annuus*	Holambra - SP
1094	*Brassica chinensis*	Mogi das Cruzes - SP
1110	*Syngonium podophyllum*	Holambra - SP
1114	*Menta arvensis*	Campinas - SP
1175	*Murraya exótica*	Holambra - SP
1209	*Chrysanthemum morifolium*	Holambra - SP
1235	*Lactuca sativa*	Holambra - SP
1247	*Cichorium endivia*	Monte Alegre do Sul - SP
1262	*Philodendron* sp.	Holambra - SP
1263	*Philodendron* sp.	Holambra - SP
1274	*Cucumis melo*	Sorocaba - SP
1297	*Arracacia xanthorrizza*	Mogi das Cruzes - SP
1414	*Lactuca sativa*	Santa Isabel, SP
1524	*Ocimum basilicum*	Campinas - SP
1525	*Ipomoea* sp.	Holambra - SP
1526	*Lactuca sativa*	São Lourenço da Serra - SP
1527	*Lactuca sativa*	Embu-Guaçu - SP

* IBSBF: Culture Collection of Laboratório de Bacteriologia Vegetal, Instituto Vegetal, Instituto Biológico, Campinas, SP, Brazil.

Biotech), 1X *Taq* buffer, 200 μM dNTPs mixture and 0.4 μM of each primer. The 16S DNAr amplifications were carried out by using an initial denaturation step at 95°C for 2 min; followed of 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 3 min; and a final extension period at 72°C for 3 min. The 16S-23S rDNA spacer regions amplification, by an initial denaturation step at 95°C for 2 min; followed by 25 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 3 min; and a final extension period at 72°C for 5 min, in a thermocycler (GeneAmp PCR system 9700; Perkin-Elmer Corporation, Norwalk, Conn). The 16S rDNA were amplified using the primers 27f and 1525r (LANE, 1991) and the 16S-23S spacer region, pHr (Massol-Deya *et al.*, 1995) and p23Suni322-anti (Honeycut *et al.*, 1995).

PCR products (5 μl) of the 16S rDNA were digested individually with each of the following restriction endonucleases *Afa* I, *Dde* I, *Hae* III, *Hinf* I and the products of the 16S-23S spacer region with *Afa* I, *Dde* I, and *Taq* I under conditions specified by the manufacturer (Amersham Biosciences). The restriction fragments were separated by electrophoresis in 3% agarose gels in 1X TAE buffer (40 mM Tris-acetate/1 mM EDTA). The gels were stained with 0.1 μg/mL of ethidium bromide and then photographed under ultraviolet transillumination using an Alpha Innotech 2200 digital system. The molecular weights of the fragments were determined by comparison with a 100 base pairs (bp) DNA ladder (Amersham Biosciences).

The DNA profiles obtained by using RFLP-16S-23S rDNA spacer regions were analyzed in a binary form. Bands were scored as present (1) or absent (0) for each strain. Molecular weights below 100 bp were not considered in the analysis. Similarity matrices were constructed by using the program similarity for qualitative data (SIMQUAL), with the Dice coefficient. A dendrogram was constructed by using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm with the NTSYS-PC program (Rolf, 1992). Cluster analysis was performed on the data generated by the PCR-RFLP analysis.

RESULTS AND DISCUSSION

The amplification of the spacer region yielded a unique band of

approximately 1,100 base pairs (bp) for all the thirty nine strains tested. The strains were grouped according to the hosts and the PCR products were digested with the restriction enzymes *Afa* I, *Dde* I, and *Taq* I. The data obtained with *Afa* I and *Taq* I are shown in the figures 1A, B and 2A, B. The fragments with weights ranging from 100 to 1,100 bp were considered to analysis.

The digestions with *Afa* I, *Dde* I, and *Taq* I generated fragments from approximately 100 to 800, 100 to 300 bp, and from 100 to 700 bp, respectively. The data obtained from the RFLP of the 16S-23S spacer regions were combined for cluster analysis and a total of 31 bands were scored. Using the algorithm UPGMA cluster analysis, several groups were identified (Figure 3). The banding profile expected should be very similar among the strains since the isolates belong to the same species but it was observed a high genetic diversity. For instance, strains isolated from different hosts and geographical regions showed identical profiles (IBSBF 233, 1053 and 1247) whereas strains isolated from the same hosts and geographical regions showed distinct patterns (IBSBF 1235 and 1526, Figure 3).

The Polymerse Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 16S-23S spacer region has been used to differentiate xanthomonads pathogenic to citrus (Destéfano & Rodrigues Neto, 2001 and 2002) and sugarcane plants (Destéfano *et al.*, 2002) suggesting that this approach is a simple, rapid and precise method for typing the strains. Our PCR-RFLP results revealed groups of strains showing banding profiles very different for the spacer region with similarity level below 70%, some of them with only 53%, indicating that this species is a very heterogeneous one.

The amplification with the primers 27f/1525r corresponding to 16S ribossomal gene was performed to confirm the taxonomic position of isolates at genus level. The fragments of 1,500 bp were digested with the restriction enzymes *Afa* I, *Dde* I, *Hae* III and *Hinf* I and the banding profiles obtained were highly similar among the isolates but not identical, confirming that all isolates belong to *Pseudomonas* genus.

The PCR-RFLP of the 16S-23S spacer region was useful to differentiate the species *P. agarici*, *P. fluorescens*, *P. toolasi* and *P. cichorii*. However, in respect of *P. cichorii* species, additional data,

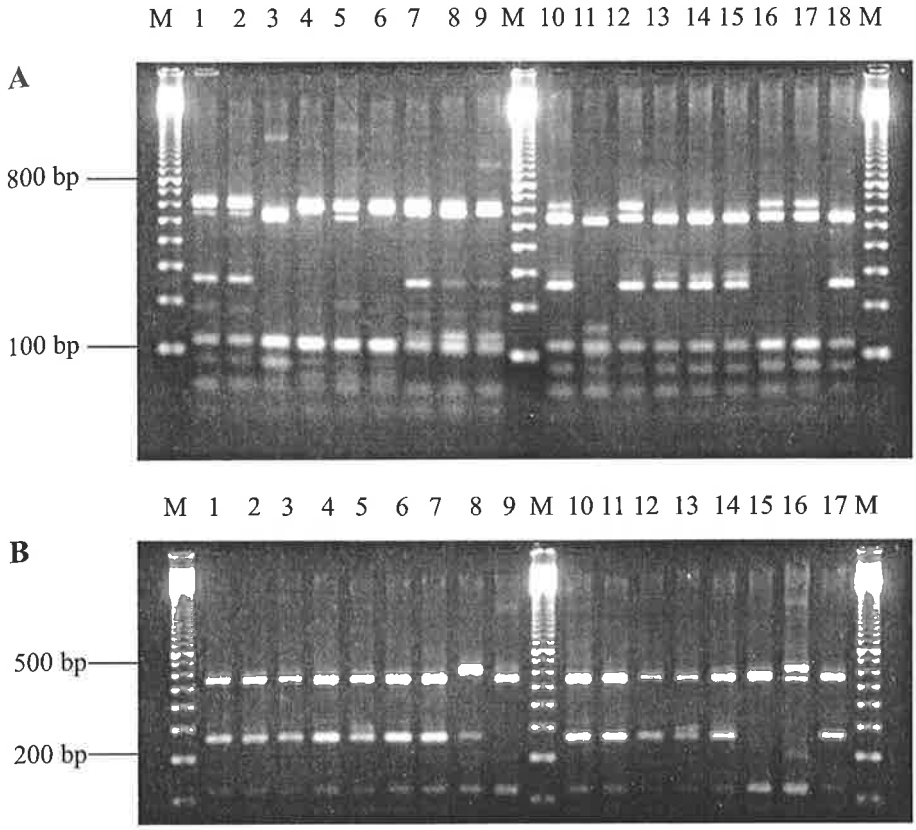


Figure 1. Restriction patterns of the 16S-23S DNAr intergenic spacer regions of *Pseudomonas cichorii* digested with *Taq* I. (**M**) Marker 100 bp (Amersham-Biosciences); (**A**) lanes (**1**) 1021; (**2**) 1414; (**3**) 234; (**4**) 1235; (**5**) 1526; (**6**) 1527; (**7**) 258; (**8**) 402; (**9**) 403; (**10**) 262; (**11**) 1209; (**12**) 508; (**13**) 1053; (**14**) 1069; (**15**) 1070; (**16**) 1059; (**17**) 1060; (**18**) 1094; (**B**) (**1**) 1061; (**2**) 1247; (**3**) 1262; (**4**) 1263; (**5**) 233; (**6**) 521; (**7**) 587; (**8**) 951; (**9**) 1013; (**10**) 1084; (**11**) 1110; (**12**) 1114; (**13**) 1524; (**14**) 1175; (15) 1274; (16) 1297; (17) 1525.

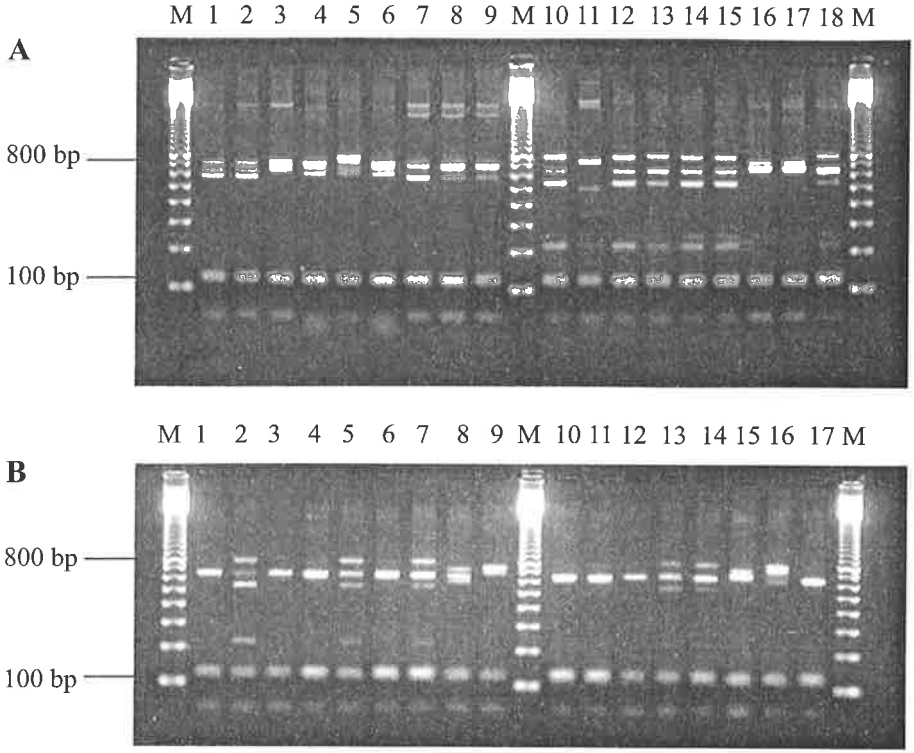
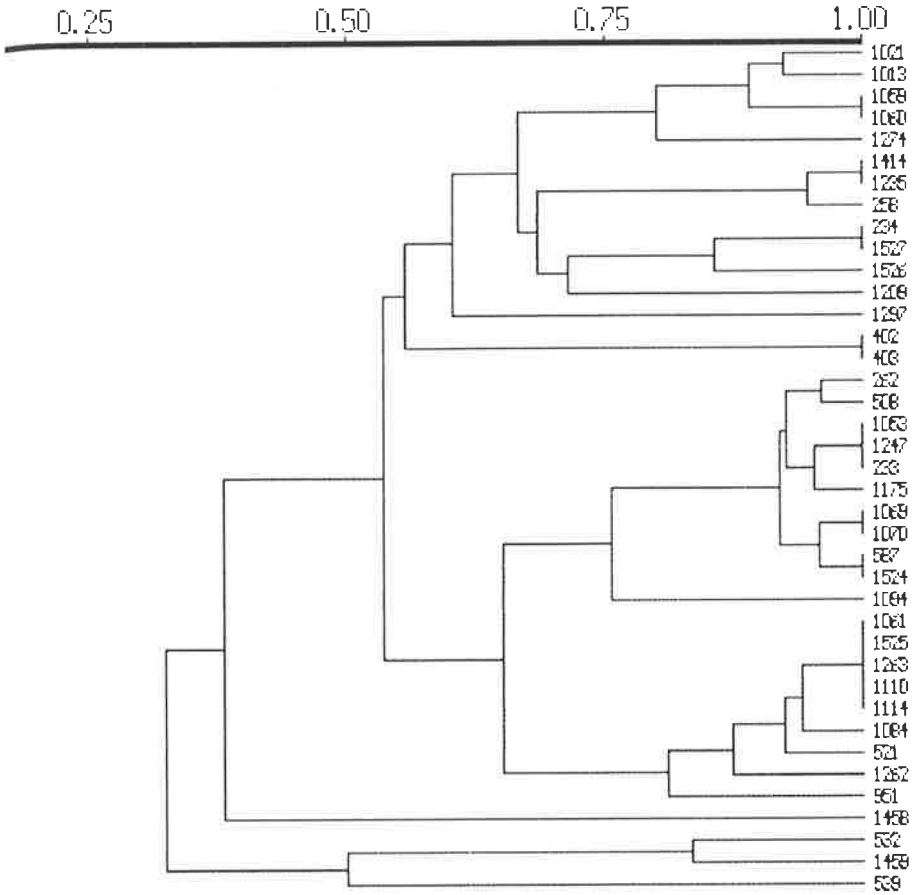


Figure 2. Restriction patterns of the 16S-23S DNAr intergenic spacer regions of *Pseudomonas cichorii* digested with *Afa* I. (**M**) Marker 100 bp (Amersham-Biosciences); (**A**) lanes (**1**) 1021; (**2**) 1414; (**3**) 234; (**4**) 1235; (**5**) 1526; (**6**) 1527; (**7**) 258; (**8**) 402; (**9**) 403; (**10**) 262; (**11**) 1209; (**12**) 508; (**13**) 1053; (**14**) 1069; (**15**) 1070; (**16**) 1059; (**17**) 1060; (**18**) 1094; (**B**) (**1**) 1061; (**2**) 1247; (**3**) 1262; (**4**) 1263; (**5**) 233; (**6**) 521; (**7**) 587; (**8**) 951; (**9**) 1013; (**10**) 1084; (**11**) 1110; (**12**) 1114; (**13**) 1524; (**14**) 1175; (15) 1274; (16) 1297; (17) 1525.

Figure 3. Dice-UPGMA cluster analysis of the Restriction Fragment Lenght Polymorphism (RFLP) patterns of the 16S-23SrDNA spacer region based on the combined restriction pattern (Afa I, Dde I, and Taq I). Scale bar indicates similarity.



including biochemical and physiological tests, and other molecular approaches, should be obtained in order to clarify the low level of similarity of the 16S-23S spacer region among the some strains.

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