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Clonal Diversity of the Taxon *Porphyromonas gingivalis* Assessed by Random Amplified Polymorphic DNA Fingerprinting

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A total of 97 strains of the periopathogen *Porphyromonas gingivalis* were collected. This collection included laboratory strains and clinical isolates of human origin with diverse clinical and geographical origins. Biological diversity was further increased by including 32 strains isolated from the oral cavities of nine different animal species. Genomic fingerprints of the 129 strains were generated as random amplified polymorphic DNAs (RAPDs) by the technique of PCR amplification with a single primer of arbitrary sequence. Four nonameric oligonucleotides were used as single primers, and the banding patterns of the DNA products separated on agarose gels were compared after ethidium ethidium bromide staining. Distance coeffients based on the positions of the major DNA fragments were calculated, and dendrograms were generated. We identified 102 clonal types (CTs) that could be assembled into three main groups by cluster analysis by the unweighted pair group method with mathematic averages. Group I (n = 79 CTs) included all 97 human strains and 6 monkey isolates. The strains in group II (n = 22 CTs) and III (n = 1 CT) were strongly differentiated from those in group I and included only strains of animal origin; they likely represent two cryptic species within the present *P. gingivalis* taxon. We observed that strains from Old World monkeys clustered together with the human genotype, whereas strains from New World monkeys clustered with the animal genotype. Our results with human strains also indicated that (i) the population structure is basically clonal, (ii) no dominant or widespread CT could be observed, and (iii) no relationship could be established between specific clusters of CTs and the periodontal status of the host. Our results corroborate previous findings by B. G. Loos, D. W. Dyer, T. S. Whittam, and R. K. Selander (Infect. Immun. 61:204–212, 1993) and suggest that *P. gingivalis* should be considered a commensal of the oral cavity acting as an opportunistic pathogen. Our results are not consistent with the hypothesis that only a few virulent clones of *P. gingivalis* are associated with disease.

Porphyromonas gingivalis (formerly *Bacteroides gingivalis*) is a strict anaerobic, nonfermentative, gram-negative coccobacillus. The association of *P. gingivalis* with periodontal disease in humans is well documented (49). The destructive potential of this species for the periodontium has been previously demonstrated by periodontal ligature experiments (for a review, see reference 41). Other studies have shown variations in virulence depending on the strains studied (12, 32, 48). In the mouth, the biotope of *P. gingivalis* is the gingival crevice (41), but it has also been recovered from acute periapical lesions (13, 44), tonsillitis (47) and odontogenic abscesses (50). Occasionally, *P. gingivalis* has been isolated from nonoral sites: a pulmonary abscess, chronic suppurative otitis media, a perforated appendix, a case of gas gangrene (34, 40), and a tubo-ovarian abscess (14).

Black-pigmented asaccharolytic bacteria identified as *P. gingivalis* have also been isolated from the oral cavities of some mammals (17), including dogs (45, 56), monkeys (3, 16), and sheep (10). With a few exceptions, the taxonomic status of these strains has not been studied by DNA-DNA homology (15, 24, 56).

In highly polymicrobial infections such as periodontal diseases, which occur in the presence of the complex flora of the dental plaque, assessing the pathogenic nature of each suspected etiologic agent (i.e., whether the disease results from the colonization of an obligate pathogen or from a modification of the proportions of commensal bacteria) is a delicate task which has received little attention.

Loos et al. (20) raised many questions regarding the natural history of infection with *P. gingivalis* that fall into three interrelated categories. The first category is the nature of the infectious agent. Is *P. gingivalis* a member of the indigenous oral flora that behaves as an opportunistic pathogen in dental infections, or is the organism exogenous and an obligate pathogen? The second category is the natural population structure. What is the extent of genotypic diversity among strains of the species? Are only a few genetic types associated with periodontitis and with other oral infections, or do a wide variety of genetic types have a pathogenic relationship with the host? The third category is epidemiology. Are individual strains widely distributed on a continental scale or even a global scale? What is the biological reservoir of human infections?

Using multilocus enzyme electrophoresis (MLEE), Loos et al. (21) were the first to study the genetic diversity of *P. gingivalis*, and they advanced several hypotheses on the population structure and on the role of this species in oral infections that need to be challenged.

Here we report an assessment of the genetic diversity and an analysis of the population structure of the *P. gingivalis* taxon by comparing random amplified polymorphic DNA (RAPD) fingerprints (54) generated by the arbitrarily primed PCR (AP-

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PCR) (52). Our results indicate that humans are infected by strains of widely different clonal origins and confirm that the actual *P. gingivalis* taxon is composed of two genospecies.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We examined 97 strains recovered from 79 human subjects and 32 strains recovered from 30 animals of nine different species. Most of the human isolates were collected from adult patients with chronic periodontitis (Table 1). All strains were identified as *P. gingivalis* by using established physiologic criteria and gas-liquid chromatographic analysis of cellular fatty acids (9).

The strains were grown in Todd-Hewitt broth (BBL, Cockeysville, Md.) enriched with hemin (10 μg/ml) and vitamin K_1 (1 μg/ml). Stock cultures were maintained on blood agar plates of the same medium enriched with 2% laked human blood. All cultures were incubated in an anaerobic chamber at 37°C. They were regularly checked by the Gram reaction and the API-ZYM (API Analytab Products, Plainview, N.Y.) and ATB 32A enzymatic tests (API System, La-Balme-les-Grottes, France).

Preparation of template DNA for AP-PCR. Cells from broth cultures (5 to 8 ml) in the logarithmic phase were first harvested by centrifugation at 3,500 × *g* for 20 min. The supernatants were discarded, and individual cell pellets were stored at −20°C without additional preparation until DNA isolation. Pure genomic DNA from all strains tested was obtained by a standard miniprep procedure (55) to which we added an RNase treatment (42). The concentration of DNA in samples was calculated by measuring the A_{260}, and the quality was estimated by A_{260}/A_{280} ratio (36), agarose gel electrophoresis, and comparison with DNA standards.

RAPD fingerprinting by AP-PCR. RAPD fingerprinting was performed as previously described (26) using four previously tested arbitrary primers (27). Amplification reactions were done in 25-μl volumes, and the amplification reaction mixtures contained 200 μM (each) dATP, dCTP, dGTP, and dTTP, 1.2 μM of primer, 25 ng of template DNA, and 1.25 units of *Taq* DNA polymerase (Pharmacia, Baie d'Urfé, Canada) in 1× PCR buffer (20 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$, 0.001% gelatin [wt/vol], 0.1% Triton X-100 [vol/vol]). The tubes were kept on ice until the beginning of the amplification procedure. A negative control without template DNA was included in each experiment. The reaction mixtures were overlaid with mineral oil and subjected to amplification in a DNA Thermal Cycler (Perkin-Elmer Cetus, Montréal, Canada) programmed for 25 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 32°C, and 2 min at 72°C, using the fastest available transition between each temperature. On completion of the PCR program, the samples were cooled to 4°C. Amplification products were compared by high-resolution horizontal electrophoresis of 8-μl samples in 1.5% SeaKemGTG agarose gel (FMC, Rockland, Maine) in Tris-acetate buffer (0.04 M Tris-acetate, 0.002 M EDTA; pH 8.5), ethidium bromide staining, and photography with a UV light transilluminator by using the Polaroid MP4 system. DNA size markers (1-kb DNA ladder; GIBCO BRL, Burlington, Canada) were included in each gel.

Analysis of RAPD. To assess overall genetic relatedness, a data matrix scoring major amplicons as 1 (present) and 0 (absent) was first compiled for each strain. Similarities between all tested strains were then estimated by using the coefficient (31) $F = (2n_{xy}/n_x + n_y)$, where n_{xy} is the number of bands common to strains *x* and *y*, and n_x and n_y are the total number of bands in each strain. These data were clustered by the unweighted pair group method with mathematic averages (UPGMA) (43) and by the neighbor-joining method (35) to construct dendrograms. Data handling was performed with the R package (18), and we used the PHYLIP program (7) for reconstructions.

RESULTS

Genetic relationships and diversity in the taxon. Four arbitrary primers were used to analyze the extent and nature of polymorphism in the *P. gingivalis* taxon. From 42 to 83 distinct amplitypes were observed, depending on the primer used (Table 2). That these multiple amplitypes were associated with 79 different human subjects and 30 animals is a first indication of the genetic diversity. The combined data from all four primers were compiled as a data matrix of 66 discrete characters (major amplicons), 61 of which were shared by two or more isolates.

A first dendrogram obtained by hierarchical clustering revealed that 128 of the 129 study isolates clustered in two major phylogenetic groups in which a total of 101 clonal types (CTs) could be resolved (Fig. 1). A third group consisted of a single strain. Group I included all 97 human isolates, seven nonhuman primate (NHP) isolates, and one canine isolate (Chien N9). Group II consisted exclusively of animal isolates including three NHP strains. Group III consisted of one animal strain isolated from a sheep with broken-mouth disease. The average genetic distances between groups were as follows: between groups I and II, 0.58; between groups I and III, 0.59; and between groups II and III, 0.78. The average genetic distance between isolates of group II (0.43) was higher than that for group I (0.32), the former reflecting the great diversity of hosts in group II. Interestingly, isolates from a carrier species (dogs, cats,...) were never seen to group in a given cluster. This result presumably reflected the higher diversity of the animal population studied (nine mammalian species).

Genetic relationships between isolates of group I. The dendrogram in Fig. 2 details the genetic relationships among the 79 CTs in group I. At a genetic distance of 0.28, the relationship between the 105 isolates could be resolved as 10 clusters of CTs and 5 lineages represented by single CTs; these clusters are labelled with letters (A to O). Eighty-five isolates of group I were recovered from subgingival lesions in adult patients with periodontitis (Table 1), three isolates were from patients with infected root canals, three isolates were from patients with gingivitis, one isolate was from patient with a tubo-ovarian abscess, and seven strains were from subjects with no destructive periodontal disease. We observed that neither the strains from cases of periodontal disease nor from healthy individuals were concentrated in a unique cluster. No CT with a predominant distribution over the globe, over a continent, over a country, or even locally was observed.

Pairs or small groups of strains from one individual or a couple most often clustered in one CT. This was the case for CTs 2, 4, 6, 11, 30, 34, 66, 73, and 79 (Fig. 2 and Table 1). The observation of strains from distinct hosts clustering in one CT suggests horizontal transmission. CTs 21 and 22, distinguished by a small genetic distance (0.12), were isolated from one periodontal pocket in one host (Table 1): they may have originated from a clonal population in which subtle changes in the genome attest an early phase in the process of selection. This observation is also valid for CTs 27 and 28. On the other hand, the genetically distant CTs 1 (strain RB22D-1 deposited as ATCC 49417) and 74 (strain RB46D-5), collected from two periodontal lesions from one patient, confirmed that polyclonality may occur in this type of infection, as previously observed (23).

Genetic variation versus phenotypic characters. The single strain of group III and all isolates of group I, except for strain Chien N9 (CT 64), belonged to the human biotype (negative reactions for catalase, β-galactosidase, and glutamyl-glutamic acid arylamidase) (9). All isolates of group II were of animal biotype (positive reactions for catalase, β-galactosidase, and glutamyl-glutamic acid arylamidase) (9). A nearly perfect correlation could thus be established between the human biotype and group I, as well as between the animal biotype and group II.

In addition, we know the fimbrial restriction fragment length polymorphism (RFLP) group for 18 isolates (19) and the infectivity potential for 8 isolates (12) (Fig. 2). Since members of diverse RFLP groups and infectivity potentials were spread across the dendrogram, the absence of a structured relationship between these phenotypic traits and specific clusters is inferred.

Second hypothesis of genetic relationships. To improve our analysis of the relationship between strains, another reconstruction of phylogeny between isolates was generated by an additive tree method (Fig. 3), the neighbor-joining method (35). Four points were observed in the resulting phylogeny: (i) animal strains from group II were grouped in a single cluster; (ii) CT 102 (group III) was integrated into a cluster of group I;

TABLE 1. *P. gingivalis* strains (n = 129, originating from a total of 111 different hosts) used in this study

Group, cluster, and CT or lineage	Isolate	Host	Infection or diagnosis	Site of isolation	Geographic origin
Group I					
Cluster A					
1	ATCC 49417[a]	Human	Periodontitis	Periodontal pocket	Québec, Canada
2	Cerco 1.1	NHP[b]	None	Subgingival	Québec, Canada
	Cerco 1.2	NHP (same animal as the preceding one)	None	Subgingival	Québec, Canada
3	295-1	Human	Periodontitis	Periodontal pocket	Umeå, Sweden
4	19.2	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	19.3a	Human[c]	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	19.5	Human[c]	Periodontitis	Saliva	Amsterdam, The Netherlands
	19.8	Human[c]	Periodontitis	Oral mucosa	Amsterdam, The Netherlands
	19.11	Human[c]	Periodontitis	Tongue	Amsterdam, The Netherlands
	19.13	Human[c]	Periodontitis	Tonsils	Amsterdam, The Netherlands
	19.16	Human (spouse)[d]	None	Subgingival	Amsterdam, The Netherlands
5	16-1	Human	Periodontitis	Periodontal pocket	Tokyo, Japan
6	13.3	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	13.6	Human[e]	Periodontitis	Oral mucosa	Amsterdam, The Netherlands
	13.17	Human (spouse)[f]	None	Subgingival	Amsterdam, The Netherlands
	13.19	Human (spouse)[f]	None	Saliva	Amsterdam, The Netherlands
	13.30	Human (child)[g]	None	Subgingival	Amsterdam, The Netherlands
7	EM-3	Human	Endodontic infection	Root canal	Buffalo, N.Y.
8	HG 760	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
9	OMG 406	Human	Periodontitis	Periodontal pocket	Kenya
10	AJW5	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
11	22.20	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	22.21A	Human (spouse)[h]	None	Saliva	Amsterdam, The Netherlands
Cluster B					
12	HG 66 (W83)	Human	Unknown	Unknown	Bonn, West Germany
	HG 76 (W50)	Human	Unknown	Unknown	Bonn, West Germany
	W50	Human	Unknown	Unknown	Bonn, West Germany
13	R 113	Human	None	Subgingival	Rennes, France
14	JKG 1	Human	Periodontitis	Periodontal pocket	Ann Arbor, Mich.
15	45-8M	Human	Periodontitis (EO[i])	Periodontal pocket	Cairo, Egypt
16	57-14D	Human	Periodontitis (EO)	Periodontal pocket	Cairo, Egypt
17	22KN6-12	Human	Periodontitis	Periodontal pocket	Tokushima City, Japan
18	A7436	Human	Refractory periodontitis	Periodontal pocket	Atlanta, Ga.
19	46-11M	Human	Periodontitis (EO)	Periodontal pocket	Cairo, Egypt
20	41-14M	Human	Periodontitis (EO)	Periodontal pocket	Cairo, Egypt
	44-11M	Human	Periodontitis (EO)	Periodontal pocket	Cairo, Egypt
	64-3D	Human	Periodontitis (EO)	Periodontal pocket	Cairo, Egypt
21	ML2g	Human	Periodontitis	Periodontal pocket	Rennes, France
22	ML2n	Human (same patient as the preceding one)	Periodontitis	Periodontal pocket	Rennes, France
23	HG 1023	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
24	20.2	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
25	BH 6/26	Human	Periodontitis	Periodontal pocket	Winnipeg, Canada
26	102	Human	Endodontic infection	Root canal	Umeå, Sweden
27	H48q	Human	Gingivitis	Subgingival	Rennes, France
28	H48s	Human (same patient as the preceding one)	Gingivitis	Subgingival	Rennes, France
29	MC 18	Human	Periodontitis (EO)	Periodontal pocket	Rio de Janeiro, Brazil
30	17 sp	Human	Periodontitis	Saliva	Amsterdam, The Netherlands
	17.1.36d	Human[j]	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
31	17.2.36d	Human (spouse)[k]	None	Subgingival	Amsterdam, The Netherlands
32	HG1022	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
33	20.15	Human (spouse)[l]	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
34	2.3	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	2.12	Human (same patient as the preceding one)	Periodontitis	Saliva	Amsterdam, The Netherlands
35	817H	Human	Periodontitis	Periodontal pocket	Malmö, Sweden
Cluster C					
36	19A4	Human	Periodontitis	Periodontal pocket	Québec, Canada
37	JH4	Human	Periodontitis	Periodontal pocket	Yokohama, Japan
38	ESO 127	Human	Periodontitis	Periodontal pocket	Okayama, Japan
39	CLN17-6-1	Human	Periodontitis (NIDDM)[m]	Periodontal pocket	Sacaton, Ariz.
40	2.16	Human (spouse)[n]	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands

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TABLE 1—*Continued*

Group, cluster, and CT or lineage	Isolate	Host	Infection or diagnosis	Site of isolation	Geographic origin
Cluster D					
41	9-14K-1	Human	Periodontitis (IDDM)[o]	Periodontal pocket	Buffalo, N.Y.
42	17-5	Human	Periodontitis	Periodontal pocket	Minneapolis, Mich.
43	17A1	Human	Periodontitis	Periodontal pocket	Québec, Canada
44	JKG5	Human	Periodontitis	Periodontal pocket	Ann Arbor, Mich.
45	13T1K-3	NHP[p]	Experimental periodontitis[q]	Periodontal pocket	Buffalo, N.Y.
46	E20-1	Human	Endodontic infection	Root canal	Buffalo, N.Y.
47	LB13D-2	Human	Periodontitis	Periodontal pocket	Québec, Canada
48	JKG 2	Human	Periodontitis (IDDM)	Periodontal pocket	Ann Arbor, Mich.
49	11	Human	Periodontitis	Periodontal pocket	Tokyo, Japan
50	I-2	Human	Periodontitis	Periodontal pocket	Tokyo, Japan
51	7B5	Human	Periodontitis[r]	Subgingival	Québec, Canada
52	OMZ 409	Human	Periodontitis	Periodontal pocket	Zürich, Switzerland
53	HG 1021	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
Cluster E					
54	BH 18/10	Human	Periodontitis	Periodontal pocket	Winnipeg, Canada
Cluster F					
55	381[s]	Human	Periodontitis	Periodontal pocket	Boston, Mass.
	ATCC 33277[s]	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
	HG 372 (=ATCC 33277)[t]	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
56	HG 91 (= 381)[t]	Human	Periodontitis	Periodontal pocket	Boston, Mass.
57	Prèl 92A	Human	Periodontitis	Periodontal pocket	Québec, Canada
58	OMG 402	Human	Periodontitis	Periodontal pocket	Kenya
Cluster G					
59	10X1K-3	NHP[p]	Experimental periodontitis[q]	Periodontal pocket	Buffalo, N.Y.
60	JKG 3	Human	Periodontitis	Periodontal pocket	Ann Arbor, Mich.
61	2J14	Human	None	Supragingival	Rennes, France
62	T22	NHP[p]	Experimental periodontitis[q]	Periodontal pocket	San Antonio, Tex.
63	3079.03	NHP[p]	Experimental periodontitis[q]	Periodontal pocket	San Antonio, Tex.
64	Chien N9	Dog	None	Subgingival	Québec, Canada
65	T7	NHP[p]	Experimental periodontitis[q]	Periodontal pocket	San Antonio, Tex.
Cluster H					
66	HW24D-2	Human	Periodontitis	Periodontal pocket	Québec, Canada
	HW24D-4[u]	Human	Periodontitis	Periodontal pocket	Québec, Canada
	HW24D-5[u]	Human	Periodontitis	Periodontal pocket	Québec, Canada
67	HG 1024	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
68	18.5	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	18.23	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
69	AJW2	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
Cluster I					
70	CR2A	Human	Gingivitis	Subgingival	Boston, Mass.
Cluster J					
71	HG 1020	Human	Periodontitis	Periodontal pocket	Buffalo, N.Y.
72	MAMA	Human	Tubo-ovarian abscess	Salpingotomy	Rio de Janeiro, Brazil
Cluster K					
73	23A1	Human	Periodontitis	Periodontal pocket	Québec, Canada
	23A4	Human (same patient as the preceding one)	Periodontitis	Periodontal pocket	Québec, Canada
74	RB46D-5	Human	Periodontitis	Periodontal pocket	Québec, Canada
Cluster L					
75	16NH2-1	Human	Periodontitis	Periodontal pocket	Yokohama, Japan
Cluster M					
76	A7A1-28	Human	Periodontitis (NIDDM)	Periodontal pocket	Sacaton, Ariz.
Cluster N					
77	HG 564	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands

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TABLE 1—*Continued*

Group, cluster, and CT or lineage	Isolate	Host	Infection or diagnosis	Site of isolation	Geographic origin
Cluster O					
78	HG 756	Human	Periodontitis	Tongue	Amsterdam, The Netherlands
79	3.2	Human	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
	3.18	Human (spouse)[v]	Periodontitis	Periodontal pocket	Amsterdam, The Netherlands
Group II					
Cluster AA					
80	B 243	NHP[w]	Experimental periodontitis[p]	Periodontal pocket	Gainesville, Fla.
81	A 1834	NHP[w]	Experimental periodontitis[p]	Periodontal pocket	Gainesville, Fla.
82	I372	NHP[w]	Experimental periodontitis[p]	Periodontal pocket	Gainesville, Fla.
83	Loup 2	Wolf	None	Subgingival	Québec, Canada
Cluster AB					
84	3492	Cat	None	Subgingival	Sydney, Australia
85	D2-9	Dog	None	Subgingival	Tokyo, Japan
86	Chien 7	Dog	None	Subgingival	Québec, Canada
87	Chien 5	Dog	None	Subgingival	Québec, Canada
Cluster AC					
88	Chien 5B	Dog	None	Subgingival	Québec, Canada
89	Chien 8 NS1	Dog	None	Subgingival	Québec, Canada
90	Chat 3(7)	Cat	None	Subgingival	Québec, Canada
91	Chien 4.2	Dog	None	Subgingival	Québec, Canada
92	Chat 1	Cat	None	Subgingival	Québec, Canada
Cluster AD					
93	Loup 1.1	Wolf	None	Subgingival	Québec, Canada
94	Coyote 1.2	Coyote	None	Subgingival	Québec, Canada
95	D2-10	Dog	None	Subgingival	Tokyo, Japan
96	BasBeagle PZ4T+	Dog	None	Subgingival	Ann Arbor, Mich.
Cluster AE					
97	Chien 6	Dog	None	Subgingival	Québec, Canada
Cluster AF					
98	Ours 3.1	Bear	None	Subgingival	Québec, Canada
Cluster AG					
99	Chat 1.1	Cat	None	Subgingival	Québec, Canada
	Chat 1.2	Cat (same animal as the preceding one)	None	Subgingival	Québec, Canada
100	Chat 3.1	Cat	None	Subgingival	Québec, Canada
Cluster AH					
101	3318	Cat	None	Subgingival	Sydney, Australia
Group III					
102	T90	Sheep	Broken-mouth disease	Periodontal pocket	Dunedin, New Zealand

[a] Isolated as strain RB22D-1.
[b] *Cercopithecus nictitans.*
[c] Same patient as the one who provided isolate 19.2.
[d] Spouse of patient who provided isolates 19.2 to 19.13.
[e] Same patient as the one who provided isolate 13.3.
[f] Spouse of patient who provided isolates 13.3 and 13.6.
[g] Child from couple who provided isolates 13.3 and 13.6 and isolates 13.17 and 13.19, respectively.
[h] Spouse of patient who provided isolate 22.20.
[i] EO, early onset.
[j] Same patient as the one who provided isolate 17 sp.
[k] Spouse of patient who provided isolates 17 sp and 17.1.36d.
[l] Spouse of patient who provided isolate 20.2
[m] Patient with non-insulin-dependent diabetes mellitus (NIDDM) (Native American of the Pima Indian Reservation, Sacaton, Ariz.).
[n] Spouse of patient who provided isolates 2.3 and 2.12.
[o] Patient with insulin-dependent diabetes mellitus (IDDM).
[p] Cynomolgus monkey (*Macaca fascicularis*).
[q] Periodontitis induced by subgingival tooth ligature.
[r] Isolate recovered from a healthy site in a patient with periodontitis.
[s] Laboratory strain used in Québec, Canada.
[t] Laboratory strain used in Amsterdam, The Netherlands.
[u] Isolate recovered from the site as HW24D-2.
[v] Spouse of patient who provided isolate 3.2.
[w] Squirrel monkey (*Saimiri squirrius*).

TABLE 2. Base sequences of primers and number of amplitypes identified with each primer among 129 *P. gingivalis* strains

Primer no.	Sequence	No. of amplitypes identified with primer
910-05	5′-CCGGCGGCG-3′	83
910-09	5′-CCGGGCCGC-3′	79
940-11	5′-GTCTCGGGG-3′	42
970-11	5′-GTAAGGCCG-3′	83

(iii) several clusters were unchanged; (iv) a cluster of taxon special cases grouping geographically and phenotypically strains CT 25 and CT 54 (these strains are slime producing), CT 102 mentioned above, CT 72 (the unique strain from an extraoral infection) and CT 45 (the single NHP strain in cluster D). Integration of CT 102 with other CTs of human biotype is not surprising. In fact, results from the distance matrix were corrupted by the UPGMA clustering because the median distance between groups III and I was smaller than the distance between groups III and II.

Two genospecies inside the taxon. A priori we hypothesized the existence of a phyletic separation between strains of human and animal biotypes. The data collected by RAPD allowed the testing of this hypothesis by the Mantel test (25). We stated the null hypothesis that animal and human strains belong to a single phylum and the alternative hypothesis that they are distinct. We tested this statement by comparing the experimental data (the distance matrix) to a model corresponding to the alternative hypothesis (the model matrix) (Fig. 4). The model matrix of the alternative hypothesis was constructed as follows: each strain was coded as a member of one or the other group; the model matrix contained 1's for pairs of strains that were of the same group (maximum similarity) and 0's for pairs that were of the other group (null similarity). This model matrix is represented as Y in Fig. 4. Sampling distribution of the Mantel statistic was obtained by repeatedly simulating realizations of the null hypothesis through 1,000 permutations of the strains (corresponding to the lines and columns) in the Y matrix. The computed Mantel statistic between the X and Y matrices was significant at $P < 0.001$, confirming the hypothesis of the phyletic division.

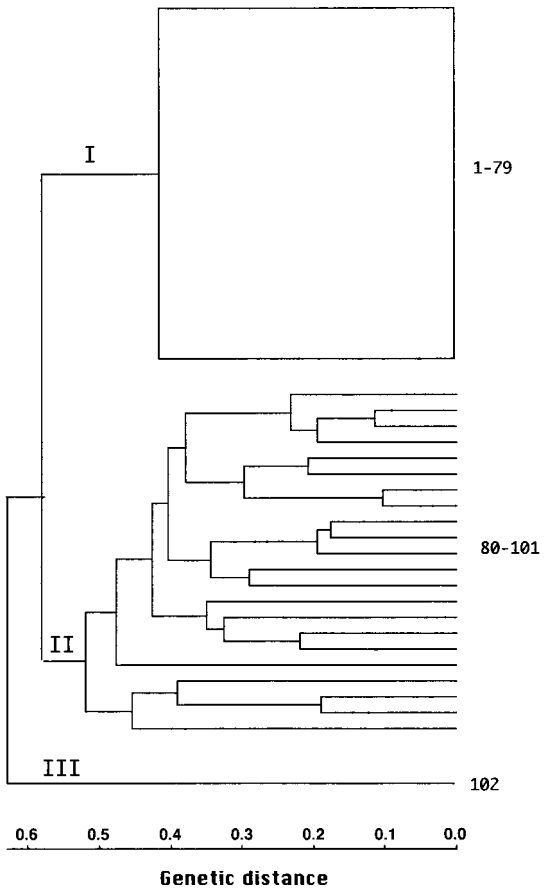

FIG. 1. Dendrogram constructed from RAPD data indicating relationships between 129 isolates of *P. gingivalis*. A distance matrix was calculated by using the Nei and Li coefficient. The tree was generated from the distance matrix by UPGMA. Group I consists of a cluster of 79 CTs for which detailed genetic relationships are presented in Fig. 2, group II consists of a cluster of 22 CTs containing 23 isolates from seven animal species, and group III consists of a single isolate from a sheep with broken-mouth disease.

DISCUSSION

Nature of the samples studied. Because *P. gingivalis* isolates were obtained from five continents and diverse biological origins, we propose that the samples are representative of the population of the taxon. We acknowledge that quite a number of strains studied were recovered from a few patients and their probands and thus may have constituted redundant CTs. We took this factor into account in the data analysis.

Biological division of the taxon. Our RAPD results corroborate the MLEE results of Loos et al. (21) in recognizing the existence of at least one cryptic phylum in the actual *P. gingivalis* taxon. As previously suggested by Fournier and Mouton (8) and Loos et al. (21), we propose that the species *P. gingivalis* sensu stricto be restricted to strains of the human biotype, pending confirmation by a DNA-DNA hybridization study.

NHP strains. Following our observation that the 10 strains isolated from NHPs were distributed into two biotypes and two groups, a reanalysis led to an unexpected result. All three strains from NHPs of the New World (America) shared characteristics of the animal biotype and clustered in group II, whereas all seven strains from NHPs of the Old World (Africa) shared characteristics of the human biotype and clustered in group I. Loos et al. (21), who studied only strains from African monkeys, also grouped their NHP strains with human strains and suggested that cynomolgus monkeys, while living in laboratory animal facilities, were infected with strains originating from their handlers. Our wider panel of strains allowed us to put forward a new hypothesis. NHP strains consist of two distinct phyla: strains from NHPs of the New World (America), demonstrating an animal biotype and that are members of the so-called animal group (II) and, on the other hand, strains from NHPs of the Old World (Africa), demonstrating a human biotype and that are members of the so-called human group (I). We challenged this hypothesis with two newly acquired strains from *Cercocebus torquatus lunulatus* (Old World) and *Ateles paniscus* (New World) and found that the results were in agreement with the hypothesis (data not shown). The recognition of these two distinct phyla suggests the intriguing possibility that the most recent ancestors of human strains of *P. gingivalis* are closely related to the Old World NHP strains or are the Old World monkey strains themselves. This possibility needs to be investigated for a larger sample of strains from a variety of NHP species indigenous to different parts of the world.

The case of Chien N9, an animal strain demonstrating an

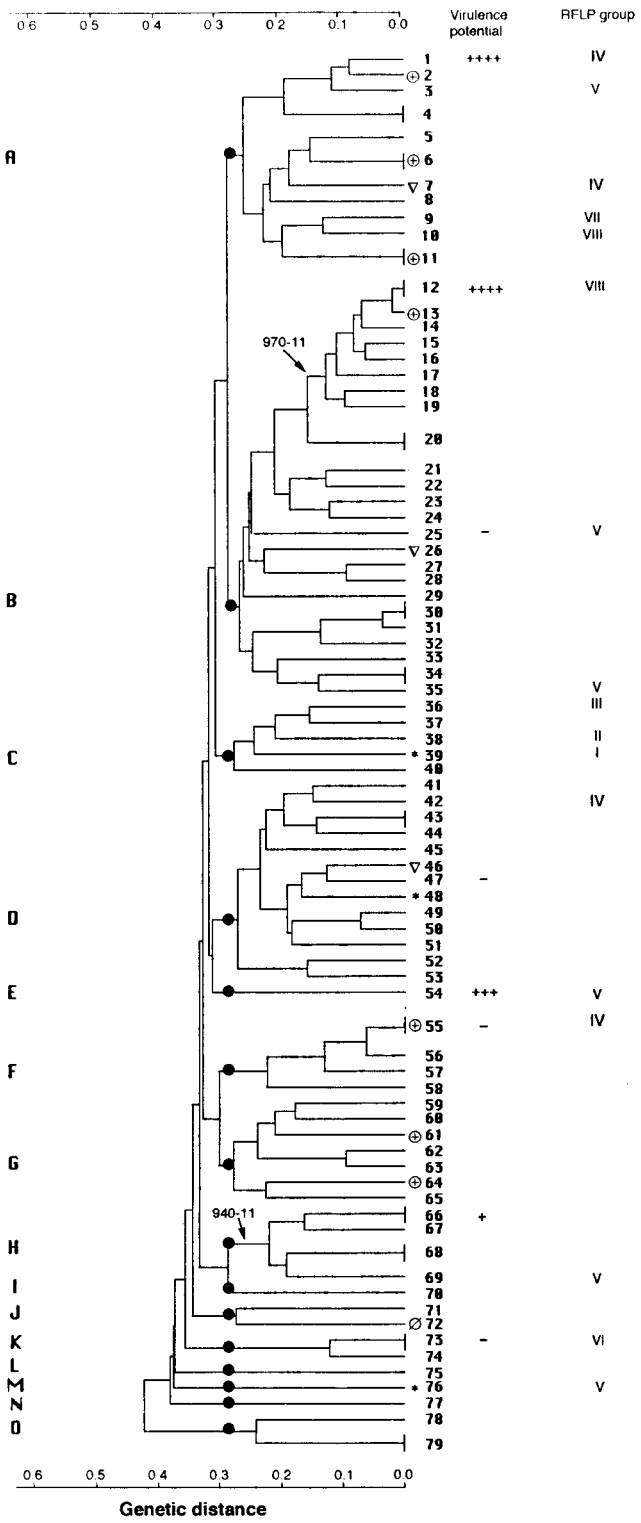

FIG. 2. Genetic relationships between the 79 CTs of *P. gingivalis* from group I. Fifteen major clusters or single lineages are each marked by a black circle in the dendrogram and indicated by a letter from A to O. CTs of isolates from a root canal infection (▽), CTs of isolates from patients with diabetes (*), CTs of isolates from periodontally healthy subjects (⊕), and the CT of an isolate from an extraoral infection (∅) (Table 1) are indicated. Two or more isolates of the same CT are indicated by a vertical line. Available information about virulence potential (11) and RFLP group (19) is indicated to the right of the figure. Virulence potential is graded from − to ++++.

animal biotype but human genotype, is paradoxical in the context of our results, although a study (56) reported strong DNA homology (75 to 100%) between ATCC 33277 and *P. gingivalis* from dogs. We observed that RAPDs of this strain included major genetic markers of the human genotype but of weaker intensity, suggesting only partial homology. The genotype of Chien N9 could be an intermediate between the two groups. Acquisition and/or selection of human-like strains would have been favored by mouth-to-mouth contact between dog and master. A subsequent selection of one or more better fitted clones would have completed this evolution.

Clonal structure and diversity. Most surveys of natural populations of animal-associated bacteria (e.g., *Actinobacillus actinomycetemcomitans* [1], *Bordetella* spp. [29], *Haemophilus influenzae* [28], *Legionella pneumophila* [39], *Staphylococcus aureus* [30], and *Shigella* spp. and *Escherichia coli* [38]) have been based on strain-specific differences in MLEE (37). These surveys, mainly using MLEE data and UPGMA reconstruction, have revealed essentially clonal structure, often with many clones detectable in the environment at large, and with just a few geographically widespread clones predominating in disease episodes. Since a large number of dendrograms and cladograms using various genetic distance formulate and various reconstruction methods can be easily generated (43), the two phylogenies that we propose should be considered an hypothesis and not the final scheme of genetic relatedness between clones of the *P. gingivalis* taxon. We used a second clustering method because UPGMA has an a priori criterion of ultrametric data that consequently increases its sensitivity to distance matrix errors (4). By assuming mean perfect data, the neighbor-joining method never yields statistically inconsistent estimation of phylogeny (6). The general agreement between the two reconstructions supports the inference that both methods reveal the same biological reality, namely, that *P. gingivalis* isolates are extremely diverse and that no particular CT could be associated with either a pathological or healthy state.

In contrast to Loos et al. (21), no CT (the restricted definition) was found to be geographically widespread. However, if we suppose that because of the sensitive methodology, a genetic distance lower than 0.1 might be considered void of meaning, then the number of CTs will markedly decrease. A single CT would replace CTs from 12 to 19. This new CT would be dispersed over Germany, France, United States, Egypt, and Japan and would better fit the extended definition of a clone (33).

A CT can be overrepresented because redundant isolates originate from the same subject or an epidemiologically related one (same family). The diversity of CTs in the same geographical area and conversely the diversity in geographical locations of clonally related strains demonstrate that the structure of the population of the *P. gingivalis* taxon is clonal, i.e., consists of multiple clones.

The diversity of the taxon *P. gingivalis* has already been demonstrated by restriction enzyme analysis (22) and MLEE (21). The diversity is largely confirmed by this AP-PCR study, which extends the succinct demonstration of the genetic heterogeneity previously reported by us (27) and others (2). Investigation of clonality cannot directly prove whether an organism is an opportunistic or exogenous pathogen, but it may provide important clues. In this study, the high level of diversity and the absence of a predominant CT associated with periodontal disease suggest that all CTs of the taxon *P. gingivalis* would be equally effective in colonizing the human host and that they share a common virulence potential, which is in strong contrast with other bacterial pathogens. Since *P. gingivalis* does not seem to demonstrate a major characteristic of

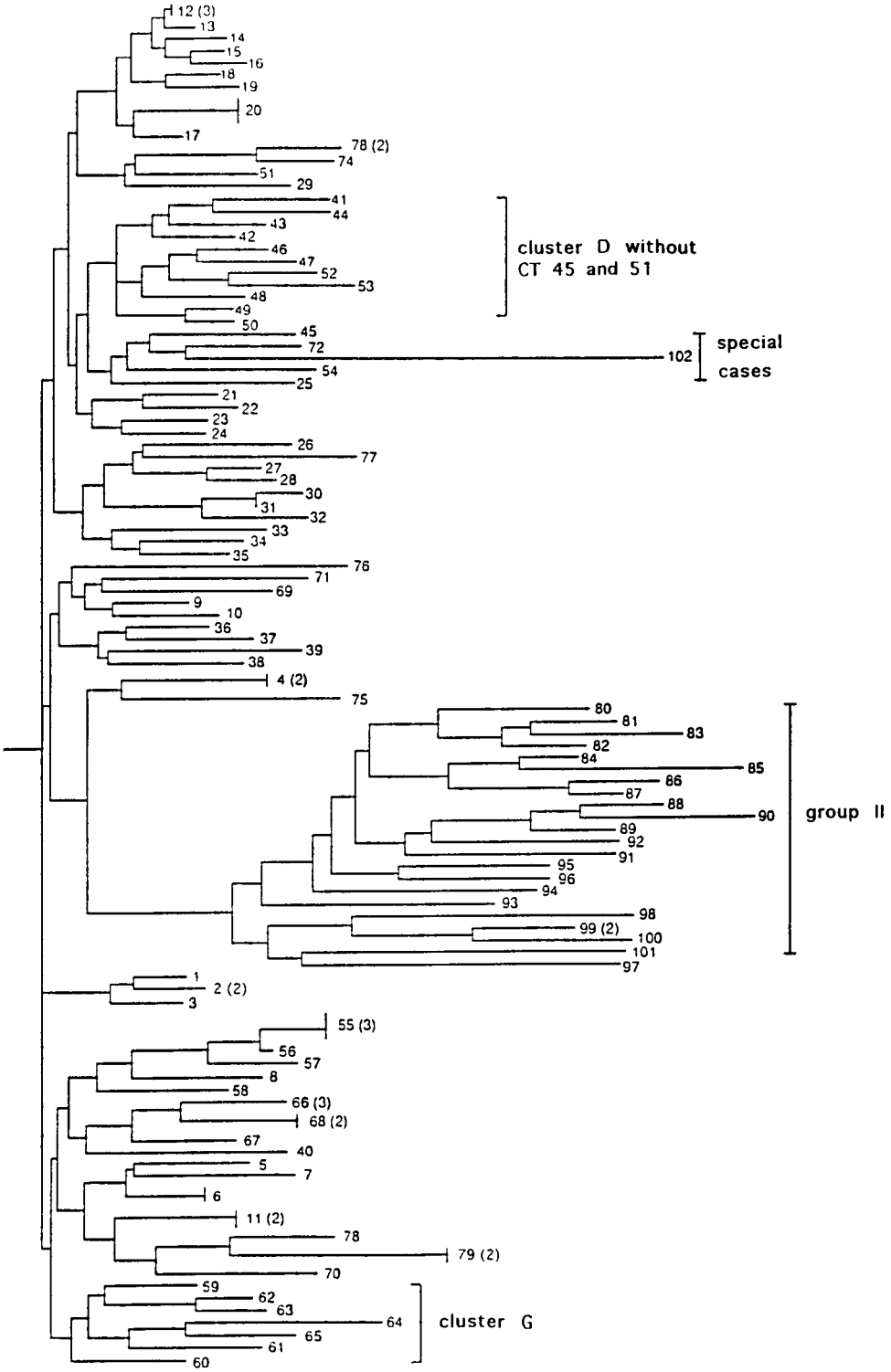

FIG. 3. Phylogenetic hypothesis inferred by a distance-based tree reconstruction (neighbor-joining) analysis. Examples of clusters, well preserved by the two reconstruction methods are given, together with a new cluster of special cases in which can be found CT 102 (representing group III) and CT 72, the isolate from an extraoral infection.

most pathogens, i.e., virulence restricted to a few clones, the hypothesis previously proposed that this species is a commensal member of the oral flora, acting mainly as an opportunistic pathogen in periodontal disease (20) rather than as an exogenous pathogen (11), acquired shortly before initiation of disease, can be further advanced. However, since RAPD fingerprinting does not allow tracing well-identified genetic markers of virulence in the analysis of clonality, we must accept that

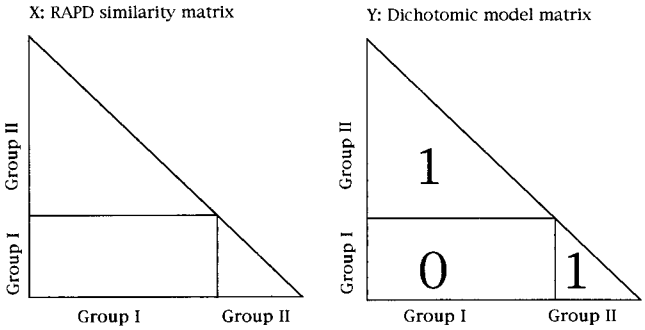

FIG. 4. Schematic representation of comparison of experimental data (matrix X) to the model (matrix Y), to test the hypothesis of phylogenetic dichotomy between groups I and II by the Mantel test.

there is a limitation in our genome-based study of clonality in assessing virulence. A final observation was that it would appear that animals other than NHPs from the African continent are excluded, as previously stated (8), as a potential biological reservoir of human infection with *P. gingivalis*.

The high level of diversity reported here, one of the most important ever found in the bacterial world, and the indirect demonstration of homoplasy by lack of association between a particular RFLP group (19) and a specific cluster raise two fundamental questions. First, is RAPD fingerprinting too sensitive to provide a realistic picture of the genetic diversity of a given bacterial species? Second, what is the origin of homoplasy? Indeed, it may be considered that real clonality is never absolute (5), since all organisms, even in the absence of selective pressure, are genetically unstable because of mutation, deletion, and acquisition of new material from generation to generation, thus providing an infinite number of clones. In spite of evidence that clonality is antagonistic to interstrain recombination, there is, therefore, no reason to assume that a clonal structure is incompatible with horizontal transfer at some degree that could generate homoplastic characters. This is particularly true in the case of a high level of diversity, which seems difficult to achieve only by divergence without sex. On the other hand, many think that a perfect demonstration of clonality is impossible and can be approached only by increasing the confidence with highly discriminatory methods of characterizing isolates. RAPD has been previously used for a phylogenetic study of bacteria (53) and also in clonal analysis (2, 46). A higher sensitivity for discriminating among related strains of a species (51) and a general agreement between MLEE and RAPD data have also been observed, which point to the value of RAPD analysis in such studies.

In conclusion, the observed genetic heterogeneity of *P. gingivalis* isolates in the human population might reflect the following four features: (i) the diversity of the species worldwide; (ii) the absence of a significant environmental reservoir of infectious organisms; (iii) the rarity of extrafamilial person-to-person spread and, thus, relatively strong competition among clones that would select clones that are most fit to ensure posttransmission survival in the new host; and (iv) evolutionary changes, including periodic selection in a clone during its long-term residence in its human host.

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