# Diversity of *Phytophthora megakarya* in Central and West Africa revealed by isozyme and RAPD markers

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*Phytophthora megakarya* is an important pathogen of cocoa in Africa. We used isozyme and RAPD markers to estimate the genetic diversity and structuring among 161 isolates, from the known distribution area of the fungus which corresponds to the cocoa belt in Ghana, Togo, Nigeria, Cameroon, Gabon and Sao Tome. Thirty six and 44 multilocus patterns were identified with isozymes and RAPDs, respectively. Patterns were separated into two highly differentiated genetic groups with both types of markers, one located in Central Africa and the other in West Africa. This distribution coincides with two major biogeographical domains which may reflect an ancient evolution of *P. megakarya* in this part of Africa. The genotypic diversity was lower in West Africa as compared to Central Africa. Inside Central Africa, isolates from Gabon and Sao Tome were highly differentiated based on RAPDs. Four intermediate marker patterns corresponding to isolates sampled near the border between Nigeria and Cameroon were putatively derived from genetic exchanges between the two major groups. The mating type determination permitted to confirm the high prevalence of A1 over A2. Although clonal multiplication seems to be the rule, indices of other reproduction means have been detected.

*Phytophthora megakarya* Brasier & M. J. Griffin is a diploid heterothallic oomycete. It is one of the causal agents of black pod rot, the most severe disease affecting cocoa (*Theobroma cacao* L.) in Africa. Black pod rot is a worldwide disease of cocoa that was first thought being due to a single species, *Phytophthora palmivora* (Butler, 1919). At the end of the 1970s, fine morphological and karyotype analyses permitted the identification of four species, *P. capsici*, and *P. citrophthora*, already described on other crops, *P. palmivora* and *P. megakarya* (Brasier & Griffin, 1979). *P. megakarya* is clearly distinguished from the three other species based on its karyotype. The distinction between *Phytophthora* species has also been shown to be efficient with isozymes (Blaha, 1994) and with molecular tools based on repetitive DNA (Panabières *et al*., 1989), species-specific primers designed to amplify the ribosomal internal transcribed spacer (Lee *et al*., 1993), restriction of PCR amplified ribosomal internal transcribed spacer (Cooke & Duncan, 1997) and species-specific anonymous RFLP probes (Sackey & Luterbacher, 1994).

*P. megakarya* has only been found in Africa (Ortiz-Garcia *et al*., 1994). It coexists with *P. palmivora* in Ghana, Togo, Nigeria, Gabon and Sao Tome (Brasier & Griffin, 1979; Zentmyer, 1988). In West Africa, *P. megakarya* is in extension towards West but it has not been identified yet in Ivory Coast (Djiekpor *et al*., 1982; Dakwa, 1988; Luterbacher & Akrofi, 1994). In Cameroon, the morphological characterization of more than 2000 *Phytophthora* isolates (unpublished results) seems to indicate that only *P. megakarya* is responsible for pod rot although *P. palmivora* was mentioned from this country at the end of the 1970s (Bakala, 1981).

*P. megakarya* is the most destructive *Phytophthora* on cocoa. When no treatment is used, losses are as high as 80% in Cameroon (Despréaux *et al*., 1988; Berry & Cilas, 1994) and in Gabon (Anon., 1990), and 100% in Ghana (Dakwa, 1988). Comparatively, the incidence of *P. palmivora* pod rot is often limited to 20–30%. Chemicals used to control *P. megakarya* are expensive. The emergence of cheap control strategies requires a better understanding of the epidemiology, the genetic diversity and the reproduction process of the pathogen. This will be of particular importance to characterize host-pathogen interaction and develop efficient breeding schemes for resistance.

Currently the genetic diversity of *P. megakarya* has only been poorly characterized. An isozyme study was conducted on 15 isolates (Oudemans & Coffey, 1991) and mitochondrial DNA polymorphism was investigated based on 12 isolates (Förster *et al*., 1990). Both studies strongly differentiated isolates from Cameroon and Nigeria. In this paper we present a more comprehensive study of the genetic diversity of *P. megakarya*, based on 161 isolates collected from six countries of Central and West Africa, as revealed by isozyme markers, RAPD markers and mating type.

**Table 1.** Origin and details of isolates used

| Location | N | Year | Mating type[1] | Isozyme phenotypes[1] | RAPD phenotypes[1] |
|---|---|---|---|---|---|
| Cameroon (72, 19, 28)[2] | | | | | |
| Fako (FK) | 7 | 1995 | A1 | I33 (3), I35 (2), I36 (2) | R7, R8 (3), R28 (2), R29 |
| Haut Nyong (HN) | 5 | 1995 | A1 | I31 (5) | R17 (4), R18 |
| Haute Sanaga (HS) | 6 | 1995 | A1 | I31 (6) | R17 (6) |
| Manyu (MA) | 5 | 1995 | A1 | I31 (4), I34 | R1, R31, R33, R34 (2) |
| Mbam (MB) | 15 | 1994, 1995 | A1 | I1, I4, I9, I10, I14, I31 (5), I32 (4), I33 | R11, R12, R13, R14 (3), R15, R17 (4), R18, R20, R27, R39 |
| Mefou (MEF)[3] | 5 | 1990, 1994 | A1 | I1 (5) | R17 (5) |
| Meme (MM) | 9 | 1994, 1995 | A1 | I3, I7, I31 (3), I37 (4) | R28 (4), R30, R31, R32, R33 (2) |
| Mfoundi (MF) | 4 | 1989, 1994 | A1 (3), A2 | I1 (3), I2 | R17 (2), R21, R26 |
| Ndé (NDE) | 1 | 1994 | A1 | I7 | R22 |
| Ndian (ND) | 3 | 1995 | A1 | I37 (3) | R28 (3) |
| Ntem (NT) | 1 | 1994 | A1 | I8 | R24 |
| Nyong et Kellé (NK) | 1 | 1994 | A1 | I1 | R19 |
| Nyong et Mfoumou (NM) | 6 | 1995 | A1 | I31 (6) | R17 (5), R18 |
| Nyong et So'o (NS) | 1 | 1994 | A1 | I1 | R17 |
| Océan (OC) | 2 | 1990, 1994 | A1 | I5, I11 | R25, R44 |
| Unknown (isolate 184) | 1 | — | A2 | I15 | R16 |
| Gabon (11, 5, 6)[2] | | | | | |
| Koulamoutou (KO) | 2 | 1982 | A1 | I12, I20 | R40, R41 |
| Makokou-Est (MA) | 3 | 1982 | A1 | I12 (3) | R41, R42, R43 |
| Oyem-CM (OYC) | 1 | 1982 | A1 | I17 | R43 |
| Oyem-Est (OYE) | 5 | 1982 | A1 | I12 (2), I18, I19 (2) | R23, R24, R41 (3) |
| Sao Tome (14, 4, 4)[2] | | | | | |
| Clara Dias (CL) | 1 | 1994 | A1 | I21 | R35 |
| Pedroma (PE) | 3 | 1994, 1995 | A1 | I21 (2), I30 | R36 (3) |
| Poto (PO) | 7 | 1994, 1995 | A1 | I7, I9, I21 (2), I30 (3) | R35 (2), R36 (4), R38 |
| Queluz (QU) | 3 | 1995 | A1 | I30 (3) | R35, R37 (2) |
| Nigeria (50, (9, 8)[2] | | | | | |
| Ibeku (IBE) | 9 | 1995 | A1 | I22 (9) | R1 (9) |
| Ibule (IBU) | 6 | 1995 | A1, A2 (5) | I24, I25, I26, I27 (3) | R4 (5), R5 |
| Idi-Ayunre (ID) | 15 | 1995 | A1 | I22 (13), I28 (2) | R1 (10), R2, R3 (2), R10 (2) |
| Ikom (IK) | 6 | 1995 | A1 | I22 (6) | R1 (6) |
| Owena (OW) | 3 | 1995 | A1 | I22, I28, I29 | R1, R6, R9 |
| Uhonmora (UH) | 10 | 1995 | A1 | I22 (9), I23 | R1 (10) |
| Unknown (isolate P1663) | 1 | — | A1 | I13 | R1 |
| Ghana (10, 2, 2)[2] | | | | | |
| Ashanti (AS) | 1 | 1994 | A1 | I13 | R4 |
| Brong Ahafo (BR) | 5 | 1993 | A1 | I13 (5) | R1 (5) |
| Volta (VO) | 2 | 1994 | A1 | I16 (2) | R1 (2) |
| Western (WE) | 2 | 1994 | A1 | I13 (2) | R1 (2) |
| Togo (4, 1, 2)[2] | | | | | |
| Kloto (KL) | 2 | 1988, 1991 | A1 | I13 (2) | R1 (2) |
| Litimé (LI) | 2 | 1991 | A1 | I13 (2) | R1, R2 |

[1] Number in brackets corresponds to the number of isolates for a given phenotype when there is more than one. For mating type, this is valuable only when type A2 is present.

[2] $(x, y, z)$; $x$ = Total no. of isolates; $y$ = no. of isozyme phenotypes; $z$ = no. of RAPD phenotypes.

[3] Includes isolates from *Cola nitida*.

## MATERIALS AND METHODS

***Isolate collection.*** One hundred and fifty six isolates have been collected from naturally infected cocoa pods in the field between 1982 and 1995 (Table 1). The distribution of collection areas is shown on Fig. 1. In Cameroon the whole cocoa producing area was sampled. In Nigeria sampling was at six agronomic stations with the helpful collaboration of Drs O. A. Olunoyo, K. Badaru and E. B. Esan from Cocoa Research Institute of Nigeria. Isolates from Ghana and Togo were randomly collected from several fields in the cocoa producing area. They have kindly been provided by Drs S. T. Sackey (Cocoa Research Institute of Ghana) and E. K. Djiekpor (Institut de Recherche du Café et du Cacao, Togo), respectively. Two reference strains of *P. megakarya* with a known mating type were included in this study, 309 (A1) collected in Cameroon in the area of Mbam and 184 (A2) which collection site in Cameroon is unknown (Blaha, 1995). One isolate from Nigeria, P1663 (IMI, Kew) and two, NS130 and NS131, collected in Cameroon from naturally infected fruits of cola (*Cola nitida*), were also added.

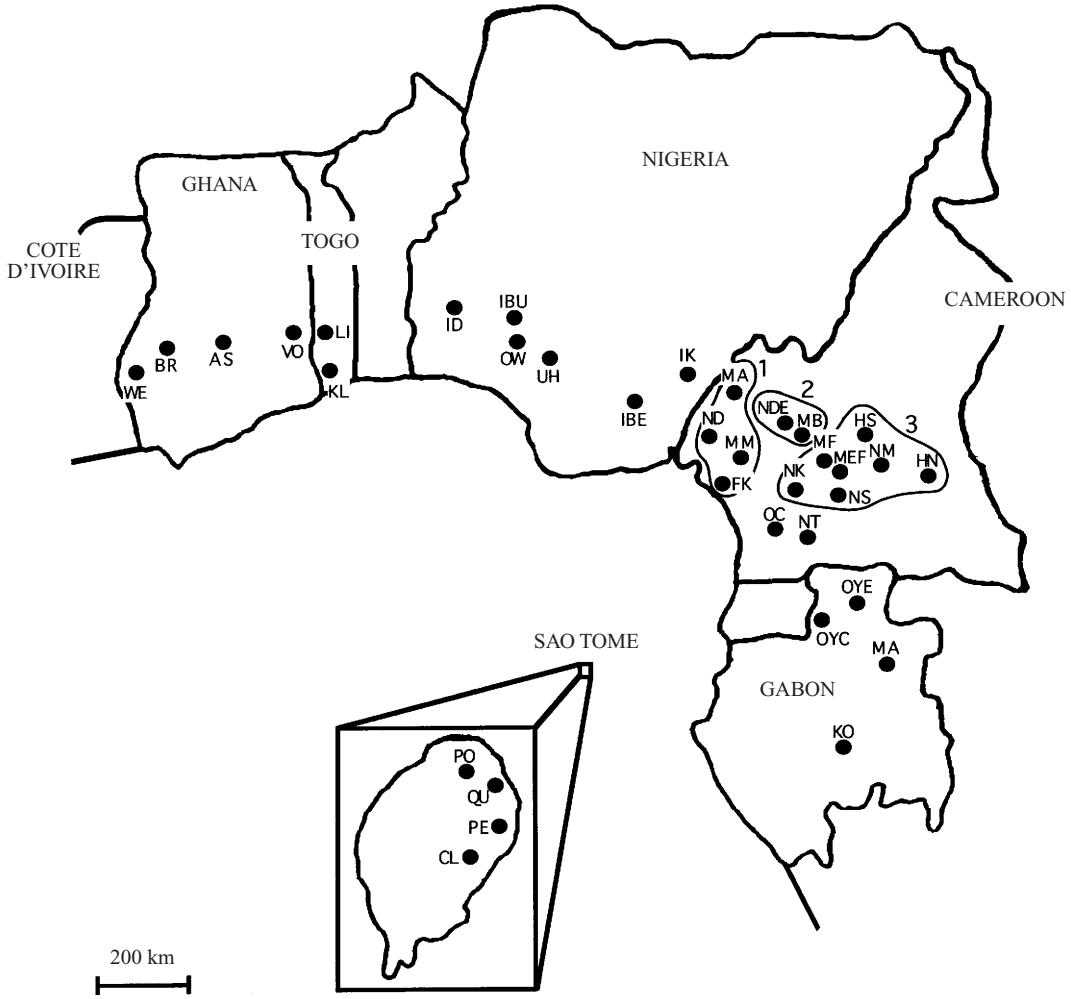


**Fig. 1.** Map showing isolate collection sites. Site initials are shown in Table 1. Explanations about the three marked regions in Cameroon are given in the text.

***Mating type determination.*** The isolates were paired on carrot agar medium (Ribeiro, 1978) with the A1 and A2 reference strains, to determine the mating type. The presence of oospores was scored from 15–30 d after incubation.

***Isozyme and RAPD analysis.*** Plich agar medium (Bonnet *et al*., 1985) was used to produce mycelium for isozyme and RAPD analysis. Methods for enzyme extraction, starch gel electrophoresis and enzyme staining are described in Pasteur *et al*. (1987).The enzymes used were isocitrate dehydrogenase (ICD), malate dehydrogenases (MDH1 and MDH2), glucose-6-phosphate dehydrogenase (G6PDH) on Tris citrate buffer at pH 8·0; phosphoglucose isomerase (PGI), mannose phosphate isomerase (MPI), phosphoglucomutase (PGM) on histidine buffer at pH 6·0; hexokinase (HK), fumarase (FUM), phosphogluconate dehydrogenase (PGD), adenosin deaminase (ADA), peptidase (PEP) on Tris phosphate buffer at pH 7·4. Peptidase has been used on two substrates, leucine-tyrosine (L-T) and glycine-leucine (G-L).

For RAPD analysis, DNA extraction was performed by grinding 300 mg of dry mycelium to a fine powder in liquid nitrogen and incubating at 65 °C for 90 min in 5 ml of extraction buffer (0·16 M sodium citrate, 60 mM $Na_2EDTA$, 1·2% (w/v) SDS, 10 mM sodium sulphite). The extract was centrifuged at 10000 ***g*** for 20 min. The supernatant was removed and mixed with the same volume of isopropanol. The resulting DNA precipitate was spooled out and resuspended in 1 ml TE buffer (10 mM Tris-HCl pH 8, 1 mM $Na_2EDTA$). RAPD analysis was performed as described by Williams *et al*. (1990) using 5 ng of DNA template, with a DNA Thermal Cycler (PE Applied Biosystems, New Jersey, U.S.A.) programmed for an initial denaturation of 4 min at 94°, followed by 45 cycles of 45 s at 93°, 1 min at 35°, and 2 min at 72°. The PCR-amplified DNA fragments were then separated by electrophoresis on a 2% (w/v) agarose gel run at 16 V $cm^{-1}$ for 3 h in TAE buffer, and stained with ethidium bromide. We used nine primers, OPR04, OPR05, OPR06, OPR11, OPR14, OPM04, OPM06, OPM07 and OPM17 from Operon Technologies (California, U.S.A.) kits previously screened for reliability and polymorphism among 40 primers.

***Data analysis.*** For each isozyme system, the different patterns observed were considered as the different modalities of a same genetic descriptor since a genetic interpretation allowing to identify locus and alleles was not possible for most of them. For RAPDs, each band was assumed to

represent a single genetic locus. Each polymorphic band was scored as 1 for presence and 0 for absence among isolates.

Similarities between isolates were calculated for all possible pairwise comparisons using the Simple Matching index. Computations were made with the NTSYS computer package (Rohlf, 1993). The correspondence between the isozyme and the RAPD similarity matrices was examined with the test of Mantel (1967). Significance level was computed over 500 permutations. Distances between multilocus patterns or phenotypes were summarized using two statistical techniques. First, Factorial Analyses of Correspondences (FAC) (Benzécri, 1973; Lebart *et al*., 1984) were performed using the ADDAD software (Anon. 1983). Secondly, cluster analyses were performed with NTSYS using the simple matching similarity index and the UPGMA clustering method. The confidence limits of the nodes produced by the dendrograms were evaluated using bootstrapping with 2000 iterations with the software Winboot (Yap & Nelson, 1996).

A measure of the genotypic diversity in each geographical region was given by:

$$G = 1/\Sigma p_i^2,$$

Where $p_i$ is the frequency of the $i$th multilocus pattern in that region (Stoddart & Taylor, 1988). For a given region, $G$ is equal to one when all isolates have the same genotype and is equal to the number of isolates when all isolates have a unique pattern. The percentage of maximum possible diversity ($G/N$) was also given to compare regions with different sample sizes (McDonald *et al*., 1994; Drenth *et al*., 1996).

## RESULTS

***Identification of two major genetic groups.*** Thirteen isozyme systems were used on the 161 isolates. Ten of them gave between two and four different banding patterns or modalities and three systems were monomorphic (Fig. 2). This gave a total of 28 different patterns or modalities for the 10 polymorphic isozyme systems. This permitted the resolution of isolates into 36 multilocus isozyme patterns or phenotypes numbered I1–I37 (Table 2). Three phenotypes were highly frequent with a corresponding number of isolates of 38, 29 and 13, respectively. Eighteen isolates had a unique phenotype.

Nine RAPD primers were used on the same 161 isolates. This revealed 33 reliable polymorphic amplified products or bands. The size of the selected bands ranged from 0·5 to 3·0 kb. The 33 bands resolved isolates into 44 multilocus RAPD patterns or phenotypes, R1–R44. Examples of RAPD patterns are given in Fig. 3. Two phenotypes were highly frequent, corresponding to 49 and 27 isolates, respectively. Twenty six isolates had a unique phenotype.

FAC and UPGMA cluster analysis were performed separately over the isozyme and RAPD multilocus patterns. With both types of markers two major groups were separated along the first axis of the FACs (Fig. 4), one formed by patterns mostly corresponding to isolates from West Africa (Togo, Ghana, Nigeria) and the other formed by patterns exclusively corresponding to isolates from Central Africa (Sao Tome, Gabon, Cameroon). Several isozyme modalities (Table 3) and RAPD bands were specific to each group. On the UPGMA dendrogram performed with RAPDs, bootstrap node values of the West and Central African groups were 76% and 46% respectively (Fig. 5). The node values of the two groups on the dendrogram performed with isozymes were 43% and 46%, respectively. Interestingly, they were boosted to 83% and 76% when genotype I34 was removed from the analysis (see below).

Five isolates did not respect this general scheme. They were from Manyu and Fako in West Cameroon, near the border with Nigeria. They had intermediate multilocus banding patterns between the two groups, for either isozymes or RAPDs: (i) One Manyu isolate has the isozyme genotype I34. This genotype appears as intermediate between the two main groups discriminated on the first plane of the FAC (Fig. 4). It has a banding pattern specific to Central Africa for three systems and a banding pattern otherwise specific to West Africa, for two systems (Table 3). This isolate has RAPD multilocus pattern R1, unique in Central Africa but frequent in West Africa. (ii) Three isolates from Fako have the RAPD pattern R8 and one has pattern R7. Patterns R7 and R8 are closely related to the West African group based on the FAC (Fig. 4) and UPGMA dendrogram (Fig. 5), but they also have four bands specific to the Central African multilocus patterns (data not shown). Those four isolates have either isozyme patterns I35 or I36 which are included in the Central African

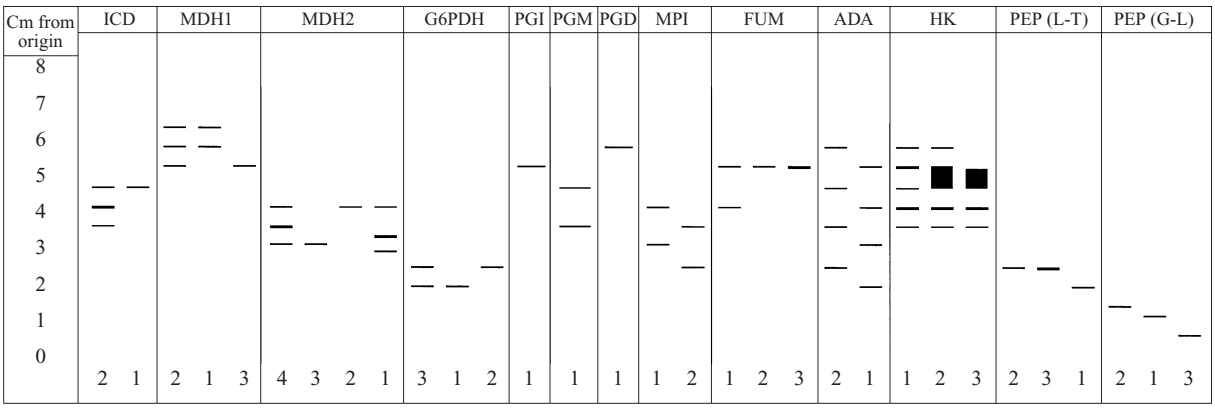


**Fig. 2.** Representation of the different possible modalities observed for each of the 13 isozyme systems used in the present study, among 161 *Phytophthora megakarya* isolates.

**Table 2.** Isozyme multilocus patterns of 161 *P. megakarya* isolates. Isozyme modality numbers for each isozyme system correspond to those in Fig. 2

| Pattern | Icd | Mdh1 | Mdh2 | G6pdh | Mpi | Fum | Ada | Hk | Pep-lt | Pep-gl |
|---|---|---|---|---|---|---|---|---|---|---|
| I1 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| I2 | 1 | 3 | 4 | 1 | 1 | 1 | 1 | 1 | 3 | 1 |
| I3 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 | 2 | 3 |
| I4 | 1 | 2 | 3 | 1 | 1 | 3 | 1 | 1 | 3 | 1 |
| I5 | 1 | 1 | 4 | 1 | 2 | 1 | 1 | 1 | 3 | 3 |
| I7 | 1 | 1 | 4 | 1 | 1 | 1 | 1 | 1 | 2 | 2 |
| I8 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| I9 | 1 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 2 | 2 |
| I10 | 1 | 2 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| I11 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| I12 | 1 | 1 | 4 | 1 | 2 | 1 | 1 | 1 | 2 | 2 |
| I13 | 2 | 1 | 2 | 2 | 1 | 2 | 2 | 3 | 2 | 2 |
| I14 | 1 | 2 | 3 | 1 | 1 | 3 | 1 | 1 | 2 | 2 |
| I15 | 1 | 2 | 2 | 1 | 1 | 2 | 1 | 1 | 1 | 1 |
| I16 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 3 | 2 | 2 |
| I17 | 1 | 1 | 4 | 1 | 2 | 2 | 1 | 1 | 2 | 2 |
| I18 | 1 | 2 | 4 | 1 | 2 | 1 | 1 | 1 | 2 | 2 |
| I19 | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 |
| I20 | 1 | 1 | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 2 |
| I21 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 2 | 2 |
| I22 | 2 | 1 | 2 | 2 | 1 | 1 | 2 | 3 | 2 | 2 |
| I23 | 2 | 1 | 2 | 2 | 2 | 1 | 2 | 3 | 2 | 2 |
| I24 | 1 | 1 | 2 | 3 | 2 | 1 | 2 | 2 | 2 | 2 |
| I25 | 2 | 1 | 2 | 3 | 2 | 1 | 2 | 2 | 2 | 2 |
| I26 | 2 | 1 | 2 | 3 | 1 | 1 | 2 | 2 | 2 | 2 |
| I27 | 1 | 1 | 2 | 3 | 1 | 1 | 2 | 2 | 2 | 2 |
| I28 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 3 | 2 | 2 |
| I29 | 2 | 1 | 2 | 3 | 2 | 1 | 2 | 3 | 2 | 2 |
| I30 | 1 | 2 | 4 | 1 | 1 | 1 | 1 | 1 | 2 | 2 |
| I31 | 1 | 1 | 4 | 1 | 2 | 1 | 1 | 1 | 1 | 1 |
| I32 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 1 | 1 | 1 |
| I33 | 1 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| I34 | 2 | 2 | 2 | 1 | 1 | 1 | 2 | 3 | 1 | 1 |
| I35 | 2 | 2 | 4 | 1 | 2 | 1 | 1 | 1 | 1 | 1 |
| I36 | 1 | 2 | 4 | 1 | 2 | 1 | 1 | 1 | 1 | 3 |
| I37 | 1 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 3 |

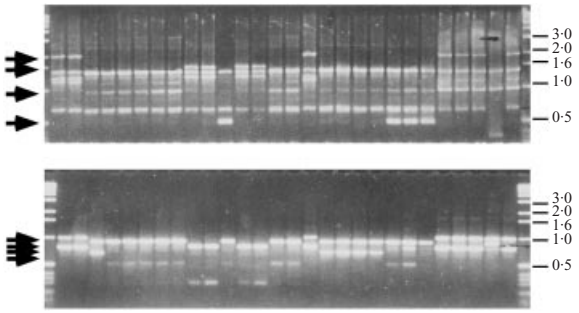


**Fig. 3.** Examples of RAPD pattern corresponding to primers OPM07 (above) and OPR11 (below) observed on a sample of 28 isolates of *P. megakarya*. Arrows indicate bands selected for scoring. One band scored for primer OPM07 is not observable on this sample. Size of selected bands may be deduced from the ladder given on the right (kb).

group. Interestingly, I35 is the only pattern from Central Africa with I34 that has isozyme modality ICD-2, otherwise common in West Africa (Table 3).

The intermediate genetic patterns observed for those five isolates are in favour of possible genetic exchanges between the two main genetic groups. This is corroborated by their geographical origin since they have all been collected at the geographical frontier between the two main genetic groups.

***Within group differentiation.*** RAPDs permitted three subgroups to be identified in Central Africa. A first subgroup formed by four multilocus patterns from Gabon and one from Océan in Southern Cameroon was discriminated along axis 2 of the FAC (Fig. 4). A second subgroup formed by the four patterns from Sao Tome was discriminated along axis 3 which explains 12% of the variability (data not shown). A third subgroup formed by four patterns from Mbam in Central

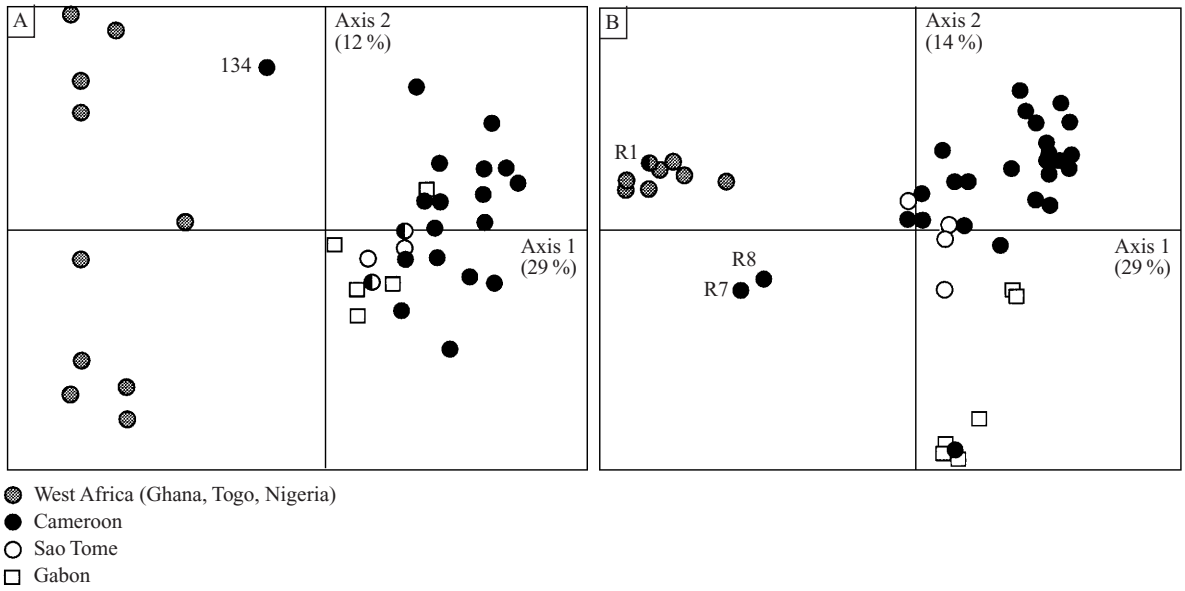


**Fig. 4.** Plots of the first two axes generated by the Factorial Analysis of Correspondence conducted on the isozyme (A) and the RAPD (B) multilocus patterns.

**Table 3.** Frequency of isozyme modalities of all polymorphic isozyme systems in West and Central Africa. For each system, modality frequencies are significantly different ($P < 0{\cdot}0001$) between WA and CA with Fisher exact test

| Isozyme system | Isozyme pattern | Pattern frequency | |
|---|---|---|---|
| | | WA | CA |
| ICD | ICD-1 | 0·14 | 0·97 |
| | ICD-2[1] | 0·86 | 0·03 |
| MDH1 | MDH1-1 | 1 | 0·6 |
| | MDH1-2 | 0 | 0·39 |
| | MDH1-3 | 0 | 0·01 |
| MDH2 | MDH2-1 | 0 | 0·01 |
| | MDH2-2 | 1 | 0·05 |
| | MDH2-3 | 0 | 0·26 |
| | MDH2-4 | 0 | 0·68 |
| G6PDH | G6PDH-1 | 0·05 | 1 |
| | G6PDH-2 | 0·84 | 0 |
| | G6PDH-3 | 0·11 | 0 |
| MPI | MPI-1 | 0·89 | 0·49 |
| | MPI-2 | 0·11 | 0·51 |
| FUM | FUM-1 | 0·77 | 0·92 |
| | FUM-2 | 0·23 | 0·02 |
| | FUM-3[2] | 0 | 0·06 |
| ADA | ADA-1 | 0 | 0·99 |
| | ADA-2[3] | 1 | 0·01 |
| HK | HK-1 | 0 | 0·99 |
| | HK-2 | 0·09 | 0 |
| | HK-3[3] | 0·91 | 0·01 |
| PEP(L-T) | PEP(L-T)-1 | 0 | 0·68 |
| | PEP(L-T)-2 | 1 | 0·29 |
| | PEP(L-T)-3 | 0 | 0·03 |
| PEP(G-L) | PEP(G-L)-1 | 0 | 0·61 |
| | PEP(G-L)-2 | 1 | 0·28 |
| | PEP(G-L)-3 | 0 | 0·11 |

[1] Pattern restricted to genotypes I34 and I35 in Central Africa.
[2] Pattern specific to genotypes I4, I14 and I32 from Mbam, Cameroon.
[3] Pattern restricted to genotype I34 in Central Africa.

Cameroon was discriminated along axis 4 which explains 11% of the variability (data not shown). Interestingly, for three of those multilocus patterns, corresponding isolates have a specific common isozyme modality for FUM (Table 3). On the UPGMA cluster analysis, the bootstrap node values associated with those three subgroups were 76%, 88% and 86%, respectively (Fig. 5). The multivariate analysis performed on the isozyme data did not reveal those subgroups. This may be due to the small number of polymorphic isozyme systems in each of the two main genetic groups. With isozymes, West African genotypes were slightly differentiated along axis 2 of the FAC. This was due to a significant association (Fisher exact test, $P < 0{\cdot}05$) between modalities of PGD and HK in the West African group which may reflect linkage disequilibrium between corresponding loci.

The two isolates collected on *Cola nitida* have exactly the same multilocus pattern, for both isozymes and RAPDs. They are also identical to the other isolates collected in the same area on cocoa.

***Genotypic diversity.*** The simple matching similarity index between isolates computed with isozymes and RAPDs have been compared with the test of Mantel. The normalized Mantel statistic was $r = 0{\cdot}82$ ($P < 0{\cdot}005$) indicating a good general concordance between the two sets of data. The correspondence between isozyme and RAPD multilocus patterns is shown on Fig. 6. The total number of different overall multilocus patterns based simultaneously on both types of markers was 63, 47 corresponding to isolates from Central Africa and 16 to isolates from West Africa. The genotypic diversity index $G$ and $G/N$ were computed on combined multilocus isozyme and RAPD patterns. It showed that the genetic variability is clearly lower in West Africa as compared to Central Africa (Table 4).

In West Africa the low variability is due to the high frequency of two related multilocus patterns, (I13, R1) and (I22, R1) since I13 and I22 are differing for a single isozyme system, FUM (Table 2). In Ibule and Owena, the variability seems higher than anywhere else although few isolates have been sampled.

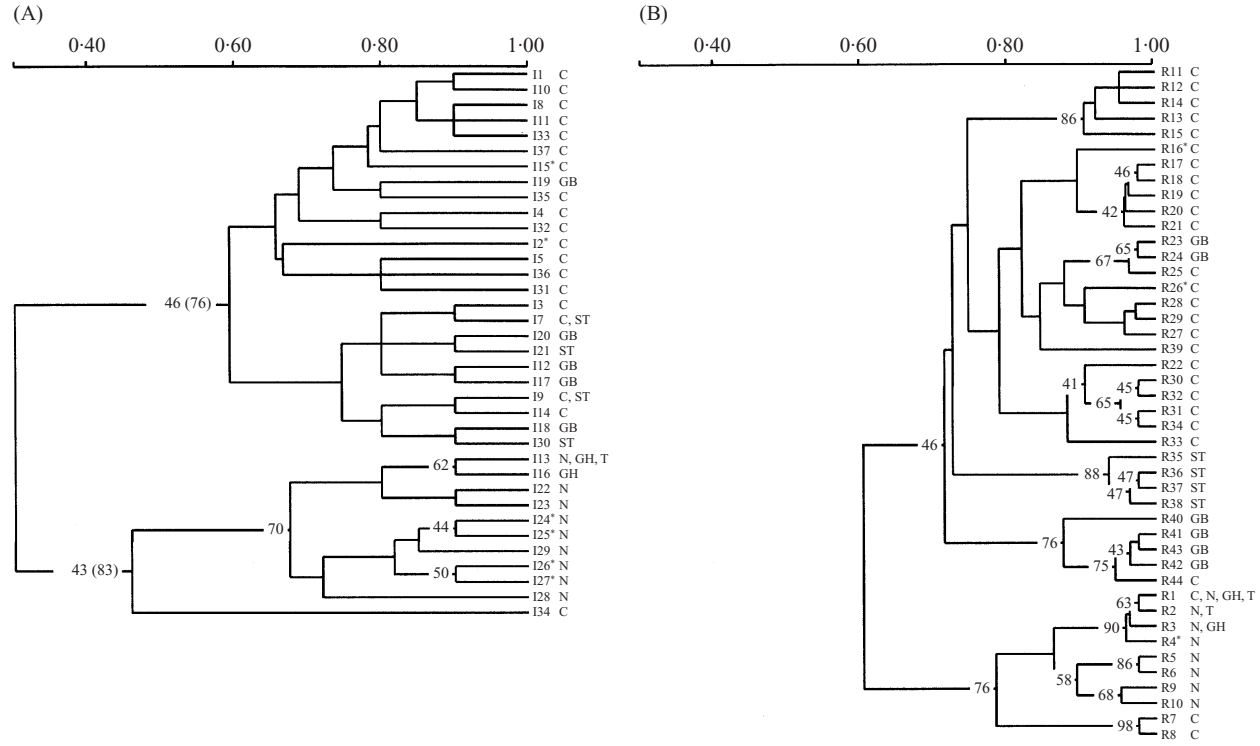


**Fig. 5.** UPGMA dendrograms performed on the isozyme (A) and RAPD (B) multilocus patterns. Bootstrap node values are given when they exceed 40%. Countries where patterns have been observed are indicated as follow; C: Cameroon, GB: Gabon, GH: Ghana, N: Nigeria, ST: Sao Tome, T: Togo. Patterns including A2 isolates are identified with an asterisk. Values in brackets on the isozyme dendrogram are obtained when I34 is removed from the analysis.

In Central Africa the diversity is higher in Cameroon, Sao Tome and Gabon (Table 4). In Cameroon, where the number of isolates collected is the highest, the distribution of the variability seems heterogeneous. To demonstrate this, we grouped collecting areas into 3 different regions with approximately equal numbers of isolates. Océan and Ntem, in Southern Cameroon, were excluded from this exercise regarding the small number of isolates available and their affinity with populations from Gabon (Fig. 6). Region 1 corresponded to the mountainous area of West Cameroon, region 2 to the East of the Bamiléké plateau and region 3 to the East of the Sanaga river basin (Fig. 1). The genotypic diversity was much higher in regions 1 and 2 compared to region 3 (Table 4). In region 3, two related multilocus patterns, (I31, R17) and (I1, R17) were highly frequent.

***Mating type distribution.*** Among the 159 isolates evaluated for mating type, 153 had the A1 type and six had the A2 type. This confirms previous results obtained by Maddison & Griffin (1981), indicating the predominance of the A1 mating type in *P. megakarya*. This is in favour of a general predominancy of asexual reproduction in this species. One of the A2 isolates was collected in Cameroon, the five others in Nigeria. The A2 isolate from Cameroon was not closely related to any A1 isolate, either with isozymes or with RAPDs (Fig. 5 and 6). The A2 isolates from Nigeria were all from Ibule. Six isolates have been collected in this location, so the A2 type is thus locally predominant. The five A2 type isolates have the same RAPD multilocus pattern, R4, but have four different isozyme patterns, I24, I25, I26 and I27. Among those, only two systems are polymorphic, ICD and MPI (Table 2). Those systems have two possible modalities each (Fig. 2). The four multilocus patterns thus corresponded to the four possible pairwise modality combinations between the two systems. The A1 type isolate has the same isozyme pattern, I27, as one of the A2 type isolates and has closely related RAPD pattern, R5. This gives some evidences for a possible sexual reproduction in this location.

## DISCUSSION

In the present study, the within-species genetic variation of *P. megakarya* was investigated with isozymes and RAPDs, based on 161 isolates collected from six countries of West and Central Africa.

The confrontation between cocoa and *P. megakarya* may be a recent event and black pod rot may result from a transfer of the fungus from a native African host plant species to cocoa. Several arguments favour this hypothesis: (i) Cocoa originated in South and Central America and was brought to Africa by Man during the 19th century; the current geographical distribution of *P. megakarya* is restricted to Africa and it has never been reported from America. (ii) This study indicates that the host range of *P. megakarya* may not be restricted to cocoa. It was isolated from fruits of *Cola nitida*, a species of

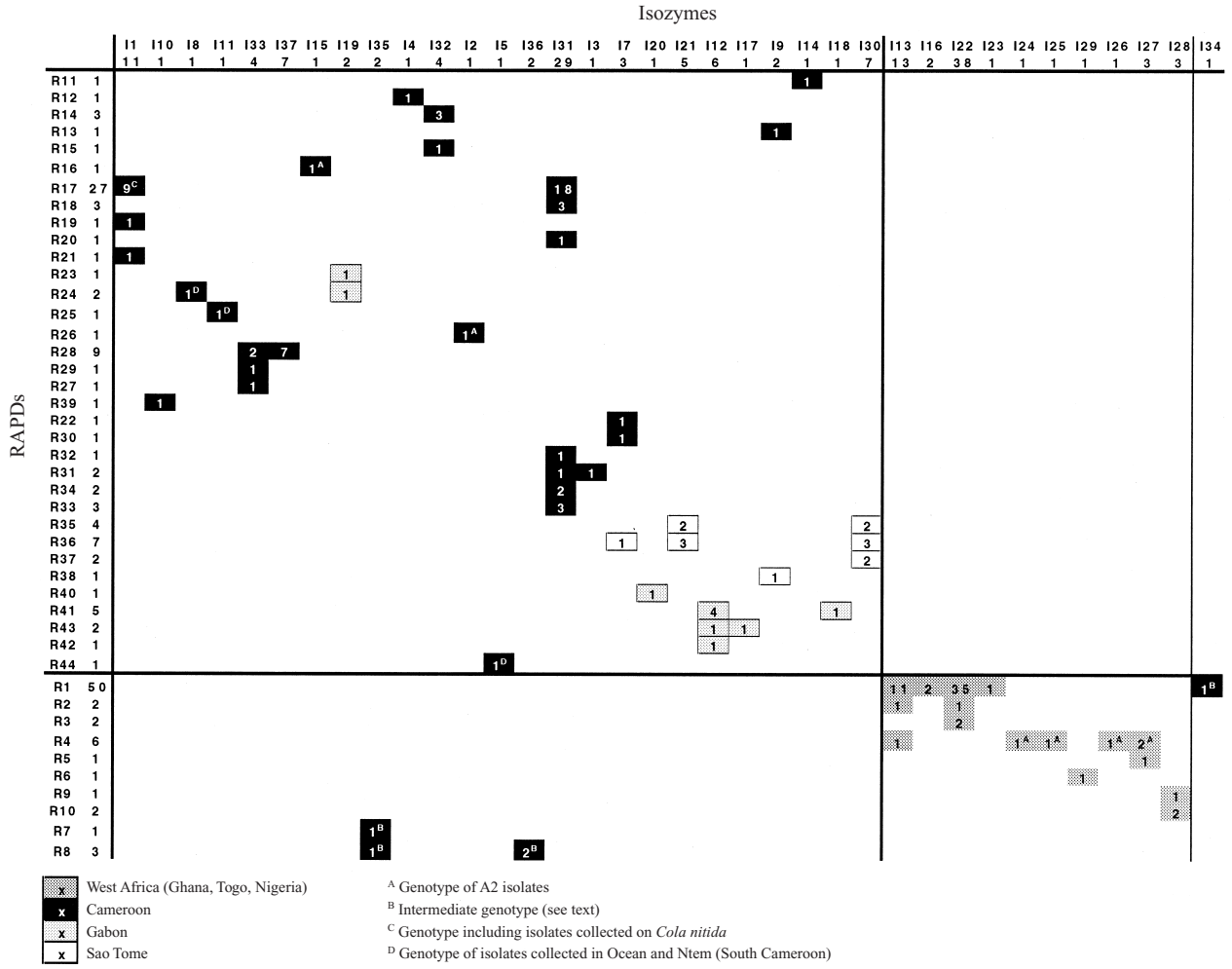


**Fig. 6.** Correspondence between isozyme and RAPD multilocus patterns. Isozyme patterns are shown on the first row and RAPD patterns on the first column. Patterns are ordered according to UPGMA dendrograms of Fig. 5. The number of isolates corresponding to a given isozyme or RAPD pattern is given on the second row or second column, respectively. The number of isolates corresponding to a given pair of isozyme and RAPD patterns is given at the point of intersection between corresponding row and column. Bold lines indicate the two main clusters observed on each dendrogram. A light line separates genotype I34 according to the isozyme dendrogram.

the Sterculiaceae to which cocoa also belongs. Moreover, many *Phytophthora* species have a broad host range. (iii) The two host species are not associated with different marker patterns of the fungus. Isolates collected on *C. nitida* had the same RAPD and isozyme patterns than the ones collected on cocoa in the same area. (iv) Many plant species are potential candidates for being the ancestral host of *P. megakarya*. *C. nitida* is native to West Africa and wild species of the Sterculiaceae are numerous in the African rain forests.

The hypothesis of an African origin for *P. megakarya* is reinforced by the population genetic structure observed within this species. Two highly differentiated genetic groups were identified with geographical distributions roughly delimited by the Cameroon–Nigeria border, one corresponding to isolates from West Africa and the other to isolates from Central Africa. This geographical structure is in agreement with the results of Oudemans & Coffey (1991) based on isozymes and the results of Förster *et al.* (1990) based on mitochondrial DNA, on samples of isolates from Cameroon and Nigeria. A tentative explanation for the separation of *P. megakarya* into two genetic groups could be found in the refugia theory (Maley, 1996). During the Quaternary era, glacial cycles induced arid climates in the tropics which reduced the African rain forest to a series of refuges. This led to a fragmentation of the distribution of the native species and their pathogens, conducting to an isolation and a possible differentiation of the populations. In the area covered by our study, two main biogeographical domains resulting from those climatic past events are distinguished based on the repartition of plant (White, 1979) and animal (Moreau, 1966) species. Highly differentiated populations corresponding to those two domains have also been reported for a native coffee, *Coffea canephora*, in a study based on isozymes (Berthaud, 1984). The limit between the two domains stands in eastern Nigeria, near the Cameroonian border and thus approximately corresponds to the geographical limit between the two main groups identified in *P. megakarya*. Furthermore, as for *P. megakarya*, a general higher level of diversity has been noticed in Central Africa as compared to West Africa. The convergence observed for the repartition and the diversity distribution of

**Table 4.** Genotypic diversity *G* and maximum possible diversity (G/N) of geographical samples of *P. megakarya* observed at different scales. Computation of *G* is based on the multilocus isozyme and RAPD genotypes

| | N[1] | n[2] | *G* | *G*/N (%) |
|---|---|---|---|---|
| Central Africa | 97 | 47 | 15 | 16 |
| Cameroon | 72 | 32 | 10 | 14 |
| Region 1[3] | 24 | 13 | 7·4 | 31 |
| Region 2[3] | 16 | 12 | 9·2 | 57 |
| Region 3[3] | 28 | 6 | 2·6 | 9 |
| Gabon | 11 | 8 | 5·3 | 48 |
| Sao Tome | 14 | 7 | 6·1 | 44 |
| West Africa | 64 | 16 | 3 | 5 |
| Nigeria | 50 | 13 | 2 | 4 |
| Ibeku | 9 | 1 | 1 | 11 |
| Ibule | 6 | 5 | 3 | 50 |
| Idi-Ayunre | 15 | 4 | 1·3 | 9 |
| Ikom | 6 | 1 | 1 | 17 |
| Owena | 3 | 3 | 3 | 100 |
| Uhonmora | 10 | 2 | 1·2 | 12 |
| Togo | 4 | 2 | 1·6 | 40 |
| Ghana | 10 | 3 | 1·9 | 18 |

[1] Number of isolates.
[2] Number of multilocus genotypes.
[3] See text and Fig. 1.

different species of the fungus, plant and animal kingdoms in this part of Africa is a novel argument in favour of an ancient evolution of *P. megakarya* in that region.

The West African population of *P. megakarya* may have originated in Nigeria. The overall genotypic diversity observed in West Africa was low, except in two locations from Nigeria. In one of those locations, Ibule, the occurrence of sexual reproduction was suspected. The presence of the fungus seems to be recent in Togo and Ghana. It was first reported in 1982 in Togo (Djiekpor *et al*., 1982) and 1985 in Ghana (Dakwa, 1988; Luterbacher & Akrofi, 1994). It has not been identified yet in Ivory Coast. Few genotypes have been detected in either Ghana or Togo with either isozymes or RAPDs and all of those genotypes were present in Nigeria except one. The extension of the fungus is probably mostly due to the clonal multiplication of a small number of individuals.

In Central Africa the overall genotypic diversity was higher than in West Africa. It was particularly high in Western Cameroon (regions 1 and 2 on Fig. 1) which corresponds to an important refuge during the late Quaternary arid periods (Maley, 1987). With isozymes, no clear structuring was detected and genotypes from Sao Tome and Gabon were closely related to those of Cameroon. With RAPDs, several populations appeared as clearly differentiated. This was the case for the isolates collected in Sao Tome, some isolates collected in Gabon and Southern Cameroon and some isolates collected in the area of Mbam, Cameroon. Interestingly, several Quaternary refuges have been reported for Southern Cameroon and Gabon (Maley, 1987). The difference of structuring observed with isozymes and RAPDs may likely reflect sampling errors due to the difference in the number of loci targeted. Only seven isozyme systems were polymorphic in Central Africa against 30 RAPD markers. This may also reflect a difference in the mutation rate of targeted loci. Isozymes reveal polymorphisms at expressed genes and RAPDs represent variation at all types of DNA sequences including repeated sequences.

This study confirms the global predominance of the A1 mating type observed by Maddison & Griffin (1981), in both genetic groups. This is in favour of a predominancy of clonal reproduction which is corroborated by the high frequency of a few multilocus genotypes over large geographical ranges in West Africa and Cameroon. Nevertheless other reproduction means cannot be excluded: (i) in Ibule, Nigeria, the presence of both mating types, the nature of polymorphism observed with isozymes and the small genetic distance observed between sympatric A1 and A2 isolates may indicate that sexual reproduction occurs in this area. (ii) The five isolates with intermediate genotypes originating from Western Cameroon may indicate the possibility of genetic exchanges between the two groups either resulting from sexual reproduction or from heterocaryosis.

The diversity scheme observed here for *P. megakarya* may offer some guidelines for future prospects. The occurrence of sexual reproduction in the wild may be further examined by concentrating efforts in the locations where is it suspected. The epidemiology of the fungus may be investigated by taking into account the possible role of native wild plant species. The choice of isolates to characterize host–pathogen interactions may be rationalized by taking into account the diversity structure revealed by neutral markers. In this perspective, the availability of genetic maps for cocoa (Lanaud *et al*., 1995) could be helpful for a comparative analysis of partial resistance genes between different isolates.

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