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Variability in the phloem restricted plant trypanosomes (*Phytomonas* spp) associated with wilts of cultivated crops

Isoenzyme comparison with the lower trypanosomatids

EMMANUELLE MULLER[1], DANIEL GARGANI[1], VALÉRIE SCHAEFFER[1], JAMIE STEVENS[2], CARMEN FERNANDEZ-BECERRA[3], MANUEL SANCHEZ-MORENO[3] and MICHEL DOLLET[1]*

[1] *Laboratoire de Phytovirologie des Régions Chaudes, CIRAD, BP 5035, 34032 Montpellier cedex 01, France;* [2] *Insect Biology Group, School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK;* [3] *Grupo de Investigacion en Bioquimica y Parasitologia Molecular, Universidad de Granada, E-18071 Granada, Spain;* * *Author for correspondence*

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Abstract. Plant trypanosomatids (*Phytomonas* spp) have been isolated from the crude sap of coconut trees and oil palm trees affected with Hartrot or Marchitez diseases in South America and *Alpinia purpurata* affected with wilt in the Caribbean. They are also commonly isolated from the fruits and latex of Euphorbiaceae or Asclepiadaceae. Thirty-four *Phytomonas* stocks were studied by isoenzyme electrophoresis (11 loci) in order to investigate genetic variability in the intraphloemic group. Our results showed that variability in phloem restricted *Phytomonas* is very high, and that Marchitez or Hartrot associated trypanosomatids are not readily separated into two distinct groups. Moreover, the two isolates from *Alpinia* are very close to the other intraphloemic isolates. The results confirm the existence of several distinct groups, comprising of at least 2 groups of phloem restricted trypanosomatids, not related to the host species, 3 groups of latex trypanosomatids, and one group of fruit trypanosomatids.

Introduction

When plant trypanosomatids were discovered [Lafont, 1909], a new genus – *Phytomonas* – was suggested in order to distinguish them from trypanosomatids occurring in man or animals [Donovan, 1909]. These plant parasites are transmitted by insects, in which they multiply as it was described in some cases [McGhee and Hanson, 1964].

Biological and molecular biological studies have shown a high degree of variability among plant trypanosomatids: firstly, differences were identified among *Phytomonas* populations based on the types of tissue or cell which they colonize (latex, phloem or fruit) [Dollet, 1994]. Only intraphloemic flagellates are specifically associated with diseases occurring in Latin America: Hartrot of coconut, Marchitez sorpresiva of oil palm and phloem necrosis of coffee [Stahel, 1931; Parthasarathy et al., 1976; Dollet et al.,

1977; Dollet, 1984]. The other plant trypanosomatids parasitize plants and fruits without inducing a pathological syndrome in the plant. In fact, the first studies using isoenzymes and restriction patterns of kinetoplast DNA (kDNA) showed clear differences between *Phytomonas* from latex and phloem restricted *Phytomonas* [Ahomadegbe et al., 1990; Guerrini et al., 1992; Ahomadegbe et al., 1992].

In the present work, we investigate isoenzyme variability in the intraphloemic *Phytomonas* group in order to study the epidemiology of the Hartrot and Marchitez diseases, and to confirm the distinction between these trypanosomatids and others originating from fruits or latex. Two isolates from Grenada island associated with another wilt (*Alpinia purpurata*, Zingiberaceae) are included in this study [Gargani et al., 1992]. We also include isolates from other genera of the lower trypanosomatid group [Wallace et al., 1992]: *Crithidia, Herpetomonas* and *Leptomonas* in order to estimate the level of separation possible by this technique.

Materials and methods

Table 1 summarizes the origin of the 22 primary isolates of *Phytomonas* and the 12 clones which were also characterized. Isolates were obtained from various hosts across a broad ecogeographic range, providing a diverse sample. Isolates from French Guiana come from two plantations 100 km apart: Combi and Saut Sabbat. Details of trypanosomatids originating from insects (*Crithidia, Herpetomonas* and *Leptomonas*) are presented in Table 2.

Laticiferous plant and fruit isolates were cultured in axenic Grace medium [Grace, 1962] (Gibco) supplemented with 10% heat inactivated foetal calf serum (FCS). Insect isolates were cultured in axenic Grace medium supplemented with 10% FCS, 1% yeast extract, 1% fraction V of bovine albumin. The intraphloemic isolates were adapted to culture as previously described in Grace medium with 10% FCS, in the presence of insect haemocyte cells [Ménara et al., 1988, Yunker et al., 1967].

Enzyme electrophoresis was performed on cellulose acetate plates (Helena Laboratories, Beaumont TX) according to the procedure described by Ben Abderrazak et al. [1993]. Ten enzyme systems were assayed: alanine aspartate transaminase (E.C.2.6.1.2., ALAT), glucose 6 phosphate dehydrogenase (E.C.1.1.1.49., G6PDH), glucose phosphate isomerase (E.C.5.3.1.9., GPI), glutamate oxaloacetate transaminase (E.C.2.6.1.1., GOT), isocitrate dehydrogenase (E.C.1.1.1.42., ICD), malate dehydrogenase (E.C.1.1.1.37., MDH), malic enzyme (E.C.1.1.1.40., ME), mannose phosphate isomerase (E.C.5.3.1.8., MPI), peptidase 1 (E.C.3.4.11 or 13, PEP1, substrate leucyl-leucyl-leucine), peptidase 2 (E.C.3.4.11 or 13, PEP2, substrate leucyl-L-alanine). These correspond to eleven loci.

Table 1. Phytomonas stock origins

Stock	Host	Isolation date	Country	Locality
HART 1	*Cocos nucifera*	1986	French Guiana	Saut Sabbat
HART 1 cl. 1	*C. nucifera*	1986	French Guiana	Saut Sabbat
HART 1 cl. 2	*C. nucifera*	1986	French Guiana	Saut Sabbat
HART 3	*C. nucifera*	1987	Brazil	Moju (Para)
HART 3 cl. 1	*C. nucifera*	1987	Brazil	Moju (Para)
HART 3 cl. 2	*C. nucifera*	1987	Brazil	Moju (Para)
HART 4	*C. nucifera*	1987	French Guiana	Saut Sabbat
HART 6	*C. nucifera*	1990	French Guiana	Combi
HART 9	*C. nucifera*	1990	French Guiana	Organabo
HART 10	*C. nucifera*	1990	French Guiana	Combi
HART 11	*C. nucifera*	1990	Venezuela	Irapa (Sucre)
HART 12	*C. nucifera*	1991	Venezuela	San Augustin (Sucre)
HART 13	*C. nucifera*	1992	Brazil	Moju (Para)
MAR 1	*Elaeis guineensis*	1989	Colombia	La Cabaña (Meta)
MAR 1 cl. 1	*E. guineensis*	1989	Colombia	La Cabaña (Meta)
MAR 1 cl. 2	*E. guineensis*	1989	Colombia	La Cabaña (Meta)
MAR 2	*E. guineensis*	1989	Ecuador	Shushufindi (Oriente)
MAR 2 cl. 1	*E. guineensis*	1989	Ecuador	Shushufindi
MAR 2 cl. 2	*E. guineensis*	1989	Ecuador	Shushufindi
MAR 2 cl. 3	*E. guineensis*	1989	Ecuador	Shushufindi
MAR 2 cl. 4	*E. guineensis*	1989	Ecuador	Shushufindi
MAR 4	*E. guineensis*	1991	Colombia	Acacias (Meta)
MAR 5	*E. guineensis*	1992	Colombia	Meta
MAR 6	*E. guineensis*	1992	Venezuela	Zulia
MAR 7	*E. guineensis*	1993	Venezuela	Maturin
ALP I	*Alpinia purpurata*	1991	Grenada	Grand Etang
ALP II	*A. purpurata*	1991	Grenada	Grand Etang
TOMA cl. *	*Lycopersicon esculentum*	1992	Spain	Andalousia
CHERI *	*Anona cherimolia*	1993	Spain	Almuñecar (Andalousia)
TREFL *	*Trifolium* sp.	1993	Spain	Almuñecar (Andalousia)
ASCL 1	*Asclepias curassavica*	1991	Venezuela	Irapa (Sucre)
ASCL 3	*A. curassavica*	1993	Venezuela	Irapa (Sucre)
E HI GU	*Euphorbia hirta*	1987	French Guiana	Saut Sabbat
EM1 cl.	*Euphorbia pinea*	1980	France	Montpellier

cl. = clone * isolated by Sanchez-Moreno et al.

Since allelic interpretation was often difficult, a non-allelic reading of zymograms was used: each distinguishable and reproducible pattern was considered as a distinct genotype, the precise allelic composition of which remained unknown.

The level of polymorphism was calculated for each set of stocks corresponding to a given host or geographic origin (Table 3). This parameter is estimated by the following ratio: number of polymorphic loci/total number of loci surveyed.

The relationships among the stocks were estimated according to

Table 2. Insect trypanosomatid origins

Stock	Host	Source
Herpetomonas samuelpessoai	*Zelus leucogrammus*	Institut G. Roussy, Villejuif, France Professor G. Riou ATTC[1] n° 30252
Herpetomonas megaseliae	*Megaselia scalaris*	ATCC n° 30209
Herpetomonas muscarum	Blow fly, Kenya	Liverpool School of Tropical Medicine Professor D. H. Molyneux[3]
Crithidia oncopelti	*Oncopeltus fasciatus*	University of Bordeaux T. Baltz[4]
Crithidia luciliae	ND[2]	University of Bordeaux T. Baltz[4]
Crithidia acanthocephali	*Acanthocephala femorata*	ATCC n° 30251
Crithidia deanei	*Zelus leucogrammus*	ATCC n° 30255
Leptomonas seymouri	*Dysdercus suturellus*	ATCC n° 30220
Leptomonas mirabilis	*Cynomyopsis cadaverina*	ATCC n° 30263

[1] ATCC American Type Culture Collection
[2] ND not done
[3] provided by Swiss Tropical Institute, Basel, Switzerland
[4] provided by Bernhard-Nocht Institute, Hamburg, Germany.

Table 3. Level of polymorphism

Stock origin	Level of polymorphism
Total sample	1
Intraphloemic sample	0.91
Coconut sample	0.91
Oil palm sample	0.64
Intraphloemic French Guiana sample	0.91 (from oil palm and coconut)
Intraphloemic Venezuela sample	0.36 (from oil palm and coconut)
Crithidia	1
Leptomonas	1
Herpetomonas	0.84

their phenetic distances: this parameter was estimated using Jaccard's distance.

$$Dij = 1 - \frac{C}{Ni + Nj - C}$$ where C is the number of bands in common

between the i^{th} and j^{th} stocks, and Ni and Nj are the total numbers of bands in each stock. This value is calculated for each enzyme and the average is reported in the distance matrix. The Unweighted Pair Group Method of Analysis (UPGMA) [Sneath and Sokal, 1973] was used to yield a phenetic tree summarizing the distance matrix obtained [Stevens and Cibulskis, 1990, Fig 1].

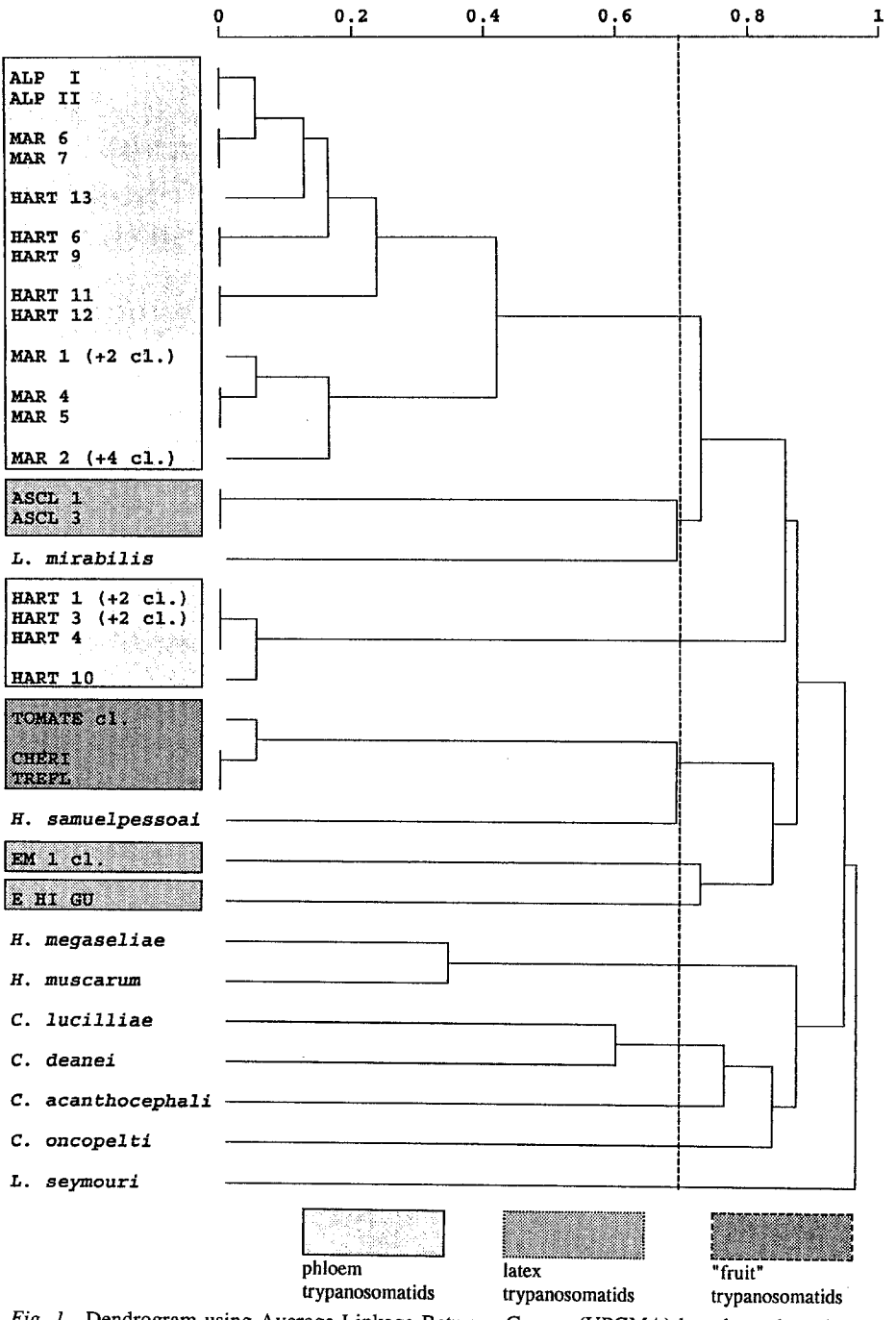

Fig. 1. Dendrogram using Average Linkage Between Groups (UPGMA) based on phenetic distances of Jaccard. Beyond the approximate value of 0.7, great distance values render tentative any attempt of hierarchization. We consider only the lower clustering levels to be reliable. cl. = clone.

Results and discussion

Our results indicate a close relationship between a) the two intraphloemic isolates from *Alpinia* and b) the other intraphloemic *Phytomonas*. This relationship is somewhat unexpected in regard to the distant geographic origins and the different host families of these isolates (*A. purpurata* originates from Grenada island in the Caribbean, the other intraphloemic isolates originate from South America).

Moreover, Marchitez and Hartrot associated *Phytomonas* could not be separated into two distinct groups. Therefore, intraphloemic isolates cannot be classified according to host species, geographic origin or vector species. Nevertheless, two isolates transmitted, presumably by two different vectors, in two different places appear to be very similar: HART 1 transmitted by *Lincus croupius* in French Guiana and HART 3 suspected to be transmitted by *Ochlerus* [Dollet et al., 1993] in Para state in Brazil (see dendrogram, Fig. 1).

The intraphloemic isolates form a heterogeneous group in which the level of polymorphism is very high (0.9; Table 3). Within this group, two subgroups can be distinguished. The first consists of four isolates (HART 1, 3, 4 and 10 from Brazil and French Guiana), while the second includes the remaining isolates (isolates of Hartrot, Marchitez and those from *A. purpurata* from widely separated areas of South America & the Caribbean: French Guiana, Venezuela, Colombia, Ecuador and Grenada island). Isolates originating from latex (from South America or the Mediterranean) belong to other distinct groups: ASCL 1 & 3; EM1; E HI GU. The similarity between the three isolates originating from Spain is of particular note: two isolates originate from fruits (*Lycopersicon esculentum* and *Anona cherimolia*) and one originates from the stem of *Trifolium sp.* which showed symptoms of stripe. The location of this isolate in the plant is not yet elucidated. Both isolates from *Trifolium* sp. and from *A. Cherimolia* come from the same orchard.

As in the previous study [Guerrini et al., 1992 with 30 isolates of *Phytomonas*], hierarchization of the upper subdivisions of this set of stocks must be considered cautiously. Thus, the results do not permit the groups to be considered as linked other than at the lower clustering levels.

The results of the current research broadly confirm the differences observed between plant trypanosomes in the study of Guerrini et al. [1992]; for example, we obtain almost the same distance between the isolate from the latex of *Euphorbia hirta* originating from French Guiana and the isolate from the latex of *Euphorbia pinea* originating from France. In addition, this study shows the necessity of using a large number of isolates; isolates of Hartrot appeared different from those of Marchitez in the previous study because only four Hartrot isolates and two Marchitez isolates were tested. Indeed, all of the Hartrot isolates studied previously were in the same subgroup as identified in this work. For some enzymes, results were not

reproducible and were not included in the analysis. Consequently, we were not able to use exactly the same enzymes as in the previous study and thus the lower subdivisions between the isolates in the two studies are not identical.

The close relationship between *A. purpurata* isolates of the Windward Islands and other intraphloemic isolates from South America is probably explained by the transport of *Alpinia* rhizomes from one place to another. The presence of wilt of *A. purpurata* on the continent remains to be confirmed. The high variability of intraphloemic isolates reflects the high variability of the French Guiana coconut sample which is also the largest sample. Three different species of *Lincus* were identified at the beginning of our studies [1983] at the experimental plantation of Saut Sabbat in French Guiana: *Lincus appolo, L. dentiger* and *L. croupius* [Dolling, 1984]. *L. lamelliger* was also found in another plantation. Thus, this variability could be due to the presence of these different species of vectors. However, it seems that since about 1985, only *L. croupius* has remained in Saut Sabbat [Louise et al., 1986, Dollet not published]. Similarly, it should be noted that the isolates HART 3 and HART 13 originating from the same coconut plantation in Moju (Para state, Brazil), where the only suspected vector is *Ochlerus* sp., belong to two very distant groups.

In accordance with Guerrini et al., our results may indicate the existence of a clonal structure for intraphloemic *Phytomonas*. Three groups of stocks (ALP I & II, MAR 6 & 7, HART 6, 9, 11, 12 & 13; MAR 1, 2, 4 & 5; HART 1, 3, 4 & 10) each appear homogeneous. The isolation of identical multilocus genotypes over large geographic areas and at intervals of several years apart (HART 1 & 3; MAR 1, 4 & 5) is a strong indication of clonal propagation [Tibayrenc et al., 1991; Tibayrenc and Ayala, 1991].

The two *Leptomonas* appear very different (distance 0.95 from each other); *Leptomonas seymouri* seems to be very distant from all other isolates, whereas *Leptomonas mirabilis* is relatively close to some isolates of *Phytomonas* (distance 0.70 from ASCL 1 & 3, MAR 1, 2, 4 & 5). This agrees with data from restriction analysis of ribosomal DNA and kinetoplast DNA and patterns of arginine cycle enzymes, which show great heterogeneity in the genus *Leptomonas* [Camargo et al., 1992]. In addition, *L. mirabilis* is more difficult to culture than *L. seymouri*. Indeed, *Phytomonas* isolates, and especially intraphloemic isolates, are difficult to culture [Ménara et al., 1988]; however, this is not the case for *Crithidia, Herpetomonas* or *Leptomonas* in the same medium. Furthermore, *Leptomonas* as well as *Phytomonas* have generally only promastigote forms.

It is interesting to note that *Crithidia, Herpetomonas* and *Leptomonas* are well separated by this technique. However, according to the results obtained in the distance matrix, we observe that ASCL 1 & 3, MAR 1, 2, 4 & 5 are more closely related to *L. mirabilis* than to HART 1, 3, 4 & 10. Thus isolates from *L. esculentum* and *A. cherimolia*, both originating from

fruit, and the isolate from *Trifolium* sp. may be similarly more closely related to *Herpetomonas samuelpessoai* than to other plant trypanosomes.

Conclusions

This study suggests that variability in the intraphloemic *Phytomonas* group is very high. In this group of intraphloemic isolates, we can distinguish two further homogeneous subgroups. But this segregation is neither related to the geographic origin nor the host species. However, a large number of isolates, or investigations using other markers, such as kDNA, could possibly lead to confirmation of this variability.

However, we are able to confirm the existence of several groups of plant trypanosomatids: at least 2 groups of phloem restricted trypanosomatids, 3 groups of latex trypanosomatids, and one group of 'fruit' trypanosomatids.

Furthermore, it appears that Hartrot of coconut, Marchitez of oil palm and the wilt of *A. purpurata* should also be considered not as three different diseases, but should probably be regrouped as slightly different forms of the same disease. Of course, symptoms may be dependent on the host and on climatic conditions [McCoy, 1982].

In future studies, it will be interesting to include intraphloemic isolates originating from *Coffea* [Stahel, 1931] in order to ascertain if phloem necrosis of Coffee can be regrouped with the other diseases.

In conclusion, it seems that, at least for the present, we were unable to associate particular groups to particular species and it appears necessary to confirm the groupings with other techniques (RFLP of the kDNA, size of the minicircles of kDNA, Random Amplified Polymorphic DNA-RAPD-studies of the total genome).

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