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A Somatic Incompatibility Study of Genetic Variation within and between Biological and Geographic Populations of the Cocoa Pathogen *Crinipellis perniciosa* (Stahel) Singer

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ABSTRACT. *Somatic incompatibility studies showed the amount of genetic variability existing within and between biological and geographic populations of the basidiomycete fungus Crinipellis perniciosa. This fungus causes witches' broom disease of cocoa (C-biotype) and is found in association with a similar disease of Solanum species (S-biotype) and a symptomless infection of liana species (L-biotype). The C-biotype of this fungus was found to be clonal within sites at Belem (3 separate sites), Camaca and Urucuca in Brazil and at Pichilingue in Ecuador but greater genetic diversity occurred at Manaus (Brazil) where 5 somatic compatibility groups (SCGs) were identified in one plantation and 3 groups at one other site. Representative C-biotype isolates from different geographic sites were all somatically incompatible. The S-biotype population studied at Manaus (Brazil) consisted of at least 2 SCGs and one S-biotype isolate from Bahia in Brazil was incompatible with both of the Manaus SCGs. As in previous studies the Ecuadorian L-biotype was found to be highly variable with a minimum of 24 and 23 SCGs identified at Pichilingue and Jauneche, respectively. The consequences of variability in populations of the cocoa pathogen, C. perniciosa, are discussed.*

INTRODUCTION

The basidiomycete fungus *C. perniciosa* (Stahel) Singer is a hemibiotrophic pathogen causing witches' broom disease of cocoa (*Theobroma*

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cacao) and related species throughout South America (Baker and Holliday, 1957; Wood and Lass, 1985; Rudgard, 1989).

Genetic variation in *C. perniciosa* was first studied in populations of the fungus occurring on cocoa and related species (C-biotype) (Wheeler and Mepsted, 1982, 1984, 1988; McGeary and Wheeler, 1988; Andebrhan, 1988; Laker, 1989). *C. perniciosa* has now been found in association with plants from families other than the Sterculiaceae, such as the liana *Arrabidaea verrucosa* (Griffith, 1989), *Solanum* species (Bastos and Evans, 1985) and *Bixa orellana* (Bastos and Andebrhan, 1986); these biological groups of the fungus are referred to here as the L-biotype, S-biotype and B-biotype, respectively.

The terminology used in studies of fungal population biology and the significance of mycelial individualism in phytopathogenic fungi has been reviewed by Rayner (1991). The microscopic hyphal interactions at the contact zone of different mycelia give rise to the macroscopic features of somatic incompatibility such as clear zones between the mycelia, barrage zones and pigment production (Ainsworth, 1987 and Ainsworth and Rayner, 1989). Relatedness of individuals within a species, and the strength of incompatibility reactions between them, have been discussed by Coates *et al.* (1981) and Adams and Roth (1967). The rate of production and extent of the antagonism zone has been related to ecological strategies (Rayner *et al.*, 1984). The physiological expression of somatic incompatibility is under complex genetic control; (Croft and Dales, 1984; Dales and Croft, 1990; Kay and Vilgalys, 1992). The number of somatic compatibility (sc) loci involved in bringing about somatic incompatibility has been estimated by a number of workers (Esser, 1974; Perkins *et al.*, 1976; Puhalla and Spieth, 1985; Croft and Dales, 1984) and there is evidence for the allelic nature of these genes (Brasier, 1984; Croft and Dales, 1984).

Studies of somatic incompatibility in a population of a basidiomycete will reflect the type of sexual reproduction occurring in the population or the accumulation of genetic types which have arisen over time and been propagated by vegetative reproduction (or homothallic "sexual" reproduction). There is strong evidence for a homothallic breeding strategy in the C-biotype of *C. perniciosa* whereas the L-biotype exhibits a tetrapolar heterothallic system (Griffith and Hedger,1994a). Less extensive studies indicate that the S- and B-biotypes also have a primarily homothallic breeding system similar to that in the C-biotype (Griffith and Hedger, 1994a).

The aim of this project was to make comparisons of *C. perniciosa* genetic variation from a range of hosts at different geographic locations (both

on a local and continental scale). It was hoped that the range of variation in the *C. perniciosa* populations would give an indication of the extent of genetic recombination occurring in this fungus under field conditions. The occurrence of new genotypes of *C. perniciosa* is an important consideration in the long term planning for breeding witches' broom disease resistant cocoa.

MATERIALS AND METHODS

The C- and S-biotypes of *C. perniciosa* were collected from sites in Brazil (October to December 1990) and C- and L-biotypes in Ecuador (February to March 1992) (Wilson, 1995).

Mycelial cultures of *C. perniciosa* were isolated from basidiocarps and infected plant material using a selective agar medium (Griffith and Hedger, 1994b). Cultures were maintained under sterile liquid paraffin on malt yeast extract agar (MYEA) slopes stored at 15°C. These mycelia were subcultured at six monthly intervals.

If less than 10 isolates were available from a population they were paired against each other in all combinations. Based on the results of initial pairings tester isolates were chosen to represent the SCGs in each population and then paired against each other. Where large numbers of isolates were available from one population pairings were carried out in two or three rounds with tester isolates from the first round being used in the second round and so on.

Paired isolates were grown on a V8 juice medium prepared as described by Griffith and Hedger (1994b) with slight modifications. To 1l of V8 juice (Campbell's soups Ltd., Norwich, U.K.) 15 g of calcium carbonate were added and the mixture shaken for 5 min before centrifuging at 5,000 g for 20 min. The clarified V8 juice (= supernatant) was diluted to 20% with distilled water before adding 2 g agar and 2 g sucrose per 100ml. The medium was autoclaved (20 min at 121°C, 15 psi) before dispensing into sterile 9 cm plastic petri dishes (25 ml per plate). Inoculations were carried out using 5 mm plugs taken from the growing margin of 7 to 14 day-old-cultures grown at 25°C on MYEA. Up to 5 isolates were paired against each other in all combinations in one petri dish using the system devised by Griffith (1989). Where ambiguous results were obtained, or the fast rate of growth of one or more isolates prevented the meeting of 2 isolates in the arrangement, the pairings were repeated as necessary. Plates were sealed with insulating tape and incubated upside down in a 25°C constant temperature room with low level

background lighting. Examination of plates was initially carried out after 2-3 weeks and again after 6-8 weeks.

RESULTS AND DISCUSSION

C-biotype

The incompatibility reactions occurring between C-biotype isolates obtained within a geographic area were relatively weak. No pigmentation formed but slight barrage zones and relatively clear zones of appressed mycelium formed between the incompatible isolates. Although the C-biotype isolates collected from Belem sites 1, 2 and 3 were obtained from cocoa and related species (Table 1) no incompatible pairing reactions were found from isolates within these sites. Similarly no incompatible reactions were observed after within site pairings of isolates from Urucuca or Camaca in Bahia, Brazil. C-biotype isolates from Pichilingue Ecuador were all somatically compatible with each other. The results of the present study of the C-biotype SCG distribution agrees in general with previous studies (McGeary and Wheeler, 1988; Andebrhan, 1988; Griffith, 1989) finding the fungus clonal within geographic sites and incompatible between geographic sites (the exception being the results for Manaus sites 1 and 2).

Representative isolates from the sites at Urucuca and Camaca in Bahia (Brazil) were incompatible with each other and with isolates from the other geographic sites studied here. With respect to the origin of the recent WBD outbreaks reported in Bahia (Pereira, 1989) it would appear that the fungus is once again clonal in nature, within restricted geographic sites, suggesting that if infection of cocoa in this state occurred only recently the two sites studied were each colonised independently by different SCGs of the C-biotype. It would seem that the introduction of the disease to Bahia was by man but the somatic incompatibility of representative isolates from Urucuca and Camaca with isolates from Belem, Manaus, and Ecuador gave no indication of the possible origin of the infection propagules which cannot be identified without further extensive comparisons of *C. perniciosa* from this and other regions where WBD occurs.

Previous somatic incompatibility studies of isolates from Manaus (Brazil) detected no incompatibility groups within this region (McGeary and Wheeler, 1988). The present study of a larger group of isolates from 2 sites at Manaus detected 8 SCGs. The majority of isolates at Manaus site 1 belonged to SCG C6 and the other SCGs had only a few representatives (Table 1). The

Table 1. Number and origin of SCGs of C-, S- and L-biotypes of *C. Perniciosa*.

SCG	[1]Biotype	[2]Plant host or substrate from which mycelia were isolated	[4]Origin	Number of solates
C1	C	*T.c,T.sp, T.g, T.su, T.o, H.*	Brazil, Belem site 1	55
C2	C	*T.g, T.su, T.o*	Brazil, Belem site 2	39
C3	C	*T.c*	Brazil, Belem site 3	37
C4	C	*T.c*	Brazil, Urucuca	19
C5	C	*T.c*	Brazil, Camaca	7
C6	C	*T.c*	Brazil, Manaus site 1	46
C7	C	*T.c*	Brazil, Manaus site 1	5
C8	C	*T.c*	Brazil, Manaus site 1	5
C9	C	*T.c*	Brazil, Manaus site 1	1
C10	C	*T.c*	Brazil, Manaus site 1	1
C11	C	*T.c*	Brazil, Manaus site 2	2
C12	C	*T.c*	Brazil, Manaus site 2	5
C13	C	*T.c*	Brazil, Manaus site 2	1
C14	C	*T.c*	Ecuador, Pichilingue site 1	12
S1	S	*S.r, S.c*	Brazil, Manaus site 3	25
S2	S	*S.r*	Brazil, Manaus site 3	3
S3	S	unknown	Brazil, Bahia	1
L1	L	not recorded	Ecuador, Pichilingue site 2	1
L2	L	living liana	Ecuador, Pichilingue site 2	3
L3	L	living liana	Ecuador, Pichilingue site 2	6
L4	L	not recorded	Ecuador, Pichilingue site 2	1
L5	L	living liana	Ecuador, Pichilingue site 2	1
L6	L	dead liana	Ecuador, Pichilingue site 2	1
L7	L	dead liana, debris	Ecuador, Pichilingue site 2	5
L8	L	liana, debris	Ecuador, Pichilingue site 2	12
L9	L	living liana, dead liana, debris	Ecuador, Pichilingue site 2	1
L10	L	dead liana	Ecuador, Pichilingue site 2	1
L11	L	debris	Ecuador, Pichilingue site 2	1
L12	L	living liana, dead liana, debris	Ecuador, Pichilingue site 2	8
L13	L	debris	Ecuador, Pichilingue site 2	1
L14	L	debris	Ecuador, Pichilingue site 2	2
L15	L	debris	Ecuador, Pichilingue site 2	1

Cont'd..

SCG	[1]Biotype	[2]Plant host or substrate from which mycelia were isolated	[4]Origin	Number of solates
L16	L	debris	Ecuador, Pichilingue site 2	1
L17	L	debris	Ecuador, Pichilingue site 2	1
L18	L	living liana	Ecuador, Pichilingue site 2	1
L19	L	dead liana	Ecuador, Pichilingue site 2	15
L20	L	dead liana	Ecuador, Pichilingue site 2	1
L21	L	dead liana	Ecuador, Pichilingue site 2	1
L22	L	dead liana	Ecuador, Pichilingue site 2	5
L23	L	dead liana	Ecuador, Pichilingue site 2	1
L24	L	living liana, debris	Ecuador, Pichilingue site 2	3
L25	L	dead liana	Ecuador, Jauneche	1
L26	L	dead liana	Ecuador, Jauneche	1
L27	L	dead liana	Ecuador, Jauneche	2
L28	L	dead liana	Ecuador, Jauneche	1
L29	L	dead liana	Ecuador, Jauneche	1
L30	L	dead liana	Ecuador, Jauneche	1
L31	L	[3]living liana (*P.r*), dead liana	Ecuador, Jauneche	5
L32	L	living liana, debris	Ecuador, Jauneche	3
L33	L	debris	Ecuador, Jauneche	1
L34	L	debris	Ecuador, Jauneche	1
L35	L	dead liana	Ecuador, Jauneche	1
L36	L	dead liana	Ecuador, Jauneche	1
L37	L	debris	Ecuador, Jauneche	1
L38	L	debris	Ecuador, Jauneche	5
L39	L	debris	Ecuador, Jauneche	1
L40	L	debris	Ecuador, Jauneche	2
L41	L	debris	Ecuador, Jauneche	1
L42	L	debris	Ecuador, Jauneche	1
L43	L	debris	Ecuador, Jauneche	3
L44	L	living liana, debris	Ecuador, Jauneche	2
L45	L	debris	Ecuador, Jauneche	1
L46	L	not recorded	Ecuador, Jauneche	1

1 Abbreviations of biotypes : C = C-biotype; S = S-biotype; L = L-biotype
2 Abbreviations of host species names : *T.c = Theobroma cacao; T.sp = Theobroma speciosum; T.g = Theobroma grandiflorum; T.su = Theobroma subincanum; T.o = Theobroma obovatum; H. = Herrania (species unknown); S.r = Solanum rugosum; S.c = Solanum crinitum* .
3 Most living and dead liana material at Pichilingue was of the species *Arrabidaea verrucosa* but *C.perniciosa* was isolated from other species of liana at Jauneche one of which was identified as *Prestonia rotundifolia.*
4 More detailed descriptions of site locations are given in Wilson (1995).

distribution of SCGs at Manaus site 1 was very similar to the situation found within the Ecuadorian Amazon population studied by Griffith (1989) who found that in the Amazon region, 2 main SCGs existed and one of these was compatible with the Ecuadorian coastal SCG. Other SCGs in the Amazon region occurred at low frequency leading to the suggestion that infected cocoa material from the coastal regions had been moved to the Amazon region and recombination between the coastal and Amazon SCGs had produced new C-biotype genotypes (Griffith, 1989).

Coates *et al.* (1981) considered that mating between primary mycelia of outcrossing species was permitted by the suppression of somatic incompatibility mechanisms which prevent successful hyphal fusion between secondary mycelia. It is possible that a similar situation occurs between homokaryons of genetically distinct homomictic mycelia in the time between spore germination and autodikaryosis. Experiments by Wheeler and Mepsted (1984) showed that putative new genotypes of *C. perniciosa* (forming new C-biotype SCGs) could be isolated from brooms produced on cocoa seedlings following inoculation with spores of different C-biotype SCGs. Such SCGs would arise if anastomosis between genetically different homokaryons resulted in a rare C-biotype heterokaryon capable of undergoing meiosis and recombination. Griffith (1989) has speculated that the less well represented SCGs present in C-biotype populations of Amazonian Ecuador could have arisen by coinfection of cocoa meristems by spores from the 2 major SCGs present. This argument may apply to the range of SCGs found at Manaus site 1. Three SCGs were found at Manaus site 2 in a smaller sample (8 isolates) but these were gathered over a wider area from scattered host trees and may reflect a more random sample of a larger *C. perniciosa* population compared with Manaus site 1. Secondary heterokaryosis between offspring of the first rare event and the parental genotypes could give rise to even greater genetic diversity in the population and could explain the relatively high number of SCGs found at Manaus site 2.

S-biotype

Incompatible reactions between the S-biotype isolates at Manaus site 3 (Brazil) were similar in form to those between C-biotype isolates from within a given geographic area. Two S-biotype SCGs were identified at this site. SCG S1 was the most widespread and included isolates obtained from *Solanum crinitum* Lam. (Table 1). Multiple SCGs occurred on a single host plant (examples of S1 and S2 were isolated from different brooms on single *Solanum*

bushes) suggesting that S1 and S2 occur at random in the S-biotype *C. perniciosa* population.

It is possible that the genetic variation in the population of this homomictic biotype is due to rare recombination (see discussion of C-biotype populations). If this is the case then at least 3 genetic groups should exist in the population (2 parental genotypes and at least one recombinant offspring). It is possible that the sample of isolates obtained was not representative of the population as a whole and hence only 2 SCGs were found. Alternatively two or more genetically distinct populations of S-biotype *C. perniciosa* may exist at this site with no genetic recombination occurring.

The existence of the S-biotype in two distant locations (Amazonas and Bahia in Brazil) poses the question of whether these populations have evolved allopatrically. The sole representative isolate from Bahia exhibited a slower growth rate than those from Manaus (observed on pairing plates) but otherwise was very similar in morphology with a relatively sparse and appressed white mycelium. The somatic incompatibility reactions between the Bahia isolate and the Manaus isolates was very clearly defined by a wide clear zone but no barrages or pigments were formed, indicating a moderate incompatibility response identical to that obtained between incompatible pairings of isolates from within Manaus site 1. Unfortunately as only one isolate was available from Bahia the variation of the S-biotype population in this region could not be studied and it is not known if this isolate is representative of the population. As a result of this lack of information it is not possible to speculate on the ecology or evolution of the S-biotype in the Bahia region.

L-biotype

In comparison to the relatively weak somatic incompatibility reactions within the C- and S-biotypes incompatible pairings between the majority of L-biotype isolates were very clearly defined. Clear zones and/or barrage zones occurred between the paired mycelia and pigment formation often occurred. The 74 L-biotype isolates obtained from the Pichilingue site (Ecuador) formed 24 SCGs and at Jauneche (Ecuador) 37 L-biotype isolates were placed into 22 SCGs (Table 1). These 46 SCGs were defined during the initial round of pairing isolates from within the Pichilingue and Jauneche sites. Representative isolates from each of these SCGs were chosen to be paired against each other in all combinations. All of the SCGs defined in the first round of pairing were upheld by the results of the second round. The exceptions to this were 2

combinations of mycelia apparently intermingling suggesting that somatic incompatibility was intransitive in these specific cases.

The distribution of L-biotype SCGs at Pichilingue and Jauneche (Ecuador) were very similar to those described by Griffith (1989) and discussed by Griffith and Hedger (1994b). SCGs were restricted to within a 5 m radius and multiple SCGs were often found in very close proximity. Continuity of SCGs along the length of both living and dead lianas was found at different sites. During this study *C. perniciosa* L-biotype was found along approximately 30 cm of a living liana of *Prestonia rotundifolia* K. Schum. ex Woods. Griffith and Hedger (1994b) discussed the possibility of the L-biotype being specific to *Arrabidaea verrucosa* (Standl) A. Gentry and forming latent infections of this species. The presence of *C. perniciosa* on a living liana of another species may indicate that the L-biotype is not as specialised as previously thought. However, these isolates were also compatible with two isolates from an associated unidentified dead liana. In this case the invasion of the living liana may have been an opportunist infection of the bark layer and not a specific association.

Pairings of isolates of different biotypes and geographic regions.

A subset of C-, S-, and L-biotype SCGs from different geographic regions were chosen to be paired against each other in all combinations. All of the pairings between biotypes produced incompatible reactions but these varied, in some cases, more than previously recorded. As with previous studies the interactions between C-biotype isolates were weak to medium within sites and generally moderately antagonistic between sites with the exception of coastal Ecuadorian isolates which produced strong reactions with all of the Brazilian isolates tested. Reactions between C- and L-biotype isolates were also strong, as found by Hedger *et al.* (1987) and Griffith (1989). The most surprising result was the very weak reaction between a Manaus S-biotype isolate and one of the Jauneche (Ecuador) L-biotype isolates. The S-biotype isolates produced no "very strong" reactions with any of the other L-biotype or C-biotype isolates tested.

As discussed in the introduction, the characteristics of somatic incompatibility expression have been found to be related to the genetic similarity of the mycelia paired (Ainsworth and Rayner, 1989). Hyphal fusions are generally restricted to interspecies pairings. A weak response between two opposing mycelia may be due to the sc loci of each being very similar, resulting in only a very weak response following hyphal fusion, or so different that the

hyphal fusion does not occur. Although no hyphal anastomoses between paired C-biotype isolates of different SCGs have been described, the generally weak reactions (characterised by clear zones) have been assumed to be an indication of the limited differences in sc genes occurring in this homomictic biotype. The stronger response elicited by the coastal Ecuadorian C-biotype has been ascribed to its greater divergence from the rest of the C-biotype population possibly during an early stage of adaptation to *T. cacao* as a host.

It is still unclear how the relatively weak reactions of the S-biotype place it in respect to the C- and L-biotypes. Logically it would seem that the S-biotype appears to have sc genes which are intermediate between those of the C- and L-biotypes and other studies of genetic diversity in *C. perniciosa* indicate more similarity between the S- and L-biotypes than between either of these and the C-biotype (Griffith *et al.*, 1994).

CONCLUSIONS

With respect to sc genes the C-biotype of *C. perniciosa* is variable within local populations in the Amazon basin region of Brazil but clonal within other geographic populations in Brazil and Ecuador. Variation in sc genes also occurs within and between geographic populations of the S-biotype and this biotype is recorded here as occurring on a previously unknown host (*Solanum crinitum* Lam.). L-biotype is the most genetically variable biotype with many SCGs existing within small areas (5 m radius). The L-biotype of *C. perniciosa* may not be specifically associated with the liana *A. verrucosa* as previously thought.

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