Vegetative Compatibility Groups and Parasexual Segregation in *Colletotrichum acutatum* Isolates Infecting Different Hosts

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ABSTRACT

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Heterokaryosis is an important mechanism which provides genetic variability increase in filamentous fungi. In order to assess the diversity of vegetative compatibility reactions existing among *Colletotrichum acutatum* isolates derived from different hosts, complementary *nit* mutants of each isolate were obtained and paired in all possible combinations. Vegetative compatibility groups (VCG) were identified among the isolates according to their ability to form viable heterokaryons. Seven VCGs were identified among the isolates, one of which contained isolates from different hosts. VCGs 2 and 6 contained two and three members, respectively; VCG-3

contained four members, and four VCGs (1, 4, 5, and 7) contained a single one. This study shows, for the first time, the isolation and the parasexual segregation of a heterozygous diploid sector derived from the heterokaryon formed with *nit* mutants from VCG-6. Diploid, named DE-3, showed *nit*⁺ phenotype and growth rate similar to the parental wild isolate. When inoculated in the presence of the haploidizing agent benomyl, the diploid strain produced parasexual haploid segregants exhibiting the *nit* phenotypes of the crossed mutants. Since viable heterokaryons and diploid may be formed among vegetative compatible isolates of *C. acutatum*, this study suggests that the parasexual cycle may be an alternative source of genetic variability in *C. acutatum* isolates.

Additional keywords: anthracnose, diploid formation.

Colletotrichum Corda, anamorphic *Glomerella* Spauld. and H. Schrenk (sordariomycetes) comprises both plant pathogenic and saprophytic species. Pathogenic species cause typical disease symptoms, known as anthracnose, characterized by sunken necrotic lesions that produce orange conidial masses under favorable environmental conditions (14,36). Anthracnose has been reported to be the preharvest and postharvest cause of severe financial loss to crops in tropical, subtropical, and temperate regions. The disease may occur on leaves, stems, and fruit of a wide range of host plants, such as strawberry (*Fragaria* × *ananassa*) (12,13), almond (*Prunus dulcis*) (1), anemone (*Anemone coronaria*) (16), and citrus (*Citrus* spp.) (42).

Whereas many species of *Colletotrichum* may infect a single host, a single *Colletotrichum* species may infect several ones. Examples of single species of *Colletotrichum* affecting multiple hosts include *C. acutatum* and *C. gloeosporioides* which infect a wide range of fruit crops, such as strawberry, apple, and avocado (2,12,14,18).

The characterization of *Colletotrichum* species which cause disease epidemics is crucial for developing and implementing effective management strategies. While traditional methods are not always adequate for reliable differentiation among *Colletotrichum* species, the use of molecular techniques, such as polymerase chain reaction (PCR) methods, using DNA species-specific primers designed for the taxonomic identification of *Colletotrichum* species, has provided new insights into the

differentiation of the species infecting a variety of crops (2,36,39).

Vegetative compatibility grouping is an important tool extensively employed for the analysis of fungal populations of *Colletotrichum* species, including *C. lindemuthianum* from common-bean, *C. gloeosporioides* from almond, and *C. acutatum* from strawberry (9,13,20,30). Vegetative compatibility phenotypes are naturally occurring genetic markers of phytopathogenic fungi controlled by the rigid system of vegetative (or heterokaryon) incompatibility (31). Control is mediated by vegetative self/nonself recognition, impairing the formation of viable heterokaryons after the cell fusion events or anastomosis. Heterokaryons, the coexistence of different nuclear types in a common cytoplasm, however, are formed only by compatible isolates, i.e., strains genetically similar to *vic* (for vegetative incompatibility) or *het* (for heterokaryon incompatibility) genes. Such compatible strains are classified as members of the same vegetative compatibility group (VCG) (23,32). On the other hand, isolates that differ at one or more *vic* or *het* loci are named incompatibles and programmed cell death is triggered when the cells of incompatible strains fuse (7,9,40).

Genetically distinct haploid nuclei inside the heterokaryon mycelium weld into each other and originate heterozygous diploid nuclei. The latter give rise to paternal haploids or recombinants through successive processes of mitotic chromosome nondisjunction (4,5,28,29).

Since heterokaryosis is an important mechanism which provides genetic variability increase in filamentous fungi (25,30,41), the purpose of the present study is first to assess the diversity of vegetative compatibility reactions existing among *C. acutatum* isolates infecting strawberry, guava, Tahiti lime, sweet orange, peach, and green pepper; and second, to obtain heterozygous

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diploid sectors by employing genetically complementary *nit* mutants, i.e., mutants that do not reduce nitrate.

MATERIALS AND METHODS

Fungal isolates and media. Isolates of *C. acutatum* employed in this study were obtained from different host plants with anthracnose symptoms: strawberry (*Fragaria × ananassa*), Tahiti lime (*Citrus latifolia*), sweet orange (*Citrus sinensis*), peach (*Prunus persica*), green pepper (*Capsicum annuum*), and guava (*Psidium guajava*) (Table 1). The reference isolates, CGA (*C.*

TABLE 1. Host plants, geographic location, molecular characterization, and vegetative compatibility groups (VCG) of *Colletotrichum* isolates employed in current study

Isolate	Host plant	Location	Tissue	Molecular characterization ^a	VCG
A-1	Strawberry	São Paulo	Fruit	<i>C. acutatum</i>	1
A-2	Strawberry	Paraná	Fruit	<i>C. acutatum</i>	2
B	Tahiti lime	São Paulo	Fruit	<i>C. acutatum</i>	2
C-1	Sweet orange	São Paulo	Flower	<i>C. acutatum</i>	3
C-2	Sweet orange	São Paulo	Fruit	<i>C. acutatum</i>	3
C-3	Sweet orange	São Paulo	Flower	<i>C. acutatum</i>	3
C-4	Sweet orange	São Paulo	Fruit	<i>C. acutatum</i>	3
D-1	Peach	São Paulo	Fruit	<i>C. acutatum</i>	4
D-2	Peach	São Paulo	Fruit	<i>C. acutatum</i>	5
E-1	Green pepper	São Paulo	Fruit	<i>C. acutatum</i>	6
E-2	Green pepper	São Paulo	Fruit	<i>C. acutatum</i>	6
E-3	Green pepper	Rio de Janeiro	Fruit	<i>C. acutatum</i>	6
F	Guava	São Paulo	Fruit	<i>C. acutatum</i>	7
CAA ^b	Sweet orange	São Paulo	Fruit	<i>C. acutatum</i>	3
CGA ^b	Peach palm	Paraná	Fruit	<i>C. gloeosporioides</i>	–

^a Molecular characterization using polymerase chain reaction methods with DNA species-specific primers.
^b Reference isolates.

gloeosporioides) from peach palm (*Bactris gasipaes*) and CAA (*C. acutatum*) from sweet orange (*Citrus sinensis*), were donated, respectively, by D. J. Tessmann and by W. Mário de Carvalho Nunes (Departamento de Agronomia, Universidade Estadual de Maringá PR, Brazil). Culture stocks were maintained at 25°C in amber flasks with Basal medium (BM). Complete medium (CM) and BM have been previously described by Castro-Prado et al. (9).

Isolation of *nit* mutants. Mycelium plugs, 5 mm in diameter, were removed from the periphery of *C. acutatum* colonies cultivated in CM and transferred to petri plates with BM + NaNO₃ (0.2%) + KClO₃ (3.5 to 4.0%). Plates were incubated at 22°C for 15 days. Fast-growing sectors emerging from the restricted colonies were transferred to petri plates containing BM and incubated at 22°C for 5 days. Colonies exhibiting a thin and expanding mycelium in BM were classified as unable to use nitrate as a nitrogen source (*nit* mutants). In order to determine the phenotypes of *nit* mutants, they were transferred to petri plates with BM, supplemented with different nitrogen sources: sodium nitrate (0.2%), potassium nitrite (0.085%), hypoxanthine (0.01%), and ammonium tartrate (0.092%). Plates were incubated at 22°C for 4 to 10 days, and *nit* mutants were characterized in three distinct phenotypic classes: *nit1* (nitrate nonutilizing), *Nit3* (nitrate and nitrite nonutilizing), and *NitM* (hypoxanthine and nitrate nonutilizing; deficient in the molybdenum cofactor) (6).

Obtaining heterokaryons and diploids. Mycelium plugs (5 mm) of complementary *nit* mutants were paired equidistantly apart (approximately 1.0 cm) in petri dishes containing BM + NaNO₃. After 12 to 30 days of incubation at 22°C, prototrophic heterokaryon growth was observed in the contact area of the paired mutants (Fig. 1A). When a sterilized coverslip was placed between the two mutant colonies at the interaction zone, no prototrophic growth occurred (Fig. 1B). The need for physical contact between the pairing mutants for heterokaryon formation was thus indicated. Fast-growing sectors, suggestive of possible

Fig. 1. A, Pairing of complementary *nit* mutants of the isolates from green pepper (E-1 to E-3) and sweet orange (C-1). Heterokaryons (arrows) were formed only between vegetative compatible isolates. B, Pairing between *Nit3* (A-2) and *nit1* (B) mutants, respectively, from strawberry and Tahiti lime, separated by a coverslip. Heterokaryons (arrows) did not occur beneath the coverslip which impairs direct contact between the pairing mycelia. C, Heterokaryotic colony [E-1 (*Nit3*)/E-2 (*nit1*)] showing mitotic segregation of the crossed *nit* mutants (arrows). D, Heterokaryotic colony showing a fast-growing sector (arrow). E, Diploid segregant (DE-3) showing mitotic segregation of the crossed *nit* mutants (arrows) in BM + NaNO₃. F, Unstable diploid segregant (DE-3) growing in BM + NaNO₃ + benomyl. Bar = 3.0 mm.

diploidy, may arise spontaneously when the heterokaryons are growing in BM + NaNO₃. Whereas the heterokaryotic colonies are macroscopically compact, irregular (Fig. 1C), and slow-growing, the diploid ones are homogeneous, morphologically distinct from the heterokaryon (Fig. 1D to F), and have a growth rate similar to wild-type isolates. When growing in the presence of benomyl, the haploidizing agent, the diploid colonies produce mitotic sectors exhibiting the auxotrophic phenotypes of the parental strains. Putative diploids derived from heterokaryons were isolated and transferred to BM + benomyl (0.5 µg/ml) to detect the presence of auxotrophic sectors (or parental segregants). All experiments were repeated at least three times.

Vegetative compatibility grouping. The formation of a viable heterokaryon between two complementary *nit* mutants assigned their wild parent isolates to the same VCG.

Isolation of parasexual segregants from heterokaryon and diploid colonies. Parasexual segregants (or sectors), showing *nit* phenotype, were isolated directly from heterokaryon and diploid colonies grown in BM + NaNO₃. Mycelium plugs (5 mm) of parasexual segregants were later transferred to BM supplemented with different nitrogen sources (sodium nitrate, potassium nitrite, and hypoxanthine) for phenotypic characterization. Phenotypic analysis was repeated at least twice.

Genomic DNA extraction. Pure cultures of *C. acutatum* were grown in CM for 5 days at 25°C. Mycelia were harvested by filtration (Whatman No. 1 filter paper) and ground to a fine powder in liquid nitrogen for DNA extraction as described by Loudon et al. (24).

Fig. 2. Amplification products in agarose gel stained with ethidium bromide for internal transcribed spacer (ITS) ribosomal DNA. **A**, Taxon-specific identification of *Colletotrichum acutatum* (primer *CaInt2* in combination with primer *ITS4*). **B**, Taxon-specific identification of *C. gloeosporioides* (primer *CgInt* in combination with primer *ITS4*). Lane *M*, molecular markers and lane *N*, negative control. Lanes 1 to 13, *C. acutatum* isolates, respectively, from strawberry (lanes 1 and 2), Tahiti lime (lane 3), sweet orange (lanes 4 to 7), peach (lanes 8 and 9), green pepper (lanes 10 to 12), and guava (lane 13). Lanes 14 and 15, reference isolates of *C. acutatum* and *C. gloeosporioides*, respectively.

PCR analysis. Species-specific PCR primers were synthesized for *C. gloeosporioides* (*CgInt*) 5'-GGCCTCCCGCCTCCGG GCGG-3' and for *C. acutatum* (*CaInt2*) 5'-GGCGCCGGCCCCG TCACGGGGG-3' from internal transcribed spacer 1 (ITS1) of the ribosomal DNA (rDNA) gene (1,2). The PCR primers for taxon-specific amplification were used with the conserved primer ITS4 5'-TCCTCCGCTTATTGATATGC-3' (38). Previously characterized isolates CAA (*C. acutatum*) and CGA (*C. gloeosporioides*) were used as positive controls, whereas amplification reaction without DNA was employed as negative control. PCR amplification was performed in a 25 µl reaction mixture containing 5 ng of DNA; 200 µM each of dATP, dCPT, dGTP, and dTTP; 500 mM KCl; 100 mM Tris-HCl (pH 9.0); 1 µM ITS4 primer; 1 µM *CgInt* or *CaInt2* primer; and 0.65 U of Taq DNA polymerase. Temperature cycling conditions were 35 cycles of denaturation (94°C, for 1 min), annealing (59°C, for 2 min), and DNA extension (72°C, for 2 min). All amplified DNA products were undertaken by electrophoresis on agarose gels (1.5%) in a Tris-HCl buffer, ethidium bromide (0.4 µg/ml), stained and photographed. All experiments were repeated at least twice.

RESULTS

Taxon-specific PCR. All isolates of *C. acutatum* obtained from strawberry, guava, Tahiti lime, sweet orange, peach, and green pepper, including the reference isolate CAA (*C. acutatum*), produced a 490-bp fragment, following PCR with primers *CaInt2* and ITS4 (Fig. 2A). On the other hand, none of the isolates, except the CGA reference isolate (*C. gloeosporioides*), produced an amplicon of 450-bp following PCR with the *C. gloeosporioides*-specific primers *CgInt* and ITS4 (Fig. 2B).

VCG characterization of isolates and diploid formation. Stable *nit* mutants, exhibiting thin and expansive colonies in BM + NaNO₃, were obtained from the wild *C. acutatum* isolates when grown in MB + NaNO₃ + KClO₃. Mutants were screened for their ability to utilize different nitrogen sources so that their phenotypes might be determined. In the case of each of the 13 wild isolates, the predominant phenotypic class was *nit1* mutant followed by *Nit3* and *NitM* mutants (Table 2).

Pairings of *nit* mutants derived from the 13 isolates in all possible combinations identified seven VCGs. Heterokaryons exhibiting prototrophic phenotypes and appreciably slower growth rates than those of the wild isolates in BM were produced in *nit* mutants pairings from isolates A-2 (from strawberry) and B (from Tahiti lime), which were allocated on VCG-2. Isolates from sweet orange (C-1 to C-4) and green pepper (E-1 to E-3) were allocated, respectively, on VCGs 3 and 6, whereas isolates from strawberry (A-1), peach (D-1 and D-2), and guava (F) were allocated, respectively, on VCGs 1, 4, 5, and 7, all of which contained a single member (Table 1, Fig. 1A).

TABLE 2. Frequency of *nit* mutants of *Colletotrichum acutatum* isolates

Isolates	Frequency (%) of auxotrophic <i>nit</i> mutants		
	<i>nit1</i>	<i>Nit3</i>	<i>nitM</i>
A-1	61	25	14
A-2	89	12	06
B	68	09	23
C-1	87	13	0
C-2	61	41	0
C-3	75	19	06
C-4	81	19	0
D-1	73	19	08
D-2	68	17	15
E-1	71	17	12
E-2	89	09	02
E-3	32	67	01
F	09	55	36

Although isolates A-2 and B (VCG-2) have been carrying out anastomosis among themselves (data not shown), the pairing of their *nit* mutants did not form viable heterokaryon in BM + NaNO₃ when the paired mutants were separated by a sterilized coverslip (Fig. 1B). The heterokaryon formation only occurred where the mycelium of the two paired mutants came in direct contact beyond the edges of the coverslip. Results demonstrate the need for physical contact between mutant hyphae (anastomosis) for heterokaryon formation. Furthermore, heterokaryotic colonies, growing on plates containing BM + NaNO₃, gave rise spontaneously to parasexual auxotrophic segregants which exhibited the parental phenotypes of the crossed mutants (Table 3, Fig. 1C). Hypoxanthine nonutilizing mutants, such as D-2 (*NitM*_I) and D-2 (*NitM*_{II}), exhibiting complementation in BM + NaNO₃ through the production of a prototrophic heterokaryon, were assumed to be nonallelic. This fact indicated the existence of multiple loci controlling the production of a molybdenum-containing cofactor (Table 3). Similar results have been reported in other *Colletotrichum* species, such as *C. lindemuthianum* and *C. truncatum* (9,31).

A fast-grown sector with *nit*⁺ phenotype was isolated from the heterokaryon formed by the pairing of *Nit3* and *NitM* mutants derived from isolate E-3 (from green pepper) (Fig. 1D). This sector, named DE-3, produced parasexual haploid segregants in the presence of benomyl, which exhibited the parental phenotypes of the crossed mutants (Table 3, Fig. 1E and F). DE-3 was therefore characterized as a diploid segregant.

DISCUSSION

The current study evaluates the genetic relationship among *C. acutatum* isolates characterized by ribosomal DNA analysis. Thirteen *C. acutatum* isolates derived from different hosts were grouped into seven VCGs by demonstrating heterokaryosis through complementation using *nit* mutants. Four of the isolates belonged to the largest group VCG-3. VCGs 3 and 6 included only isolates from sweet orange and green pepper, respectively. The two isolates from peach (D-1 and D-2) were neither compatible with each other nor with any other isolates. Similarly, the isolate from guava was not compatible with the isolates from other hosts. As a rule, it was observed that isolates derived from the same host were either allocated together into the same VCG or allocated separately in distinct VCGs. An exception, however, was observed for strawberry (A-2) and Tahiti lime (B) isolates which showed

vegetative compatibility between themselves and were, therefore, allocated on VCG-2. In addition, the vegetative compatibility of the isolates did not appear to be related to their geographical origin. Actually, out of the three multimember VCGs, only one (VCG-3) contained members from the same geographic area (Table 1).

Vegetative compatibility is a mechanism that controls the genetic isolation of fungal populations since vegetatively compatible isolates are expected to be similar to one another, specifically for *vic* or *het* genes (23,32). Consequently, each VCG corresponds to a multilocus haplotype with respect to vegetative compatibility-controlled loci. Moreover, genotype diversity of a sample may be represented by the ratio of the number of haplotypes to sample size (10). Seven VCGs were identified among *C. acutatum* isolates, with diversity estimates of approximately 0.54.

Studies of vegetative compatibility have been extensively used to identify subspecific genetic clones of anamorphic populations of plant pathogens, classifying them by genetic approach regardless of morphology, physiology, or pathogenicity. VCGs comprising isolates from more than one host have been previously reported in *Colletotrichum* species, such as *C. acutatum* and *C. gloeosporioides*, and in *Verticillium dahliae*, a pathogen of potato, cotton, and some vegetable crops. Israeli isolates of *C. acutatum* from strawberry and anemone showing vegetative compatibility through the production of prototrophic heterokaryons were allocated on VCG ANE-F; *V. dahliae* isolates from six different hosts, including potato, tomato, and peanut, were assigned on the same VCG (VCG 2A); whereas isolates from *C. gloeosporioides*, recovered from apple and red pepper, were allocated on the largest group, named VCG-A (3,16,20,21).

Although heterokaryosis resulting from hyphal anastomosis and vegetative incompatibility barriers have been previously reported in the plant pathogenic fungi *C. acutatum* (13,14,16), no diploid conidia or parasexual segregation have been detected from its heterokaryotic growth. Current research first demonstrates that prototrophic heterokaryons of *C. acutatum* produce mitotic sectors which are auxotrophs with phenotypes identical to one or to the other parental strain. Second, current study demonstrates that heterokaryons in *C. acutatum* may yield heterozygous diploid conidia from which haploid segregants originate. A heterozygous diploid sector, named DE-3, was spontaneously obtained from the pairing of complementary *nit* mutants from green pepper E-3 isolate. The DE-3 colonies were prototrophic and semi-stable, producing mitotic sectors with *nit* phenotype. Conidia recovered from these sectors comprised the haploid parent phenotypes. The conclusion that these sectors were haploid was supported by the observation that they were stable when growing in the presence of benomyl (data not shown).

Many fungi have a parasexual cycle in which haploid nuclei may fuse to form relatively stable diploid nuclei. Diploid cultures of mycelial fungi, which grow normally as haploids, have been proved to be important tools in conducting genetic analysis, especially in (i) the complementation and dominance testing of genetic traits; (ii) the assignment of markers to linkage groups in sexual and nonsexual fungi; and (iii) the determination of order of gene markers and estimation of the distance between them. On the other hand, the ability to form diploid cultures may be an important component of risk assessment for plant pathogen isolates used for biological control, such as *Aspergillus flavus* and *C. gloeosporioides* f. sp. *hakea*, especially those whose sexual cycles are rare or nonexistent in nature. Parasexual crosses between such isolates and the related natural populations could produce competitive progeny with more adapted genotypes and potentially capable to disseminate to new localities (5,9,11, 17,19,26,28,29).

During the vegetative multiplication of heterozygous diploid nuclei, somatic crossing-over (so-called parasexual recombination) and/or nondisjunctions leading to haploidization may occur,

TABLE 3. Phenotypic segregation of diploid and heterokaryotic colonies formed by *nit* mutants of *Colletotrichum acutatum*

Pairing of <i>nit</i> mutants		Phenotypes of parasexual segregants		
Paternal 1 ^a	Paternal 2 ^a	Paternal 1	Paternal 2	Total ^b
A-2 (<i>Nit3</i>)	/ A-2 (<i>nit1</i>) ^c	06	08	14
B (<i>Nit3</i>)	/ B (<i>nit1</i>) ^c	16	29	45
A-2 (<i>NitM</i>)	/ B (<i>nit1</i>) ^d	20	20	40
A-2 (<i>NitM</i>)	/ B (<i>Nit3</i>) ^d	182	24	206
A-1 (<i>NitM</i>)	/ A-1 (<i>Nit3</i>) ^c	53	07	60
C-3 (<i>NitM</i>)	/ C-2 (<i>nit1</i>) ^d	58	17	75
C-3 (<i>NitM</i>)	/ C-4 (<i>nit1</i>) ^d	07	29	36
D-1 (<i>NitM</i>)	/ D-1 (<i>nit1</i>) ^c	25	29	54
D-2 (<i>NitM</i>)	/ D-2 (<i>NitM</i>) ^c	20	31	51
E-1 (<i>Nit3</i>)	/ E-3 (<i>NitM</i>) ^d	73	13	86
E-2 (<i>Nit3</i>)	/ E-3 (<i>NitM</i>) ^d	45	09	54
E-1 (<i>Nit3</i>)	/ E-2 (<i>nit1</i>) ^d	57	31	88
F (<i>Nit3</i>)	/ F (<i>NitM</i>) ^c	01	17	18
DE-3 (diploid segregant)				
E-3 (<i>Nit3</i>)	// E-3 (<i>nitM</i>) ^c	16	05	21

^a Mutant strain obtained from the wild isolate.

^b Total number of mitotic segregants.

^c Intrastrain pairing of *nit* mutants.

^d Interstrain pairing of *nit* mutants.

so that conidia eventually may be obtained which have either homozygous diploid nucleus for certain gene markers or haploid nuclei containing new combinations of the parental genetic markers. The evolutionary significance of the parasexual cycle may derive from specific advantages of both ploidy levels: the extensive vegetative diploid phase of the parasexual cycle enables fungi to accumulate recessive mutations that may be recombined and tested in haploid nuclei. The vegetative organism may consequently undergo genetic adaptation, resulting in the production of better-adapted conidia (5,8,11,30,33,34,41).

The ability of *C. acutatum* isolates to form heterozygous diploid nuclei, demonstrated in present study, reveals the potential of this plant pathogen to originate undesirable conidial genetic change during its vegetative propagation. Although the life cycle of *C. acutatum* includes a sexual stage, it occurs rarely in nature (27,35). However, previous studies have shown the extensive genetic diversity and heterogeneity within this species (15,19,22). One hypothesis for this diversity is the occurrence of anastomosis and heterokaryosis among compatible isolates (37). Vegetative compatibility observed in current study between isolates from strawberry and Tahiti lime suggests that isolates from different hosts are potentially able to exchange genetic material.

Since viable heterokaryons and diploid may be formed between vegetatively compatible *C. acutatum* isolates (present results), and taking into account that independent isolates of *C. acutatum* may infect the same host (10), current research suggests that the parasexual process may act as a likely mechanism of genetic variability in this species.

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