THE USE OF DUPLICATION-GENERATING REARRANGEMENTS FOR STUDYING HETEROKARYON INCOMPATIBILITY GENES IN NEUROSPORA[1]

DAVID D. PERKINS

Department of Biological Sciences, Stanford University, Stanford, California 94305

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ABSTRACT

Heterokaryon (vegetative) incompatibility, governing the fusion of somatic hyphal filaments to form stable heterokaryons, is of interest because of its widespread occurrence in fungi and its bearing on cellular recognition. Conventional investigations of the genetic basis of heterokaryon incompatibility in *N. crassa* are difficult because in commonly used stocks differences are present at several *het* loci, all with similar incompatibility phenotypes. This difficulty is overcome by using duplications (partial diploids) that are unlikely to contain more than one *het* locus. A phenotypically expressed incompatibility reaction occurs when unlike *het* alleles are present within the same somatic nucleus, and this parallels the heterokaryon incompatibility reaction that occurs when unlike alleles in different haploid nuclei are introduced into the same somatic hypha by mycelial fusion.—Nontandem duplications were used to confirm that the incompatibility reactions in heterokaryons and in duplications are alternate expressions of the same genes. This was demonstrated for three loci which had previously been established by conventional heterokaryon tests—*het-e*, *het-c* and *mt*. These were each obtained in duplications as recombinant meiotic segregants from crosses heterozygous for duplication-generating chromosome rearrangements. The particular method of producing the duplications is irrelevant so long as the incompatibility alleles are heterozygous.—The duplication technique has made it possible to determine easily the *het-e* and *het-c* genotypes of numerous laboratory and wild strains of unknown constitution. In laboratory strains both loci are represented simply by two alleles. Analysis of *het-c* is more complicated in some wild strains, where differences have been demonstrated at one or more additional *het* loci within the duplication used and multiple allelism is also possible.—The results show that the duplication method can be used to identify and map additional vegetative incompatibility loci, without the necessity of heterokaryon tests.

GENETIC factors other than mating type that prevent or impede the formation of heterokaryons in *Neurospora crassa* were first described by GARNJOBST (1953, 1955) and by HOLLOWAY (1953, 1955). If two monokaryotic haploid strains differ in the alleles at one or more loci that govern heterokaryon incompatibility, stable heterokaryons are not formed between them. Four such incompatibility loci, *het-c*, *-d*, *-e*, and *-i*, have been identified using heterokaryons (GARNJOBST 1953, 1955; PITTENGER and BRAWNER 1961; WILSON and GARNJOBST 1966), and the mating-type alleles *A* and *a* act also as a pair of hetero-

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karyon incompatibility genes (COONRADT 1943; BEADLE and COONRADT 1944; SANSOME 1946; GARNJOBST and WILSON 1956; PITTINGER 1957; NEWMEYER, HOWE and GALEAZZI 1973). At least one other *het*-gene difference is known to be present in laboratory stocks (J. F. WILSON, personal communication). Cytological and chemical observations have been made on the heterokaryon incompatibility reactions (WILSON, GARNJOBST and TATUM 1961; WILLIAMS and WILSON 1966), whose behavior and specificity suggest that they may have a bearing on cellular recognition mechanisms in higher organisms.

Heterokaryon or vegetative incompatibility independent of sexual compatibility is also found in other fungi such as Aspergillus (JINKS *et al.* 1966), Podospora (*t* and *u* genes—ESSER and BLAICH 1973), and the slime molds (DEE 1966; COLLINS and CLARK 1968). The widespread occurrence of vegetative incompatibility differences suggests a significant selective role, the nature of which has been a subject of speculation (see JINKS *et al.* 1966; BUTCHER 1968; CATEN 1972; CLARK and COLLINS 1973; HARTL, DEMPSTER and BROWN 1975). For reviews of heterokaryosis and its genetic control see DAVIS (1966) and CATEN and JINKS (1966).

Because marker stocks and commonly used laboratory wild types of *Neurospora crassa* often differ at two or more *het* loci, a conventional genetic analysis is difficult, as with any multifactorial trait where the individual component genes cannot be distinguished phenotypically. Using the heterokaryon incompatibility associated with mating type, NEWMEYER (1965, 1970) circumvented the difficulty of analysis by constructing partial diploids, in which only one segment is present in heterozygous condition and the rest of the genome is haploid. Heterozygous partial diploids are readily obtained from crosses which involve chromosome rearrangements that generate nontandem duplications by meiotic recombination and chromosome segregation (NEWMEYER and TAYLOR 1967; TURNER *et al.* 1969; PERKINS 1971, 1972a, 1974). Each such rearrangement produces predictably a class of progeny that are duplicated for a specific chromosome segment. If the segment includes a heterokaryon incompatibility locus, and if the two parents contribute different alleles at that locus, the incompatibility is manifested in each heterozygous offspring by a characteristic incompatibility phenotype. Thus, *het* loci can be identified and studied in one region at a time. Duplications have been used in this way by MYLYK (1975a) to identify at least five new vegetative incompatibility loci in *N. crassa* strains collected from nature.

The usefulness of heterozygous nontandem duplications is not limited to studying incompatibility, and the approach used in this paper could equally well be applied to other situations where interactions are to be studied, as for example with regulatory genes (METZENBERG, GLEASON and LITTLEWOOD 1974).

The main purpose of this paper is to show that the duplication method is valid and that incompatibility observed in duplications is in fact due to heterokaryon incompatibility genes. This has been accomplished for three of the loci previously known from their effects on heterokaryons—*het-e* (VIIL), *het-c* (IIL), and mating type (IL)—using appropriate rearrangements to produce

duplications embracing each of them. Duplications covering *het-e* are reported here for the first time. Preliminary accounts were given earlier of *het-c* in duplications (PERKINS 1968, 1969, 1972b). Mating-type (*A/a*) heterozygosity has previously been studied using terminal duplications from a pericentric inversion (NEWMEYER and TAYLOR 1967) and interstitial duplications from an insertional translocation (PERKINS 1972a); these results are now extended using *A/a* duplications obtained in a third way which employs overlapping rearrangements whose breakpoints straddle the mating-type locus.

MATERIALS AND METHODS

Strains: *T(VII→IV)T54M50* originated in wild type 74A following UV in experiments of INOUE and ISHIKAWA (1970). *T(II→V)NM149* originated in wild type Em a following UV in 1964 experiments of NOREEN E. MURRAY. *T(I;V)AR12* arose in a strain of mixed parentage following UV in 1967 experiments of ALAN RADFORD. *T(I;V)P5401* was found in a stock of *his-4* (C141) received from BARBARA D. MALING in 1959. *T(I;V)47711* arose from the cross Abb 4A × 25a following UV (BEADLE and TATUM 1945). *T(II→VI)P2869* was found in a cross of *T(VI→[I;III]Y16329* × *T(I;II)P5390*. All are phenotypically wild-type and have been separated from any mutant genes that may originally have been present. Standard wild types were 74-OR23-1A and 74-OR8-1a or their derivatives OR23-1VA and ORSa (MYLYK, BARRY and GALEAZZI 1974). Standard testers for mating type (*mt*, *A/a*) and for aberration *vs.* normal sequence were the isosequential fluffy strains *fl^{P}A* and *fl^{P}a*. For information on the origin, characteristics and scoring of the markers used, see references given by BACHMANN and STRICKLAND (1965), BACHMANN (1970), or BARRATT and OGATA (1974). The meaning of locus symbols is given in the legend of Figure 1.

Media and technical methods: Duplications heterozygous for different *het* alleles are initially abnormal in morphology and growth rate, and produce brown pigment when grown on glycerol complete medium (GCP, TATUM *et al.* 1950) or on minimal medium (VOGEL 1964) with 1% sucrose plus L-phenylalanine, 0.2 mg/ml, and L-tyrosine, 0.5 mg/ml (PT). On minimal medium without these amino acids the morphology is the same but no pigment is formed. Because the pigment is useful in scoring, ascospores were usually isolated to either GCP or PT medium. Crosses were carried out on synthetic crossing medium (SC) (WESTERGAARD and MITCHELL 1947) containing 2% agar and 2% sucrose, usually in 18 × 150 mm tubes. Mating type, fertility, and presence of an aberration or duplication were scored by fertilizing 5-day-old fluffy testers in individual 10 × 75 mm tubes of SC at 25° (TAYLOR 1965). Cultures were scored as aberrant when the ascospores shot from perithecia of these test crosses were about 75% black and 25% white (defective) for duplication-producing rearrangements, and 50%, 50% for reciprocal translocations; about 95% of ascospores are black in crosses between strains that are isosequential, either both Normal or both rearranged (PERKINS 1974). Methods of crossing, ascospore isolation, and stock preservation were as described previously (PERKINS 1959, 1962).

RESULTS

Three examples of heterokaryon (vegetative) incompatibility loci will be given—*het-e*, *het-c* and mating type.

I. het-e *testers and the manifestation of* het-e *in heterozygous duplications*

Construction, verification, and use of duplication-generating het-e *testers*: *het-e*, which was identified and mapped in VIIL by WILSON and GARNJOBST (1966), is contained in duplications generated by translocation *T(*VII→IV*)-T54M50*, whose structure is shown in Figure 1A. A distal segment of VIIL

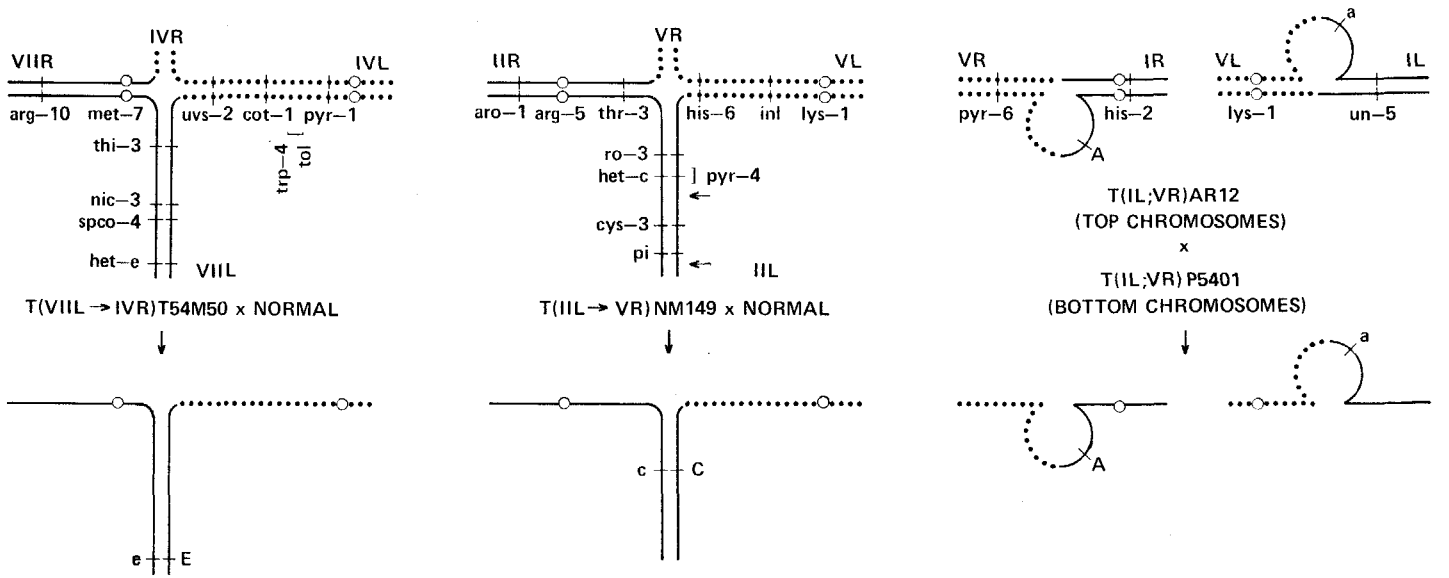

FIGURE 1.—A. Chromosome structure of *T(VIIL→IVR)T54M50* × Normal, and of *Dp(VIIL→IVR)T54M50*, used for analyzing *het-e*. Linkage Group VII segments are shown as solid lines, IV as dotted. B. Chromosome structure of *T(IIL→VR)NM149* × Normal and of *Dp(IIL→VR)NM149*, used for analyzing *het-c*. Linkage Group II segments are shown as solid lines, V as dotted. Arrows indicate limits of the segment transposed in *T(IIL→VI)P2869*. C. Origin of *A/a* duplications from an intercross between two overlapping reciprocal translocations, *T(IL;VR)AR12 and T(IL;VR)P5401*. Linkage Group I segments are shown as solid lines, V as dotted.———*Above*: Meiotic pairing in the crosses that generate duplications by independent segregation during meiosis. *Below*: Constitution of the duplications that result when appropriate centromeres go to the same pole at anaphase I. Drawings are not to scale.———One third of viable progeny are duplications. When the parents differ in *het*-genotype, duplications are mostly heterozygous. The *het*-genotypes shown would result from crossing *T(VII→IV)T54M50 het-E* × Normal *het-e*, *T(II→V)NM149 het-C* × Normal *het-c*, and *T(I;V)AR12 a* × *T(I;V)P5401 A*.———*Meaning of locus symbols*: *al*—albino, *arg*—arginine, *aro*—aromatic amino acids + *p*-aminobenzoic acid, *cot*—temperature-sensitive colonial, *cys*—cysteine, *his*—histidine, *inl*—inositol, *lys*—lysine, *met*—methionine, *mt*—mating type, *nic*—niacin, *pi*—pile (morphology), *pyr*—pyrimidine, *ro*—ropy (morphology), *spco*—spreading colonial, *thi*—thiamine, *thr*—threonine, *tol*—tolerant (suppressor of *A/a het*-incompatibility), *trp*—tryptophan, *un*—'unknown' (heat-sensitive conditional lethal), *uvs*—ultraviolet-sensitive. The relative order of *het-c* and *pyr-4* is not known.

containing *thi-3*, *nic-3*, *spco-4* and *het-e* is translocated to the right end of IV near *uvs-2*. The rearrangement behaves as a quasiterminal reciprocal translocation, such that the translocated IVR tip contains no essential gene. Meiotic pairing in a cross of Translocation × Normal would thus be as in Figure 1A, and one third of viable progeny (one quarter of the meiotic products) are duplicated for the VIIL segment and deficient for the nonessential tip. (Meiotic products complementary to the duplications are deficient and inviable.) The viable duplications are expected to be heterozygous for any covered markers that were different in the two parents, except where meiotic crossing over has resulted in homozygosis of loci distal to the exchange. The breakpoints in Figure 1A are based both on conventional mapping and on mapping by duplication coverage (PERKINS *et al.* 1969).

*T(*VII→IV*)T54M50* originated in wild type 74A, which is known to be *het-C het-d het-e* (WILSON and GARNJOBST 1966). When the original translocation was crossed to laboratory strains derived from 74A, all duplication progeny were phenotypically wild-type. In contrast, when *T(*VII→IV*)T54M50* was crossed to a Lindegren wild type such as RL3-8A, shown by WILSON and GARNJOBST to be *het-E*, a substantial number of the progeny, the heterozygous *E/e* duplications, were highly inhibited and excreted brown pigment locally into GCP medium. These inhibited cultures, which were called Tiny-Browns, escaped from the inhibition after a lag of 3 to 7 days or longer, as had previously been described for *A/a* and *C/c* duplications, and the resulting cultures were mostly barren when tested by normal-sequence testers, as is typical of duplications.

The *het-E* allele was introduced into translocation sequence by crossing *T(*VII→IV*)T54M50 nic-3 het-e a* × RL3-8 *nic-3*$^{+}$ *het-E A* and selecting fertile *T nic*$^{+}$ crossover progeny. In this way, a set of *T(*VII→IV*)T54M50 het-e* and *het-E* testers of both mating types was obtained. WILSON and GARNJOBST had constructed a set of normal-sequence strains having *het-C* and *-c*, *het-D* and *-d*, *het-E* and *-e*, and mating types *A* and *a* in all combinations (see BARRATT and OGATA 1974). When the *T(*VII→IV*)T54M50 het-e* testers were crossed to the 16 WILSON-GARNJOBST reference strains (Table 1A), the results showed that the inhibited response, indicating incompatibility, depends solely upon which allele of *het-e* is present in the parent tester strain, and not upon *het-c*, *het-d*, or mating type.

Results with some strains of previously unknown *het-e* constitution are given in Table 1B. Without exception, all the tested strains could be classed either as *het-E* or *het-e*.

Theoretically, one third of surviving progeny should be duplications. The number of inhibited progeny attributed to *E/e* heterozygosity is consistently less than one third in Table 1. This may be due in part to homozygosis resulting from meiotic crossing over proximal to *het-e*. The deficit may also be due to a lowered recovery of *E/e* ascospores, because *E/e* duplications are so very inhibited that some may be classed as nongerminants.

Characteristics of het-E/het-e *duplications*: Cultures heterozygous for *het-e* alleles are extremely inhibited when growth is initiated from germinating

TABLE 1

Diagnosis of het-e *and* het-c *genotypes by means of duplications from intercrosses between Normal-sequence and translocation-sequence parents*

A. Confirmation of genotypes of translocation testers using Normal-sequence strains whose *het*-constitution was known directly from heterokaryon tests of WILSON and GARNJOBST. B. Use of the confirmed translocation testers to identify *het-e* and *het-c* alleles in various strains of unknown *het*-genotype. The numbers given for each test cross are (inhibited duplication progeny)/(total progeny).

Normal-sequence parent			*het-e* tests			*het-c* tests		
Strain genotype or designation	Stock no.*	Known *het* type	*T(VII→IV)T54M50* tester parent† *het-E*	*het-e*	Diagnosis of normal parent	*T(II→V)NM149* tester parent‡ *het-C*	*het-c*	Diagnosis of normal parent
A. *Normal-sequence strains of known* het-*genotype.*								
al-2; pan-1 A	1423	CDE A	0/29	**5/24**	E	0/36	**9/33**	C
al-2; pan-1 a	1427	CDE a	0/59	**16/85**	E	1/48	**14/46**	C
RL 3-8 A	2218	CDE A	0/38	**12/32**	E	0/40	**15/48**	C
RL 21 a	2219	CDE a	0/55	**6/47**	E	0/44	**22/49**	C
inl A	1422	cde A	**13/47**	0/44	e	**8/27**	0/28	c
inl a	1436	cde a	**13/45**	0/44	e	**8/41**	1/44	c
inl A	1453	Cde A	**7/43**	0/45	e	1/49	**15/44**	C
inl a	1438	Cde a	**13/45**	0/48	e	0/50	**17/42**	C
74-OR23-1V a	2489	Cde A	**7/71**	0/39	e	0/47	**16/49**	C
ORS a	2490	Cde a	**9/46**	0/44	e	0/42	**20/46**	C
al-2; pan-1 A	1425	cDE A	0/33	**7/28**	E	**13/31**	**10/81**	?
al-2; pan-1 a	1429	cDE a	0/45	**9/35**	E	**15/41**	0/45	c
inl A	1454	CDe A	**11/48**	0/46	e	0/35	**10/31**	C
inl a	1439	CDe a	**9/45**	0/94	e	0/44	**13/49**	C
al-2; pan-1 A	1426	cdE A	0/30	**9/69**	E	**7/31**	0/25	c
al-2; pan-1 a	1430	cdE a	0/52	**5/33**	E	**14/30**	1/44¶	c
al-2; pan-1 A	1424	CdE A	0/34	**15/63**	E	0/41	**14/50**	C
al-2; pan-1 a	1428	CdE a	0/30	**5/37**	E	0/27	**8/36**	C
inl A	1455	cDe A	**12/48**	0/46	e	**15/45**	1/45	c
inl a	1437	cDe a	**8/45**	0/44	e	**22/68**	1/70	c
B. *Normal-sequence strains of unknown* het-*genotype.*								
fl^{P} *A*	1838		**10/63**	0/39	e	**15/48**	0/47	c
fl^{P} *a*	1690		**9/41**	0/47	e	**29/92**	0/42	c

TABLE 1 (Continued)

Normal-sequence parent			*het-e* tests			*het-c* tests		
Strain genotype or designation	Stock no.*	Known *het* type	*T(VII→IV)T54M50* tester parent† *het-E*	*het-e*	Diagnosis of normal parent	*T(II→V)NM149* tester parent‡ *het-C*	*het-c*	Diagnosis of normal parent
Em 1534.12 A	691		**5/38**	0/23	e	0/38	**14/60**	C
Em a	8044 (DP)		**7/39**	0/35	e	**8/26**	0/33	c
Lein-7 A	847		**7/39**	0/45	e	0/56	**13/54**	C
Lein-8 a	1693		**10/29**	0/38	e	0/36	**9/27**	C
Abbott 4 A	1228		0/33	**16/47**§	E	**18/51**	0/47	c
Abbott 12 a	351		0/35	**22/77**	E	**15/44**	0/41	c
Chilton a	1691		0/30	**9/65**	E	0/54	**14/46**	C
Liberia a	967		0/59	**10/43**	E	**11/42**	0/48	c
Puerto Rico 18 a	429		**9/55**	0/34	e	**18/42**	0/46	c
Adiopodoumé A	430		0/34	**8/28**	E	**12/50**	**15/54**	?
Groveland-1c a	1945		**9/59**	0/30	e	**11/60**	**7/53**	?
Panama A	1131		0/32	**4/34**	E	**11/34**	**9/45**	?
Panama a	1132		**10/31**	0/42	e	**11/38**	**15/33**	?
Costa Rica A	851		0/35	**4/27**	E	**15/53**	**11/53**	?
Marrero-1d a	2224		**5/39**	0/42	e	**14/45**	**13/47**	?
Houma-1g A	P497 (DP)		0/40	**7/37**	E	**5/48**	**19/48**	?
Mauriceville-1c A	2225		0/38	**6/45**	E	**12/42**	**21/47**	?

Where significant numbers of inhibited duplications were observed, the test results are shown in boldface type, indicating that the parents contributed different alleles of the *het* gene being tested.

* Fungal Genetics Stock Center accession numbers except where marked (DP), which signifies Perkins laboratory stock numbers.

† *T(VII→IV)T54M50 het-e A* (FGSC 2466), *T(VII→IV)T54M50 het-e a* (2467), *T(VII→IV)T54M50 het-E A* (2603), and *T(VII→IV)-T54M50 het-E a* (2604). Also, in a few tests: *T(VII→IV)T54M50 nic-3 het-e A* and *T(VII→IV)T54M50 nic-3 het-e a.*

‡ *T(II→V)NM149 het-c A* (FGSC 1483), *T(II→V)NM149 het-c a* (1482), *T(II→V)NM149 ro-3 het-C A* (2011), and *T(II→V)NM149 ro-3 het-c a* (2012).

§ These inhibited duplications differed morphologically from all other *E/e* duplications, forming larger, spreading spidery colonies that somewhat resemble *A/a* Dark-Agar duplications.

¶ In an earlier presumably identical cross five inhibited progeny were found among 34 germinants. However, these were not spreading Brown-Flat types typical of *C/c*, but tiny, very inhibited colonies more nearly resembling *E/e* duplications.

ascospores or by subculture. A few hyphae are sent out that grow only a fraction of a millimeter, while nonduplication siblings grow up to fill a culture tube. Under 20 to 40× magnification, small clots of amorphous growth can be seen along the hyphal strands (suggesting tufts of Bermuda grass along subterranean runners). After several days at 34°, macroscopically visible Tiny-Brown colonies 1 mm or less in diameter are formed by the incompatible duplications.

Heterozygosity for *het-E/het-e* in duplications is far more inhibiting than for *het-C/het-c* or *A/a*. This is consistent with the microscopical observations of haploid mycelia reported by WILSON, where the killing reaction is more rapid and severe for a *het-e* difference. Photographs of duplications heterozygous for various vegetative incompatibility loci, including *het-e* and *het-c*, have been published by MYLYK (1975a), and a photograph of *A/a* is given by NEWMEYER and TAYLOR (1967).

Effect of tol *on* het-e: An independent gene *tol* (tolerant) suppresses the vegetative incompatibility associated with mating type. Incompatibility reactions of *A* and *a* are suppressed both in heterokaryons and in duplications. The incompatibility phenotype of *het-C/het-c* duplications is not suppressed by *tol*, however (NEWMEYER 1970). Similar tests have now been carried out with *het-E/het-e* duplications. Normal-sequence *tol trp-4*; *het-e* was crossed with *T(*VII→IV*)T54M50 het-E*. The locations of *trp-4* and *tol* are shown in Figure 1A. From this cross, a nonbarren (i.e., nonduplication) *T(*VII→IV*)T54M50 trp-4*, presumably *tol* progeny was chosen and used in the experimental cross with *In(IL→IR)H4250*; *tol*; *het-e*. (*In(IL→IR)H4250* is a pericentric inversion whose duplication progeny are heterozygous for mating type—NEWMEYER 1970.)

The experimental cross proved homozygous for *tol* and heterozygous for both of the translocations and for mating type and *het-e*. No inhibited Dark-Agar phenotypes were observed among the progeny, contrary to the behavior of *A/a* duplications in the absence of *tol*. Instead, a class of phenotypically nearly normal duplications was produced which formed barren perithecia when crossed both with *A* and with *a*. In contrast, 15 of the 74 progeny showed the inhibited Tiny-Brown phenotype characteristic of *het-E/het-e* duplications, indicating that *het-e* incompatibility, like *het-c*, is not suppressed by *tol*.

The genotype of the *T(*VII→IV*)T54M50 het-E*; *trp-4 tol* parent was confirmed for *T*, *tol*, and *het-E* by examining progeny from crosses to *tol*$^+$ *het-e* strains of the three sequences *T(VII→IV)T54M50*, *In(IL→IR)H4250*, and Normal.

II. het-c *testers and the manifestation of* het-c *in heterozygous duplications*

Duplications containing *het-c* (GARNJOBST 1953, 1955; WILSON and GARNJOBST 1966) are obtained using translocation *T(*IIL→VR*)NM149*. Interest in a strain designated NM149 was originally aroused by the observation in 1966 that about one third of the viable progeny from crosses of NM149 with certain other strains had a grossly abnormal morphology, with flat aconidial growth. These abnormal cultures (called Brown-Flats) resembled *A/a* duplications in produc-

ing brown pigment on complete medium and in sectoring to give wild-type growth after a lag of several days, but they differed from *A/a* in gross morphology. Crosses of NM149 with certain other laboratory strains produced progeny that were all morphologically normal.

Strain NM149 was subsequently shown to contain an aberration that generates duplications for a left-arm segment of linkage group II, where *het-c* had been mapped by WILSON and GARNJOBST (1966). *T(II→V)NM149* had arisen in the wild-type strain Em a, now known to be *het-c*. Our normal-sequence laboratory strains with which it had given Brown-Flat progeny were in fact *het-C*, and those that had given no Brown-Flats were *het-c*.

Construction and verification of duplication-generating het-c *testers*: The probable structure of *T(II→V)NM149* is shown in Figure 1B. A long distal segment of linkage group IIL is translocated to the end of V, where the breakpoint has never been observed to recombine with the rightmost marker *his-6*. The behavior of the translocation is most economically explained if it is a quasi-terminal reciprocal translocation, such that the translocated VR tip contains no essential gene. Meiotic pairing in a cross of *NM149* × Normal would thus be as shown. One third of the viable progeny (one fourth of the meiotic products) are duplicated for the IIL segment and deficient for the nonessential tip. These duplication progeny are expected to be heterozygous for any covered markers, including *het-c*, that were different in the two parents, except where meiotic crossing over has made loci distal to the exchange homozygous. The breakpoints shown are based both on conventional mapping (BARRY and PERKINS 1969) and on mapping by duplication coverage (PERKINS *et al.* 1969).

A more complicated alternative structure is not excluded. *T(II→V)NM149* could perhaps be an insertional translocation in which a long interstitial segment with its second break far distal to *pi* in II was inserted interstitially near *his-6* in V. With either structure, duplications would be produced recurrently as one third of the viable progeny, and either structure would be equally useful for investigating included *het* genes. The order shown in Figure 1B is favored because of simplicity and the ease with which distal IIL markers can be inserted into *T(II→V)NM149*.

In the course of mapping the rearrangement, each marked Normal-sequence strain was identified either as identical to *T(*II→*V)NM149* for the *het* genes concerned (if no Brown-Flat progeny were produced), or nonidentical (if Brown-Flats appeared). It was shown that distal IIL markers were covered in the duplications. Among the marker strains used for mapping, *ro-3* carried the unlike *het* allele linked to it, and these two genes were transferred together into translocation sequence by crossing over. Translocation-sequence tester strains of both mating types were chosen, two with the original allele *het-x*, and two with *ro-3* and the second allele, *het-X*. When these *T(*II→V*)NM149* testers were crossed to the WILSON-GARNJOBST reference strains (Table 1A), Brown-Flats were regularly found from *het-x* × *het-C* but not from *het-x* × *het-c*, and from *het-X* × *het-c* but not from *het-X* × *het-C*, indicating that the original NM149 strain was *het-c* (= *het-x*) while the *ro-3* strain was *het-C* (= *het-X*). The occurrence of Brown-Flats was completely independent of *het-d*, *het-e* and mating type.

In crosses where inhibited duplications were observed from *het-c* tests, their frequencies approximated the theoretical value of one third, in contrast to the inhibited duplications described earlier from *het-e* tests, which were less frequent. This is attributed both to the location of *het-c* near the *T(*II→V*)NM149* breakpoint, minimizing homozygosis, and to the greater viability and visibility of Brown-Flat *C/c* duplications compared to the Tiny-Brown *E/e* duplications.

Only a very small number of inhibited germinants were observed in some of the Table 1 crosses that involved the *NM149 het-c* testers. These are believed not to be relevant to the diagnosis, but are attributed to *het* differences in regions other than that regularly duplicated in the progeny of *T(*II→V*)NM149*. Occasional aneuploidy of other regions might occur by 3 : 1 segregation from the translocation complex, or by nondisjunction of chromosomes not involved in the tester translocation.

One strain among the WILSON-GARNJOBST references gave exceptional results with respect to *het-c*. *al-2*; *pan-1 A*, FGSC No. 1425, supposedly *het-c*, *-D*, *-E*, gave Brown-Flat duplications as expected from crosses with *T(II→V)NM149 het-C*, but repeatedly produced many Brown-Flat progeny also when crossed with *T(II→V)NM149 het-c*. There is no evidence of another rearrangement in FGSC 1425, judging from the absence of aborted ascospores in crosses with Normal-sequence testers. Explanation of the anomalous behavior may well be related to that of certain wild strains that are incompatible with both *het-C* and *het-c NM149* testers, as will be described presently.

Use of the NM149 *translocation testers to diagnose strains of unknown* het-c *constitution*: Part B of Table 1 shows the results of crossing the confirmed *NM149* testers with strains whose *het-c* genotype had not previously been determined. The first six entries (fluffy, Emerson and Lein) are laboratory-derived strains in common use. Many other laboratory strains not shown in Table 1 were crossed with both *T(*II→V*)NM149 het-C* and *het-c* testers. Any one strain consistently gave Brown-Flat duplications with one tester or the other, but not with both, enabling it to be classified either as *C* or *c*. Brown-Flats from all such crosses were quite similar in appearance and behavior.

When wild-collected *N. crassa* strains from a variety of geographical sources were tested in the same way, a number of them resembled the laboratory strains and were typed as either *het-C* or *het-c* on the basis of their producing typical Brown-Flat duplications with one but not both of the translocation testers. The next four examples in Table 1B are of this type. (Abbott and Chilton wild types were originally collected in Louisiana.)

Other wild-collected strains behaved quite differently, giving inhibited duplications when crossed to both *het-c* and *het-C* translocation testers (Table 1B, last eight examples). With these doubly incompatible wild strains, the growth rate and morphology of most (but not all) of the inhibited duplications were characteristically different from *het-C/het-c*, depending specifically upon the wild strain involved. This behavior suggested the presence in the various wild-collected strains of either multiple *het-c* alleles or of genes at another *het* locus or loci within the duplicated segment, in addition to *het-c*.

In an attempt to clarify the basis of the dual incompatibility, additional translocation tester strains were obtained by transferring the distal IIL segment

from each of three deviant wild strains into *T(*II→V*)NM149* sequence. Strains Adiopodoumé A, Groveland-la, and Panama A were used. (FGSC Nos. 430, 1945, and 1131). The transfer of IIL was accomplished for each wild strain by crossing with *T(*II→V*)NM149 ro-3 het-C* or *T(*II→V*)NM149 pyr-4 het-C* and selecting crossover progeny that were translocation sequence and nonmutant. These new translocation tester strains, each with IIL from a different wild source, were crossed in turn with each deviant wild strain and with *het-C* and *-c*.

The results of normal-sequence × Translocation intercrosses between the five different types are given in the top half of Table 2. (The tests with *T(*II→VI*)P2869* will be discussed shortly.) Each of the five is vegetatively incompatible with the other four by these tests, which indicate the presence of *het*-gene differences within the IIL segment covered by *NM149* duplications. The bottom portion of Table 2 gives the results of crossing four other deviant wild strains with the five *T(*II→V*)NM149* Testers. Two of these resemble "PA". The others are incompatible in all five combinations, as is the deviant reference strain FGSC 1425.

At the time the dual incompatibility with *C* and *c NM149* testers was first observed, multiple-allelism at the *het-c* locus seemed the simplest hypothesis (PERKINS 1969, 1972b). Recently MYLYK (1975a) has shown that *het* loci are more numerous than was previously suspected. More specifically, he has shown the presence of one or more *het* loci in addition to *het-c* in the IIL segment covered by *NM149* duplications. Consequently, the possibility of multiple loci must now also receive serious consideration.

Use of duplications from T(II→VI)P2869 *to resolve* het *differences in strains showing dual incompatibility with* NM149 het-c *testers*: In searching for other incompatibility genes in IIL, MYLYK (1975a) employed *T(*IIL→VI*)P2869*, a duplication-generating rearrangement whose IIL breakpoints, shown by arrows in Figure 1B, differ from that of *NM149*, and whose duplications cover *pi* and *cys-3* but not *het-c*, *ro-3*, or *pyr-4*. He showed an incompatibility locus other than *het-c* to be located within *P2869* duplications, and thus also within *NM149* duplications. Differences at both *het-c* and the second locus, called *het-6*, could in part explain the results in Table 2.

Strains showing dual incompatibility with the *het-c* testers in Table 1 were crossed with *T(*II→VI*)P2839* testers containing the allele *het-6*^{OR} (MYLYK 1975a), with the results shown in the rightmost column of Table 2. *P2869*-duplication progeny were inhibited in the test crosses of five strains—Groveland, Panama, Costa Rica, Marrero, and Mauriceville. The dual incompatibility of these with both *het-C* and *het-c NM149* testers may thus be reasonably attributed to differences at *het-6* or another locus similarly placed.

P2869-duplication progeny of the remaining anomalous strains were phenotypically noninhibited. Thus their incompatibility with both *C* and *c NM149* testers cannot be due to *het-6* or another incompatibility locus within the segment covered by *Dp(*IIL→VI*)P2869*. Their behavior could be explained either by multiple alleles at *het-c* or by another heterozygous *het* locus in addition to *het-c* that is located within the limits of *Dp(*IIL→VR*)NM149* but outside

TABLE 2

Results from intercrosses between Normal-sequence and T(IIL→VR)NM149 *strains involving* IIL *segments that deviate from behavior typical of* het-C *or* het-c

The numbers given for each test cross are (inhibited duplication progeny)/(total progeny).

Normal-sequence parent*	Translocation-sequence parent†					
	T(IIL→VR)NM149					*T(IIL→VI)P2869*
	C standard	c standard	"AD"	"GR"	"PA"	*het-6^{OR}*
C standard	0/38	**14/60‡**	**10/48**	**13/56**	**14/35**	0/50
c standard	**37/118‡**	0/33	**16/58**	**15/48**	**12/36**	0/26
"AD"	**12/50**	**15/54**	0/47	**10/43**	**8/43**	1/49
"GR"	**12/130**	**7/53**	**6/37**	0/35	**14/46**	**13/48**
"PA"	**42/166**	**34/138**	**13/48**	**14/47‡**	0/47	**15/42**
Costa Rica A (851)	**15/53**	**11/53**	**12/47**	**15/46**	0/84	**18/45**
Houma-1g A (P497)	**15/125**	**19/48**	**14/45‡**	**18/44**	**17/37**	1/44
Marrero-1d a (2224)	**14/45**	**13/47**	**14/42**	**14/35‡**	0/48	**14/46**
Mauriceville-1c A (2225)	**12/42**	**21/47**	**11/45**	8/93‡	**15/50‡**	**11/44**
al-2; pan-1 A (1425)	**13/31‡**	**10/81‡**	**18/66‡**	**21/52**	**13/45**	0/46

* The following normal-sequence stocks were used: C standard: 74-OR23-1A (FGSC 987), 74-OR23-1VA (2489), 74-OR8-1a (988) or 74-ORSa (2940). c standard: Em a (692), *fl^{P}A* (295, 1838), or *fl^{P}a* (1690, 2111). "AD": Adiopodoumé A (430). "GR": Groveland-1c a (1945). "PA": Panama A (1131), or *het-c^{PA}A* (2189), or *het-c^{PA}a* (2190) (derivatives of 1131).

† The following translocation-sequence stocks were used: Standards as in Table 1, third footnote. *T(II→V)NM149 het-c^{AD}A* or *a* (FGSC 2191, 2192), *T(II→V)NM149 het-c^{GR}A* or *a* (FGSC 2193, 2194), *T(II→V)NM149 het-c^{PA}A* or *a* (FGSC 2547, 2188), *T(II→VI)P2869 het-6^{OR}A* or *a* (FGSC 1828, 1829).

‡ Inhibited duplications were all or nearly all spreading Brown-Flat phenotype, typical of *het-C/het-c*. A majority of the inhibited duplications other than those marked ‡ differ phenotypically from the spreading Brown-Flat types characteristic of *het-C/het-c*, while a minority resemble Brown-Flats. See PERKINS (1972c) for an attempt to describe and distinguish the phenotypes of various incompatible heterozygotes.

*Dp(*IIL→VI*)P2869*. These alternatives should ultimately be resolvable either by recombination or by use of other duplications which cover different parts of *Dp(*IIL→VR*)NM149*.

III. *Mating-type* het-*incompatibility in duplications from overlapping translocations*

Mating type provides a third example of a *het* locus studied both in heterokaryons and in duplications. The *het*-incompatibility associated with mating type was first bracketed between the IL breakpoints of *In(IL→IR)H4250* (whose duplications cover the locus) and *In(IL→IR)NM176* (whose duplications do not) NEWMEYER and TAYLOR 1967; TURNER *et al.* 1969). NEWMEYER, HOWE and GALEAZZI (1973) then showed that the somatic incompatibility and sexual compatibility functions of the mating-type region were not separable by crossing over, indicating that the functions are either two expressions of a single gene, or if determined by more than one gene, the factors responsible are extremely closely linked.

Previous results had led to the conclusion that whenever mating type in *N. crassa* is heterozygous in a duplication, *A/a* incompatibility is expressed as an inhibited Dark-Agar phenotype. This conclusion was based upon duplications that were either terminal, from a pericentric inversion (NEWMEYER and TAYLOR 1967), or interstitial, from an insertional translocation (PERKINS 1972a). The only exceptions involve suppression of the somatic incompatibility phenotype by the gene *tol* (NEWMEYER 1970) or by a similar factor present in *T(*I;IV*)cut* (SMITH and PERKINS 1972).

The present study demonstrates a completely different way of obtaining heterozygous duplications and shows that the same inhibited phenotype is characteristic of *A/a* duplications regardless of their mode of origin. Analysis of *A/a* heterozygotes is extended to interstitial duplications that are obtained by intercrossing rearrangements which overlap in such a way that their breakpoints bracket the mating-type region. An extension of this method should permit *het* genes to be located and studied in chromosome regions for which simpler methods of producing duplications are not available.

Origin of the duplications: If two reciprocal translocations that involve identical chromosome arms overlap so that each has one breakpoint distal and one proximal relative to the other, synapsis in an intercross between them resembles that of an insertional translocation × normal sequence, and one third of viable progeny are duplications, which survive because they contain no deficiencies (see GOPINATH and BURNHAM 1956; PERKINS 1971, 1974). The duplications include the chromosome regions between both pairs of breakpoints, and, if parents differed at any loci in these regions, these loci are expected to be heterozygous in the duplication progeny. This is illustrated in Figure 1C for *T(*I;V*)AR12* and *T(*I;V*)P5401*—two reciprocal translocations whose breakpoints involve IL and VR in such a way that duplication progeny survive. The breakpoints in IL are on opposite sides of the mating-type locus. Consequently, all duplications from the intercross are *A/a* heterozygotes with a spidery Dark-

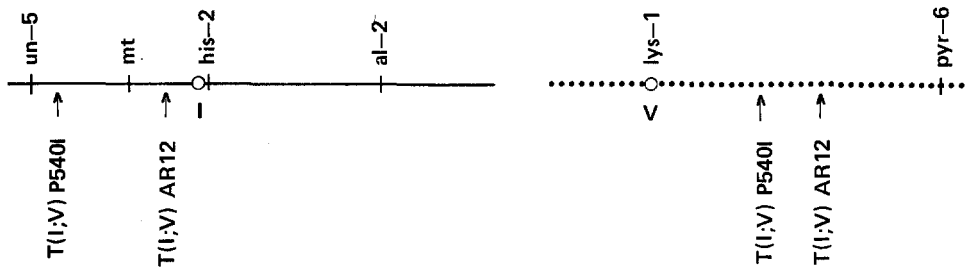

FIGURE 2.—Breakpoints of two reciprocal translocations used to study mating-type heterozygosity. Linkage Group I is shown as a solid line, V as dotted. Both are in Normal sequence. Arrows indicate the points of interchange. The location of the *P5401* breakpoint in IL is known from conventional mapping. The relative order of the other breakpoints is inferred from the fact that viable *A/a* duplications are obtained when *T(I;V)P5401* and *T(I;V)AR12* are intercrossed.

Agar phenotype typical of the vegetative incompatibility associated with mating type.

Characteristics of the Dark-Agar duplications: The inhibited progeny from intercrossing these reciprocal translocations resemble *A/a* Dark-Agar duplications from *In(IL→IR)H4250* × Normal and from *T(*I→II*)39311* × Normal, in morphology, pigment production, and escape behavior. For photographs see NEWMEYER and TAYLOR (1967).

Interchange points: *T(*I;V*)P5401* is known from mapping to be distal to mating type in IL, near *un-5* (Figure 2). *T(*I;V*)AR12* must therefore have its breakpoint proximal to mating type; otherwise the duplication progeny would not be heterozygous *A/a*. The sequence of breaks in V must be *centromere–P5401–AR12*; otherwise duplications would not be viable. If the order were *centromere–AR12–P5401*, all duplication progeny would contain lethal deficiencies.

A second IL;VR translocation, *47711*, resembles *T(I;V)AR12* in producing *A/a* duplications when it is crossed with *T(I;V)P5401*. Thus the interchange points of *T(I;V)47711* relative to *T(I;V)P5401* must be similar to those of *AR12* in Figure 2—proximal in IL and distal in VR. The order of *47711* relative to *AR12* is not known from conventional mapping, but two possible sequences can be eliminated. When *AR12* is crossed with *47711*, no viable duplications are produced. Therefore the order cannot be either *AR12–47711–centromere-I*, *centromere-V–AR12–47711*, or *47711–AR12–centromere-I*, *centromere-V–47711–AR12*, because both these sequences would result in viable duplications. The correct order must be either *AR12– 47711–centromere-I*, *centromere-V–47711–AR12*, or *47711–AR12–centromere-I*, *centromere-V–AR12–47711*.

DISCUSSION

Heterokaryon-incompatibility as one manifestation of vegetative incompatibility: The observations reported here indicate that abnormal inhibited growth of heterozygous duplications on one hand, and failure to form stable heterokaryons, on the other hand, are two different phenotypic manifestations of the same genotype for the three loci tested, *het-e*, *het-c*, and *mt*. With all three,

the inhibition is clearly due to the *het* genes and not to the duplicated condition itself, because the duplications are phenotypically normal, or nearly so, when *het* alleles are homozygous. Another line of evidence leads to the same conclusion for the vegetative incompatibility associated with mating type: an unlinked gene *tol* (tolerant) suppresses the expression of *A/a* vegetative incompatibility both in heterokaryons and in duplications (NEWMEYER 1970).

Other loci have not been tested in both duplications and in heterokaryons. *het-d* and *het-i* are known only by their effect on heterokaryons. In contrast, the six new incompatibility loci discovered by MYLYK (1975a) are known only by their effect in duplications, and it would be very laborious to demonstrate their effect in heterokaryons. From the behavior of *het-e*, *het-c* and *mt*, however, we can confidently assume that inhibition has the same underlying cause whether it is expressed in duplications or in heterokaryons.

(Since the above was written, MYLYK (personal communication) has succeeded in demonstrating directly that differences at two of the new loci which give inhibited duplications are also incompatible in heterokaryons.)

The new genes which have been detected using duplications are all predicted to show some type of heterokaryon incompatibility, but this may not be expressed identically for all loci. The incompatibility genes originally characterized in heterokaryons fall into two main classes. One type shows an immediate protoplasmic killing reaction following fusion of incompatible mycelia, as was described by GARNJOBST and WILSON (1956) and WILSON and GARNJOBST (1966) for *het-c*, *-d*, *-e*, and *mt*. The second type, exemplified by *het-i*, shows a delayed reaction when incompatible alleles are combined in a heterokaryon. No killing or protoplasmic change is observed initially when incompatible mycelia fuse, but the heterokaryotic condition is later terminated by elimination of one set of component nuclei (PITTENGER and BRAWNER 1961). Heterokaryon incompatibility genes in *Aspergillus nidulans* apparently belong to the second type (JINKS *et al.* 1966). The terms 'true incompatibility' and 'heterokaryon disadvantage' have been suggested by CATEN and JINKS (1966) for the two types showing instant and delayed reactions.

The three *het* genes tested in Neurospora duplications are all of the first, fast-acting type. It will be of interest to determine whether the slow-acting type of heterokaryon incompatibility gene exemplified by *I/i* also results in heterozygous duplications that are inhibited. If both slow- and fast-acting types show similar incompatibility phenotypes in duplications, this would suggest a basic similarity of action, and would tell against the suggestion of CATEN and JINKS (1966) that the two types may be different in origin, mechanism, and evolutionary consequences. And perhaps the anomalous tester FGSC No. 1425 may behave as it does because a slow-acting *het* gene is present, which went undetected in heterokaryon tests because only initial growth of the heterokaryon was observed, but which is fully expressed in heterozygous duplications.

The search for new vegetative incompatibility genes: MYLYK (1975a) has crossed each of five strains from different geographical regions by 14 different duplication-generating rearrangements. His results establish the existence of at

least five additional incompatibility loci that resemble *het-c*, *het-e*, or mating type when heteroallelic combinations are present in duplications. The results indicate that many other *het* loci will be found when other duplications and other wild strains are tested. Allelic differences at incompatibility loci are common not only between strains from geographically separated populations, but also within local populations (MYLYK 1975b).

Sources of duplications: Nontandem duplications that are either heterozygous or homozygous for known *het* genes have now been generated meiotically in three different ways, using (1) insertional translocation × Normal (PERKINS 1972a); (2) quasiterminal rearrangement × Normal—either pericentric inversion (NEWMEYER and TAYLOR 1967) or translocation (this paper); and (3) overlapping rearrangements—either reciprocal translocation × overlapping reciprocal translocation (this paper) or inversion × overlapping inversion (NEWMEYER, TURNER and TAYLOR, unpublished). Regardless of which way they originated, the recurrent duplications from a given duplication-generating combination of parents comprise a defined chromosome segment and provide a means of demonstrating allelic differences at whatever *het* or *veg* locus may be included, and of comparing the phenotypes of heterozygotes and homozygotes.

Over 30 different duplication-generating rearrangements are available in *N. crassa*, each of which provides a means of testing a specific chromosome segment for the presence of *het* differences. Well over half of the genome can be tested using duplications from one or another of these rearrangements (PERKINS 1962, Appendix). Additional segments of the genome can be covered using the duplications that are produced by intercrossing pairs of appropriately overlapping rearrangements. Thirty different pairs of reciprocal translocations that share the same two chromosome arms have already been shown to generate viable duplications, and other potentially overlapping combinations still remain to be tested.

Theoretically it should be possible to study vegetative incompatibility using disomics instead of duplications. This is impractical in Neurospora, however, because disomics are very unstable. Their greater length compared to intrachromosomal duplications would also increase the probability that more than one *het* locus would be covered.

Experimental uses for het^x/het^y duplications: 1. *Source of material for analyzing the incompatibility reaction.* The cellular and molecular basis of the abnormal growth of incompatible combinations is poorly understood. Material for analysis in a search for possible abnormal metabolites should be much more readily obtained in bulk from cultures of heterozygous duplications than from the cells involved in abnormal fusions between incompatible haploid strains.

2. *Somatic instability.* Duplications whose inhibition is due to heterozygosity of *het* genes provide valuable tools for investigating somatic events that lead to release from the inhibition. Escape from *het*-caused inhibition after a variable lag period is a general characteristic of duplications from many different sources. In individual instances, escape might occur by mutation, inactivation, homozygosis, or deletion of one of the *het* alleles, or by suppressor mutation (NEW-

MEYER 1970). It appears that segmental loss is a common cause (PERKINS, NEWMEYER and TURNER 1972; NEWMEYER and GALEAZZI 1974).

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Corresponding editor: R. H. DAVIS