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Reproductive Isolation and Taxonomic Differentiation of *Romanomermis culicivorax* Ross and Smith, 1976 and *R. communensis* Galloway and Brust, 1979

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Abstract: The infertility of hybrid progeny of *Romanomermis communensis* and *R. culicivorax* supports their retention as distinct species. Their taxonomic separation on the basis of morphometric data and possession of a cone-shaped spicule guide is rejected. However, differences in the enzyme patterns of peptidase and phosphoglucomutase and the restriction fragment length differences in repetitive genomic DNA provide sensitive diagnostic characters that confirm the differentiation into two species.

Key words: reproductive isolation, *Romanomermis communensis, R. culicivorax,* taxonomy.

Romanomermis communensis Galloway and Brust, 1979 and *R. culicivorax* Ross and Smith, 1976, both parasites of larval mosquitoes in North America, were recognized as distinct species on the basis of morphological differences (10). However, the validity of separating them into two species has been questioned recently 1) on morphological grounds, following analyses of the nature and extent of intraspecific morphological variation in *R. culicivorax* (2,3), and 2) by cross-mating experiments which produced viable hybrids (8). In this latter study preparasitic juveniles were obtained from the reciprocal crosses, but they developed into males only. Lack of females prevented testing of hybrid fertility.

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TABLE 1. Survival of males and females and gravid or postoviposition females from reciprocal and self-crosses of *Romanomermis communensis* (com) and *R. culicivorax* (cul) after 50, 75, and 100 days.*

Cross	Surviving ♂			Surviving ♀			Surviving ♀ gravid or postoviposition		
	50	75	100	50	75	100	50	75	100
(a) Nematodes reared at 20 C throughout experiment									
com ♀ × com ♂	89	t†	t	100	t	t	98	t	t
com ♀ × cul ♂	92	t	t	84	t	t	88	t	t
cul ♀ × com ♂	86	88	88	90	100	88	1	2.3	0
cul ♀ × cul ♂	70	100	100	100	78	84	6	7.7	4.8
cul ♀				100	94	91	0	0	0
com ♀				90	93	68	0	0	0
(b) Nematodes reared at 20 C for 50 days, followed by 27 C for an additional 50 days									
cul ♀ × com ♂	86	33	26	90	95	88	1	7.3	2.6
cul ♀ × cul ♂	70	80	t	100	100	100	t	96	t

* Expressed as percentage surviving 0–50 days, 50–75 days, 75–100 days for respective columns.
† t = experiment terminated at previous sampling data.

To help clarify the taxonomic status of these two nominal species, we report on further cross-mating trials to test hybrid fertility and on isoenzyme and genomic DNA restriction endonuclease analysis to partially characterize both genotypes.

MATERIALS AND METHODS

Postparasitic juveniles of *R. communensis* (reared from *Aedes communis* larvae collected from the nematode type locality at Goose Creek, Churchill, Manitoba) and *R. culicivorax* (reared from *Aedes aegypti* larvae, the nematode culture originating from the laboratory culture of Dr. J. J. Petersen, Lake Charles, Louisiana) were sexed and the separate sexes allowed to molt at 20 C (*R. communensis*) and 27 C (*R. culicivorax*) over a period of 2–3 weeks. The resulting adults were placed in a glass Petri dish containing acid-washed, coarse-grained (1–2-mm grain size) silica sand (1–2 cm deep) overlain with glass-distilled water (1 cm deep) in the following groupings:

R. communensis 100 ♀ × *R. culicivorax* 100 ♂
R. communensis 100 ♀ × *R. communensis* 100 ♂
R. culicivorax 100 ♀ × *R. culicivorax* 100 ♂
R. culicivorax 100 ♀ × *R. communensis* 100 ♂
R. communensis 100 ♀
R. culicivorax 100 ♀

After 50 days at 20 C, the number of survivors and of surviving gravid or postoviposition females was recorded (8). In view of the results obtained (see below) it was speculated that *R. culicivorax* females did not mate well at 20 C. To test this hypothesis, the surviving nematodes from each cross were subdivided into two groups and maintained at either 20 C or 27 C for an additional 50 days. The number of survivors and of surviving gravid or postoviposition females was recorded after 25 and 50 days (i.e., 75 and 100 days after the start of the experiment). In addition, to ascertain if sperm transfer had taken place at 20 C in the *R. culicivorax* selfcross, five females were stained in Hoechst 33258 and the spermatheca examined for the presence of spermatozoa (5).

The viability and fertility of *R. communensis* × *R. culicivorax* hybrid progeny was determined as follows: hybrid eggs were maintained at 20 C and the percentage of fully embryonated eggs recorded after 100 days (eggs were flushed from sand with distilled water). First instar of *A. aegypti* were infected with newly emerged hybrid preparasitic juveniles (approximately 5:1 ratio (2), and the number and sex of postparasitic juveniles produced was recorded. To test hybrid fertility, these hybrid postparasitic juveniles were placed in moist sand in the wells of tissue culture plates (2) at a ratio of approximately 3 ♂ to 1 ♀ per well. Numbers of surviving nematodes and numbers of gravid or postoviposition females was recorded after approximately 50

days at 20 C and again after the temperature was elevated to 27 C for 25 days.

Isoenzyme analysis of virgin adult females of each species reared at 20 C utilized standard starch gel techniques (1,9) with the inclusion of 15 mg O-dianisidine·HCl in the peptidase stain. A known *Drosophila* sample was included in each gel as a positive control for enzyme activity. Restriction endonuclease analysis of the genomic DNA was performed on each species as described by Curran et al. (6).

Results

Percentage survival of males and females and percentage of surviving females that were gravid or had laid eggs were recorded after 50, 75, and 100 days (Table 1). After 50 days at 20 C, both the *R. communensis* ♀ × *R. communensis* ♂ and *R. communensis* ♀ × *R. culicivorax* ♂ crosses produced a high percentage of gravid or postoviposition females while crosses involving *R. culicivorax* females had a low percentage of gravid or postoviposition females. This pattern continued throughout the experiment at 20 C. However, in the concurrent experiment, following elevation of the rearing temperature after 50 days to 27 C, the majority of females from the *R. culicivorax* selfcross were gravid or postoviposition 25 days later whereas the *R. culicivorax* ♀ × *R. communensis* ♂ cross produced few gravid or postoviposition females. No evidence for parthenogenesis was found in the two species.

The low percentage of gravid or postoviposition females obtained at 20 C from the *R. culicivorax* selfcross was surprising, since males and females were frequently observed *in copula*. In light of this low mating success, it was hypothesized that sperm transfer may not occur at 20 C. None of the five *R. culicivorax* females examined contained sperm within the spermatheca.

Eggs resulting from *R. communensis* ♀ × *R. communensis* ♂ and *R. communensis* ♀ × *R. culicivorax* ♂ (crosses maintained at 20 C) were examined 75 and 100 days postoviposition. At the respective sampling date, 7/200 and 5/200 of the *R. communensis* ♀ × *R. culicivorax* ♂ hybrid eggs were embryonated, compared with 194/200 and 196/200 of the *R. communensis* ♀ × *R. communensis* ♂ eggs. Too few *R. culicivorax* ♀ × *R. communensis* ♂ embryonated hybrid eggs were obtained to permit further study of their development.

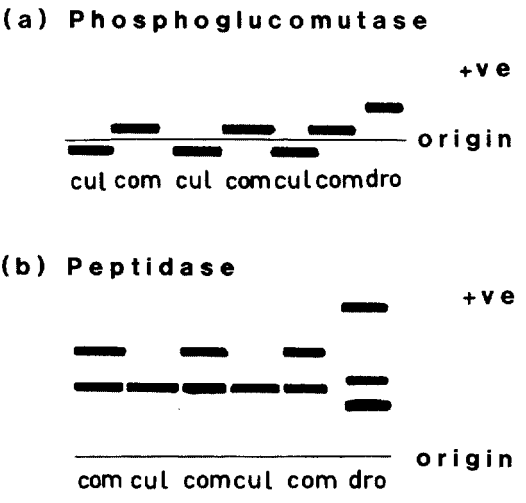

FIG. 1. Comparison of iosenzyme patterns of individual *Romanomermis culicivorax* and *R. communensis* virgin females. (a) Phosphoglucomutase. (b) Peptidase. cul = *R. culicivorax*; com = *R. communensis*; dro = *Drosophila* sp., used as positive control for enzyme activity.

The *R. communensis* ♀ × *R. culicivorax* ♂ parasitic juveniles reared in *A. aegypti* emerged over a 5-day period; 52 postparasitic juvenile males (6 dead on emergence) and 15 postparasitic juvenile females (2 dead on emergence) were collected and used in mating trials to test hybrid fertility. After 50 days at 20 C ten adult males and three females were recovered. Numerous dead postparasitic juveniles were observed, many of which had failed to complete ecdysis. The surviving males and females were placed at 27 C for an additional 25 days, but no females became gravid. *R. communensis* males and females were reared under identical conditions and after 50 days at 20 C all females were gravid.

Differences were detected between the isoenzyme patterns of virgin females of *R. culicivorax* and those of *R. communensis* (Fig. 1a, b) for phosphoglucomutase and peptidase. However, the malate enzyme-malate dehydrogenase patterns (not shown) for both species were identical. Differences in the restriction fragment lengths of repetitive DNA of *R. communensis* and *R. culicivorax* were detected (Fig. 2a, b) as can be seen by comparing the relative positions of repetitive DNA fragments (arrowed in figures).

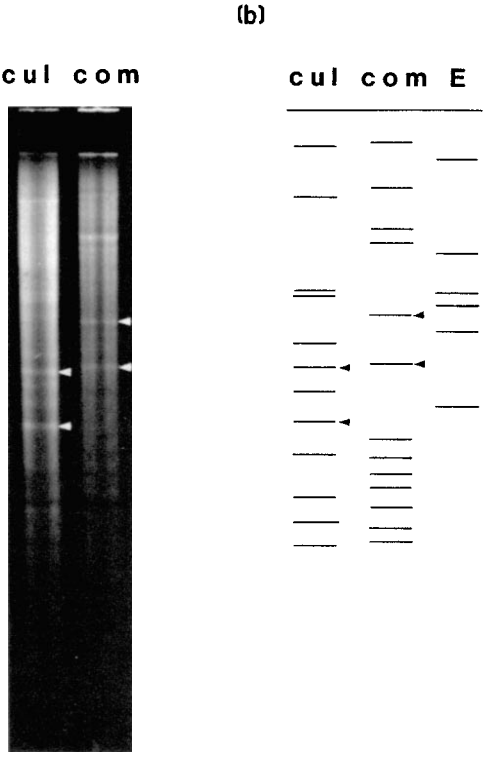

FIG. 2. Restriction fragment length differences of repetitive DNA between *Romanomermis culicivorax* (cul) and *R. communensis* (com). (a) Photograph of ethidium bromide stained UV illuminated (260 nm) 0.7% agarose gel of EcoR1 cut genomic DNA (1 μg per lane). Arrows indicate selected diagnostic repetitive DNA bands. (b) Tracing, from the negative, indicating all repetitive DNA bands, and EcoR1 cut λ DNA (Lane E) as size marker, bands at 21.7, 7.5, 5.9, 5.5, 4.9, 3.4 kilobases top to bottom.

DISCUSSION

These experiments confirm previous findings that cross-mating occurs between *R. communensis* and *R. culicivorax* (8). Furthermore, as with previous findings, more gravid females were obtained in our study from the *R. communensis* ♀ × *R. culicivorax* ♂ cross than the *R. culicivorax* ♀ × *R. communensis* ♂ cross. However, the present study has demonstrated that, despite the readiness with which mating occurred, most hybrid eggs failed to embryonate; of those that did, many of the hybrid postparasitic juveniles failed to complete ecdysis, and of the few adult males and females produced, none were fertile. This hybrid infertility supports the conclusion that *R. communensis* and *R. culicivorax* should be maintained as distinct species (8).

Originally, *R. communensis* was taxonomically separated from other species in the genus on the basis of a cone-shaped spicule guide as well as several morphometric differences (7). Later studies on intraspecific variation with *R. culicivorax* demonstrated that morphometric data was highly variable and influenced by environmental factors, and, consequently, that its use in species diagnosis within the genus was questionable (2). Furthermore, examination of adult males of *R. communensis* (provided by T. D. Galloway) revealed that the structure previously described as a spicule guide is, in fact, the lining of the cloaca. The so-called spicule guide is likely a fixation artifact because it is absent in live specimens. A similar structure can be observed in lactophenol processed *R. culicivorax* (4). Hence, the morphological characters used to differentiate *R. communensis* and *R. culicivorax* are of little diagnostic value.

The virgin females of these two species can be clearly differentiated from each other by their phosphoglucomutase and peptidase isoenzyme patterns. These biochemical characters may be subject to phenotypic variability, but by direct characterization of the genotype, using comparative restriction fragment length differences of repetitive DNA, the distinctiveness of the two species is reliably confirmed.

In conclusion, the biological, biochemical, and genetic data presented here confirms the maintenance of *R. communensis* and *R. culicivorax* as two distinct species.

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