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Congenital Myotonic Myopathy in the Miniature Schnauzer: An Autosomal Recessive Trait

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Myotonia is a clinical sign characterized by a delay in skeletal muscle relaxation following electrical or mechanical stimulation. A series of related miniature schnauzer dogs with congenital myotonic myopathy were studied. A composite pedigree of six affected litters and the results of a planned breeding between two affected animals are consistent with an autosomal recessive mode of inheritance.

Myotonia is a clinical sign characterized by a delay in skeletal muscle relaxation following the cessation of a voluntary activity or following the cessation of an electrical or mechanical stimulus (Barchi 1994). The delay in skeletal muscle relaxation is not accompanied by pain or cramping. Various biochemical defects may result in myotonia, including reduced membrane chloride conductance, alterations in the kinetics of sodium channel inactivation, and as yet undetermined membrane abnormalities (Barchi 1988, 1994). The known congenital diseases exhibiting myotonia are inherited as autosomal dominant or autosomal recessive traits and are summarized in Table 1.

In animals, myotonia has been described in the mouse, goat, horse, cat, and dog (Barchi 1994; Hickford et al. 1998). Except for congenital myotonia in the mouse and goat, due to defective chloride ion conductance across the muscle membrane (Bryant 1979; Mehrke et al. 1988), few studies characterize the biochemical defect or the mode of inheritance of the disease responsible for myotonia in animals. Defective chloride ion conductance in the goat results from a substitution of proline for alanine in the carboxyl terminus of the goat muscle chloride channel

Table 1. Congenital disease exhibiting myotonia

Disease	Defect	Mode of inheritance	Reference
Human			
Myotonia congenita			
Thomsen's myotonia	Chloride channel	Autosomal dominant	Harper (1995)
Becker's myotonia	Chloride channel	Autosomal recessive	Hudson et al. (1995)
Myotonic dystrophy	Undetermined membrane abnormalities	Autosomal dominant	Harper (1995), Hudson et al. (1995)
Sodium channel myotonia	Sodium channel	Autosomal dominant	Barchi (1995), Hudson et al. (1995)
Paramyotonia congenita	Sodium channel	Autosomal dominant	Barchi (1995), Harper (1995), Hudson et al. (1995)
Hyperkalemic periodi paralysis	Sodium channel	Autosomal dominant	Barchi (1995), Hudson et al. (1995)
Chondrodystrophic myotonia	Chloride channel	Autosomal recessive	Adams et al. (1997), Harper (1995), Swash and Schwartz (1981)
Mouse			
Arrested development of righting (ADR)	Chloride channel	Autosomal recessive	Mehrke et al. (1988)
Goat			
	Chloride channel	Autosomal dominant	Beck et al. (1996), Bryant (1979)
Horse			
Hyperkalemic periodic paralysis	Sodium channel	Autosomal dominant	Hoffman (1995)
Cat	?	?	Hickford et al. (1998)
Chow chow	?	Autosomal recessive	Jones et al. (1977)
Miniature schnauzer	Chloride channel	Autosomal recessive	Vite et al. (1997)

(Beck et al. 1996). Congenital myotonia has been described clinically in several dog breeds, however, the biochemical and molecular defect responsible for the myotonia has yet to be determined (Farrow and Malik 1981; Griffiths and Duncan 1973; Hill et al. 1995; Shires et al. 1983). Recently we discovered a miniature schnauzer with myotonia which was associated with defective chloride ion conductance across the skeletal muscle membrane (Vite et al. 1997). This present article further characterizes the clinical features and mode of inheritance of congenital myotonia in a family of miniature schnauzers.

Seventeen related miniature schnauzers were examined by the authors; nine were affected (four females; five males) and eight were unaffected with myotonia (three females, five males). In dogs with myotonia, the gait was stiff when movement was initiated, but diminished with exercise. After walking for 2 min, mild stiffness was still evident and bunny-hopping occurred when running. Any rapid change in posture associated with falling or turning quickly resulted in increased stiffness. Postural reactions, segmental reflexes, sensation, and cranial nerve examination were normal. Stridor and stertor was audible at the initiation of activity. In all affected dogs, and none of the nonmyotonic dogs, the lower jaw (mandible) was markedly shortened, resulting in superior prognathism. This condition, which occurs as a genetic defect in some dog breeds, is not common in miniature schnauzers (Patterson 1999). The mandibular canine teeth were displaced medially and the mandibular wings were narrowed. The tongue frequently protruded from the mouth at rest. Compression of the tongue resulted in dimpling which persisted for up to 30 s. Electromyographic (EMG) examination of affected dogs revealed myotonic discharges of approximately 40 s duration.

The propositus was one of two affected in a litter of six pups resulting from the mating of two related purebred miniature schnauzers. The parents, a female sibling, and three male siblings were all unaffected. A composite pedigree describing information from the family of the propositus and the results of a breeding between the propositus and an affected male miniature schnauzer are shown in Figure 1. Four affected litters of miniature schnauzers produced by breeder-directed matings were found through breeders and referring veterinarians and examined by the authors. An additional two litters with affected dogs were reported by breeders but were not examined directly at the University of Pennsylvania. All of the affected litters and their common ancestors are shown in the pedigree in Figure 1. Because of the striking and highly characteristic clinical features of the myotonia, it is likely that diagnosis of the disorder is accurate and essentially complete in the reported as well as the examined litters. However, examined and reported litters are separated in the following analysis because of possible inaccuracies in reporting the number of affected and normal animals in the litters not examined directly. In addition to the breeder-directed matings that produced affected offspring, one litter was produced by an experimental mating between affected dogs from different but related litters.

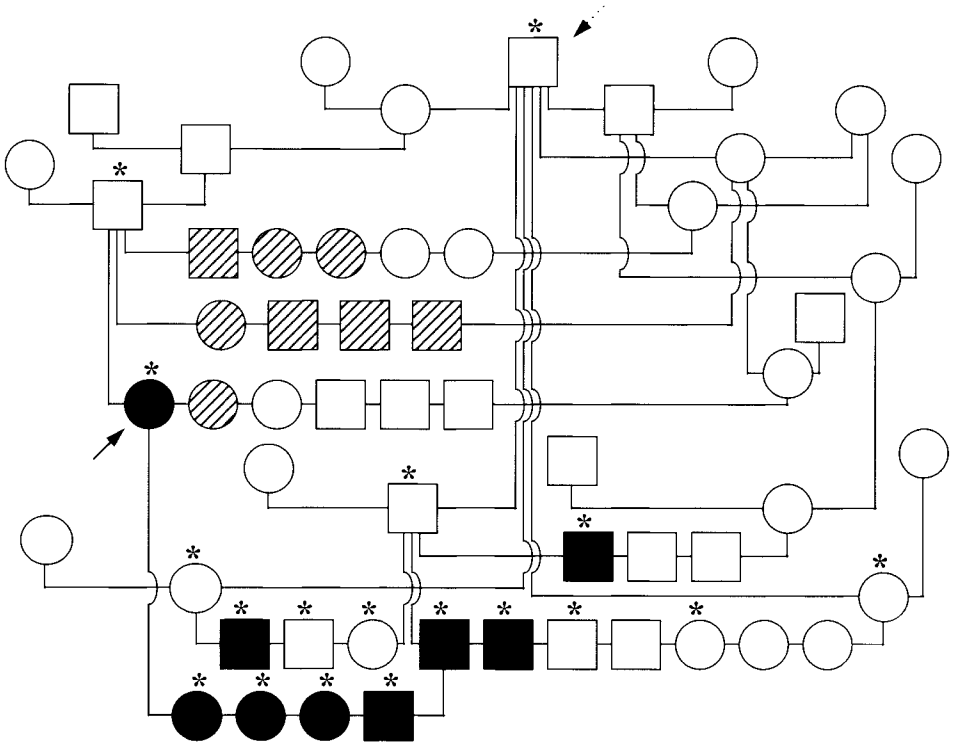

Figure 1. Pedigree information on a family of miniature schnauzers with myotonia. The litter at the bottom of the chart was a test mating between two affected dogs. Open circles and squares denote male and female clinically unaffected dogs; solid circles and squares denote clinically affected dogs, examined; striped circles and squares denote male and female clinically affected dogs, reported; asterisks denote examined animals; solid arrows denote proposita; and dotted arrows denote common ancestors.

All affected litters produced by breeder-directed matings, including those examined directly and those reported but not examined by the authors, had clinically unaffected parents. In all of these litters, the parents were related to each other through a common male ancestor reported to be unaffected. In the four affected litters examined at the University of Pennsylvania, 6 of 19 animals (31.6%) were affected, including 4 of 12 males and 2 of 7 females. One of the affected females, shown as the cross-hatched symbol in the pedigree in Figure 1, was not examined directly, but was so accurately described by the owner that its affected status was considered unequivocal. These findings suggest autosomal recessive inheritance. In the litters reported affected but not examined directly, the proportion of myotonic pups was 7 of 9 (4 of 4 males and 3 of 5 females). The apparent higher proportion of affected offspring in these litters may be due to distortion of the data by failure of breeders to accurately report the normal as well as the affected littermates. An experimental mating between a myotonic female and a myotonic male produced four affected and no unaffected offspring (three females and one male), consistent with autosomal recessive inheritance.

When examined and reported affected litters are not separated, mating of two unaffected dogs produced affected litters in which 13 affected and 15 unaffected animals were born. Eight affected males and five affected females were born, and eight unaffected males and seven unaffected females were born. The ratio of affected to unaffected animals approximates 1:1 as does the ratio of affected males to affected females. Congenital myotonic myopathy in the miniature schnauzer appears to follow

an autosomal recessive mode of inheritance.

Finally, it is interesting to note that all affected dogs exhibited superior prognathism and canine teeth that were medially displaced. This defect was not identified in any related, unaffected miniature schnauzers, and no such defect appears to have been reported in myotonia affecting other animal breeds. It is possible that this defect is due to contraction of the jaw muscles in myotonia of the miniature schnauzer; myotonia may produce a unique pleotrophic effect; or mandibular brachygnathism may be a heritable defect which segregates closely with myotonic myopathy. Based on clinical features, biochemical findings, and mode of inheritance, hereditary myotonia in the miniature schnauzer is most homologous to Becker's myotonia in man. Molecular studies on the defect in the skeletal muscle chloride channel are in progress.

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RAPD Diagnosis of the *obscura* Group Species Sympatric with *D. subobscura* in North America

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Drosophila pseudoobscura, D. persimilis, and *D. miranda,* sibling species of the *pseudoobscura* subgroup, have been characterized using RAPDs. With this technique the specimens to be analyzed can be preserved in various ways and the amount of variability that can be detected is high, which increases the probability of finding diagnostic markers. The use of RAPDs allows the unambiguous classification of any individual of the *pseudoobscura* subgroup. A total of two species-specific bands were detected in *D. pseudoobscura,* three in *D. persimilis,* and five in *D. miranda.* The amount of interspecific variation between *D. pseudoobscura* and *D. persimilis* was high; 58.38% of the total variance was due to variation between species and 41.62% to intraspecific variation.

Drosophila pseudoobscura, D. persimilis, and *D. miranda* are sibling species of the *pseudoobscura* subgroup whose distribution ranges are restricted to the western coast of North America. *D. pseudoobscura* is by far the most abundant representative of the subgroup and its distribution range includes that of the other two species (Buzzati-Traverso and Scossiroli 1955). The *pseudoobscura* subgroup species have diverged recently; the estimated times since speciation are 1 million years ago for *D. pseudoobscura* and *D. persimilis* (Wang and Hey 1996) and 2–4 million years ago for the formation of *D. miranda* (Beckenbach et al. 1993; Latorre et al. 1988; Wang and Hey 1996). Neither males nor females of these three species can be separated without laboratory studies. Traditionally the individuals of the *pseudoobscura* subgroup have been classified by sexual isolation (Dobzhansky and Epling 1944), chromosomal examination (Anderson et al. 1977), allozyme loci (Anderson et al. 1977; Prakash 1977), and mtDNA (Barrio et al. 1992; Powell 1983). Separating *D. miranda* from the other two species is easier than discerning *D. pseudoobscura* and *D. persimilis* because the differences revealed by these techniques are greater. Furthermore, the most common mitochondrial restriction patterns, and even some rare variants, are shared by *D. pseudoobscura* and *D. persimilis* collected in the same area (Powell 1983). Another disadvantage of the use of these markers in diagnosis is that live specimens and most of the time isofemale lines are needed.

Since the invasion of North America by *D. subobscura* (Beckenbach and Prevosti 1986) almost 20 years ago, it has been necessary to find markers that easily and unambiguously differentiate the Nearctic members of the *obscura* group, nowadays sympatric with the colonizing species, to monitor the pace of the colonization process. While males and females of *D. subobscura* can be classified morphologically (Beckenbach and Prevosti 1986), the endemic members of the *obscura* group cannot be separated easily. Until now, population studies of endemic and colonizing representatives of the *obscura* group in North America had classified Nearctic males of the *obscura* group as belonging to the *pseudoobscura* or *affinis* subgroup, using morphological characters (i.e., not to the species level), while the Nearctic species females were indistinguishable by morphological criteria (Pascual et al. 1993; Prevosti et al. 1989).

In a previous study the *affinis* subgroup members were characterized with allozymes, mtDNA, and randomly amplified polymorphic DNA (RAPD) (Pascual et al. 1997b). Here, having considered the advantages and disadvantages of the previous markers, we have chosen the RAPD technique as a tool for classifying the individuals of the *pseudoobscura* subgroup, since the specimens to be analyzed can be kept in different preserving materials without altering the result, and the amount of

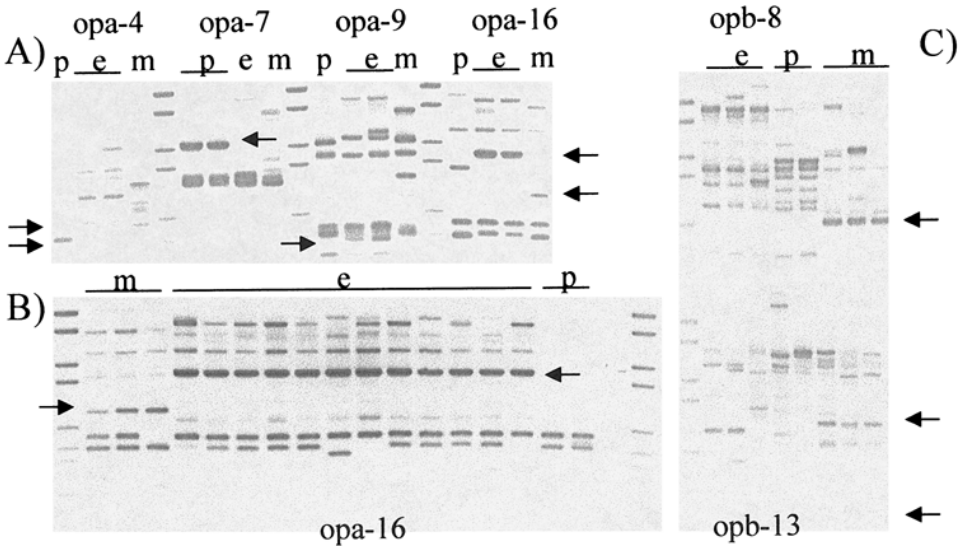

Figure 1. Amplification products of DNA that allow unambiguous classification of *D. pseudoobscura* (p), *D. persimilis* (e), and *D. miranda* (m). The arrows indicate diagnostic bands. Lanes correspond to unrelated individuals. **(A)** Primers of kit A; **(B)** opa-16; and **(C)** primers of kit B.

variability revealed by this technique is higher, which increases the probability of finding diagnostic markers. The diagnosis of all the individuals of the *obscura* group coexisting with *D. subobscura* in North America is fundamental for studying the dynamics of these species in nature. To do this it is necessary to have an easy and reliable technique available that allows the discrimination of all captured individuals.

Materials and Methods

The individuals analyzed came from different sources. Some of them were caught in the wild and kept in ethanol, others were isofemale lines that had been kept for many generations in the laboratory, and others were strains from stock centers. A total of 58 individuals were analyzed: 29 from *D. pseudoobscura,* 24 from *D. persimilis,* and 5 from *D. miranda.* The origin of the specimens is as follows: *D. pseudoobscura,* 27 from Gilroy (CA) and 2 from Salem (OR); *D. persimilis,* 3 from Gilroy (CA), 9 from Mather (CA), 2 from Winters (CA), 7 from Mt. St. Helena (CA), 1 from Eureka (CA), and 2 from stock centers; *D. miranda,* 1 from Mather (CA), 2 from Mt. St. Helena (CA), 1 from Eureka (CA), and 1 from a stock center. We tried to increase the number of *D. miranda* individuals analyzed by classifying 39 wild individuals from different localities kept in ethanol.

Genomic DNA was extracted from single individuals as described in Pascual et al. (1997a). Some of the individuals were frozen, kept in 70% ethanol, or kept in 100% ethanol. The amplification reaction was performed in 15 μl volumes, using 15 ng of genomic DNA, in a Perkin-Elmer GeneAmp PCR System 2400. The amplification program was 94°C for 5min, 35 cycles of 15 s at 94°C, 30 s at 38°C, and 1 min at 72°C; and 5 min at 72°C. Amplification products were separated on 1.4% agarose gel. Each reaction was performed twice and only bands showing clear and reproducible patterns were included in the final analysis. Subsequently a data matrix was produced, each individual being represented as a vector of 1s and 0s (1 for presence of any particular band and 0 for its absence). All of the bands scored in more than 95% of the individuals of one species and absent in those of the other two species were considered diagnostic bands. The sizes of those bands were estimated in 1.4% agarose gels. The Euclidean metric, which measures the band differences between individuals, was computed using RAPDistance (Armstrong et al. 1996) and used for studying the variance between and within species using WINAMOVA (Excoffier 1995).

Table 1. Primers used yielding species-specific bands and approximate size of bands (bp).

Primer	Sequence	*pseudo-obscura*	*persimilis*	*miranda*	*athabasca*	*azteca*
A-01	CAGGCCCTTC	—	—	—	—	650
A-03	AGTCAGCCAC	—	—	—	670	—
A-04	AATCGGGCTG	560	700	—	735/890	—
A-05	AGGGGTCTTG	—	—	—	370	—
A-06	GGTCCCTGAC	—	—	—	980	—
A-07	GAAACGGGTG	1300	—	—	—	—
A-08	GTGACGTAGG	—	—	—	540	480
A-09	GGGTAACGCC	—	480	—	—	395
A-10	GTGATCGCAG	—	—	—	—	350
A-11	CAATCGCCGT	—	—	—	350/470	640
A-14	TCTGTGCTGG	—	—	—	820	270
A-16	AGCCAGCGAA	—	1080	750	—	—
A-20	GTTGCGATCC	—	—	—	—	1000
B-08	GTCCACACGG	—	—	720/790	—	—
B-13	GTCCACACGG	—	—	370/870	—	—

Results and Discussion

To characterize the RAPD banding pattern of the three species, a preliminary screening was carried out with 20 primers (kit A, Operon Technologies) using three *D. pseudoobscura,* three *D. persimilis,* and two *D. miranda* individuals. Four primers were selected based on the clarity of the patterns obtained and the presence of species-specific bands: A-4, A-7, A-9, and A-16 (Figure 1a). A total of 64 bands, whose size ranged from 360 to 1800 bp, were scored with the four selected primers. Fifty bands were polymorphic, 13 were singleton, and 1 appeared in all individuals. The number of bands amplified per individual ranged from 12 to 24, with a mean of 17.1. Each individual yielded a different banding pattern, which demonstrates the extent of variability detected with this technique. However, when only one primer was used, the same banding pattern was observed in different individuals of the same species (Figure 1b). With the primers of kit A, a total of two diagnostic bands were detected in *D. pseudoobscura,* three in *D. persimilis,* and one in *D. miranda* (Table 1). The diagnostic bands used are in the middle molecular weight range, which usually produces more reliable results (Palacios and González-Candelas 1997). Since only one diagnostic band was detected for *D. miranda,* the 20 primers of kit B were assayed and bands appearing only in *D. miranda* individuals were scored. Primers B-08 and B-13 amplified reliable diagnostic bands in the five *D. miranda* individuals (Figure 1c, Table 1). These bands were

present in the five specimens analyzed and absent in six *D. persimilis* and seven *D. pseudoobscura* of different origin. The number of specimens analyzed is small for *D. miranda,* but since the lines used came from different localities (see Materials and Methods), it can be assumed that these bands are diagnostic. We estimated the size of species-specific bands observed by Pascual et al. (1997b) in *D. athabasca* and *D. azteca.* A total of nine bands clearly identified *D. athabasca* and seven bands identified *D. azteca* (Table 1).

The amount of variation between *D. pseudoobscura* and *D. persimilis* was estimated using the analysis of molecular variance. *D. miranda* was not included in the analysis due to the low number of individuals. Only polymorphic bands were used in the analysis. Of the overall phenotypic variation, 58.38% was due to variation between species and 41.62% to intraspecific variation. Population structure was not analyzed because the number of individuals was very low in some localities. Significant genetic differentiation between species was detected ($\phi_{ST} = 0.584$, $p < .001$). Tests of significance for variance components are based on 1,000 random permutations.

A total of 39 males and females from Bellingham (WA) and Salem (OR) kept in 70% ethanol were amplified with some of the diagnostic primers. The aim of this analysis was to increase the number of *D. miranda* in the sample. Males were morphologically classified to the subgroup level ahead of time and then the result of the RAPD amplification was cross-checked with the previous identification in order to assess the reliability of the system. A blind experiment, previously carried out with *D. azteca* and *D. athabasca* males using a morphological character and comparing the results with RAPD banding pattern has also shown the value of the technique (Pascual et al. 1997b). Bellingham (WA) individuals were classified as follows: 4 *affinis* subgroup males as *D. athabasca,* 16 *pseudoobscura* subgroup males as *D. pseudoobscura,* and 8 *obscura* group females as 7 *D. athabasca* and 1 *D. pseudoobscura.* Salem (OR) males, from the *pseudoobscura* subgroup, were classified as 7 *D. pseudoobscura* and 4 *D. persimilis.* All individuals were amplified with at least two primers, those producing species-specific bands. In each case opa-4 yielded a scorable set of bands for *D. athabasca,* opa-7 for *D. pseudoobscura,* opa-9 and opa-16 for *D. persimilis* and opb-8 for *D. miranda* (Table 1, Figure 1). Unfortunately no *D. miranda* was found and thus we could not increase the sample size of that species.

Here we describe species-specific bands of *D. pseudoobscura, D. persimilis,* and *D. miranda* that discriminate between these three sibling species. These results, along with those of a previous study using *D. azteca* and *D. athabasca* (Pascual et al. 1997b) permit classification of all the specimens collected in all samples of the distribution range of these five Nearctic species of the *obscura* group. We may thus be able to study their population dynamics and assess its influence on the colonizing success of *D. subobscura.*

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Reproductive and Mate Choice Strategies in the Hermaphroditic Flatworm *Echinostoma caproni*

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Due to the important role that mating systems play in the evolution of species, we investigate the selfing rate and mate choice in the simultaneous hermaphroditic parasite *Echinostoma caproni* (Trematoda). The echinostomes were maintained in two situations in mice: (1) double infections where the two individuals do or do not belong to the same geographic area isolate, and (2) triple infections where two of the three individuals originate from the same isolate and the third one originates from a different isolate. This experimental design permits analysis of intra- and interisolate selfing rates and of mate preference. We predict, in the first experiment, no difference between intra- and interisolate selfing rates. In the second experiment we expect a preferential outcrossing between individuals originating from the same isolate in order to avoid hybrid breakdown. The results obtained corroborate our predictions and emphasize the important and synergistic roles of selfing, inbreeding depression, and hybrid breakdown in the evolution of echinostome reproductive strategies.

Reproductive strategies constitute a major factor shaping the evolution of organisms. Indeed, mating systems influence the genetic variability as well as the genetic structure of populations (Charlesworth and Charlesworth 1987; Jarne 1995). In this context, hermaphrodites offer the opportunity to investigate the evolution of sexual reproductive modes since a single individual can self- or cross-fertilize. Compared to plants, few studies have been conducted on mating systems in animals. These works have been mostly carried out on molluscs [see Jarne et al. (1993) for a review; Doums et al. 1996; Städler et al. 1995), ascidians (Bishop 1996; Bishop et al. 1996), or free-living flatworms (Michiels and Streng 1998), and the analysis of reproductive strategies in helminth parasites has been roughly ignored for a long time. Nevertheless, studies on reproduction in parasites will provide data to fill out works on animals and to investigate if theories usually advanced to understand the evolution of mating systems in free-living organisms have a parasitic counterpart.

Some experiments have been conducted to investigate the mating behavior of the hermaphroditic platyhelminth parasite *Echinostoma caproni* (Trouvé et al. 1996). Infections of mice with two individuals, from two isolates (i.e., originating from two different geographic areas) showed that, in this condition, the echinostomes exhibit an unrestrictive mating system involving both selfing and outcrossing (Trouvé et al. 1996). Furthermore, in a mate-choice experiment where mice were infected with three individuals, two of the same isolate and one of another, we found that both related individuals produced very few outcrossed offspring with the unrelated individual (Trouvé et al. 1996). Although it was impossible to distinguish selfed from outcrossed offspring between related individuals, this result likely suggests a marked mate preference between individuals of the same isolate. This prezygotic isolation seems to be followed by a postzygotic isolation characterized by a hybrid breakdown. Indeed, it has been shown that the hybrids of the second and third generations display a significantly lower fecundity compared to both parental isolates and to the F_1 (i.e., hybrid breakdown; Trouvé et al. 1998).

In this context we can describe the forces which should drive the evolution of the echinostomes' reproductive strategies as follows. First, self-fertilization ensures the reproduction of the echinostomes, which, like many parasites, often evolve in low-density populations (Charnov et al. 1976; Ghiselin 1974; Tomlinson 1966). Second, inbreeding depression decreases the fitness of self-fertilized offspring. Third, after a period of isolated evolution, hybrid breakdown should reduce genetic exchange between different isolates.

Consequently, in the light of these predictions and our knowledge of echinostomes, we present a study on individuals originating from a new natural population, discovered in May 1996 in Mali, showing an isoenzymatic polymorphism. We investigate selfing versus outcrossing rate in (1) double infections where the two individuals do or do not belong to the same isolate; and (2) triple infections where two of the three individuals originate from the same isolate.

If our predictions are correct, we expect, in the first experiment, no difference between intra- and interisolate selfing rate. In the second experiment, we expect a preferential outcrossing between individuals originating from the same isolate in order to avoid hybrid breakdown.

Discussion

Echinostomes are simultaneous hermaphroditic Trematoda (Platyhelminth) parasitizing vertebrate intestines. The life cycle of *E. caproni* includes three successive hosts and an asexual reproduction occurring in the first intermediate host. *E. caproni* is routinely cycled in our laboratory according to standard procedures (Trouvé et al. 1996) using *Biomphalaria arabica* snails which act as first and second intermediate hosts and mice (Swiss OF1 stock) as the final host. In this study four isolates of *E. caproni* were used; they originate from Mali (Em), Egypt (El), Madagascar (Ec), and Cameroon (Ek) (Trouvé and Coustau 1998). The isolates El, Ec, and Ek are homozygous at the loci *GPI, PGM, MPI,* PheLeu peptidase, and PhePro peptidase and present at these loci, respectively, the alleles: El (aabaa), Ec (baaaa), and Ek (abcbb). Furthermore, the Em isolate has two homozygous electrophoretic forms (Em1 and Em2) for which the alleles at the previously mentioned loci are Em1 (aabaa) and Em2 (abbaa). The Em isolate also presents heterozygous individuals at the *PGM* locus.

Selfing Rate

We carried out infections of mice with two Em individuals (i.e., Em1 and Em2) to estimate the selfing rate involved within an isolate. Twenty days postinfection the intestines of the mice were opened and the worms collected. The uterus of each adult was torn to collect the eggs. Thirty larvae hatched from the eggs of each worm were individually brought into contact with one mollusc. Genetic exchanges were assessed from a "progeny-array analysis" by comparing the mother's genotype to that of her progeny at the rediae stage (larval stage in the first intermediate host). Electrophoretic analyses were performed according to the procedures described in Trouvé et al. (1996). The Em individuals showed a mixed mating system involving both selfing and outcrossing. Although variable (range 20–100%), the intraisolate outcrossing rates were very high since the total proportion of outcrossed offspring was 77% (Table 1).

Whereas our previous experiments on selfing rates (Trouvé et al. 1996) involved isolates originating from Egypt (El) and Madagascar (Ec) (interisolate situations), the present work was done on the Mali isolate for which we did not find any morphological or electrophoretic differences from the Egyptian one. In interisolate situations, the echinostomes reproduce by both self-fertilization and cross-fertilization (Table 1); the global outcrossing rate totaled 74% (Trouvé et al. 1996). The data were excluded when both individuals totally selfed (pair 5), as this could have been caused by no meeting and pairing between the mates in the host habitat.

We compared each intraisolate selfing rate to the interisolate estimates with Fisher's exact test (Sokal and Rolf 1981), using the NPstat program, and then with the sequential Bonferroni method (Rice 1989). There was no obvious difference in outcrossing rates (Table 1); also, the global statistic of the intra (mean 77%) and inter (mean 74%) outcrossing rates did not show significant p values according to Fisher's method ($p = .44$).

These results pointed out a predominance of cross-fertilization compared to self-fertilization in both inter- and intraisolate matings. However, in both cases the echinostomes partly self-fertilized. This suggests that selfing is certainly a reproductive mode used to ensure reproduction in the echinostomes. Otherwise the low probability of meeting a partner due to low mobility or population density (Charnov et al. 1976; Ghiselin 1974; Tomlinson 1966) could result in no reproduction. Furthermore, selfing is promoted by the cost of outcrossing, which refers to

Table 1. Comparison of intraisolate and interisolate outcrossing rates

Pair no.	Parent	Number of offspring analyzed	Number (%) of outcrossed offspring[a]	Pair no.	Parent	Number of offspring analyzed	Number (%) of outcrossed offspring[b]
1	Em1	13	10 (77)	1	Ec	19	11 (58)
	Em2	20	15 (75)		El	22	22 (100)
2	Em1	10	2 (20)	2	Ec	17	7 (41)
	Em2	15	4 (27)		El	24	8 (33)
3	Em1	16	11 (69)	3	Ec	21	16 (76)
	Em2	16	14 (87.5)		El	26	24 (92)
4	Em1	17	15 (88)	4	Ec	18	12 (67)
	Em2	20	17 (85)		El	25	11 (44)
5	Em1	16	10 (62.5)	5	Ec	20	0 (0)
	Em2	16	14 (87.5)		El	18	0 (0)
6	Em1	21	14 (67)	6	Ec	22	18 (82)
	Em2	19	18 (95)		El	18	18 (100)
7	Em1	24	22 (92)	7	Ec	20	20 (100)
	Em2	23	13 (56.5)		El	18	16 (89)
8	Em1	19	15 (79)				
	Em2	19	13 (68)				
9	Em1	24	10 (42)				
	Em2	24	20 (83)				
10	Em1	23	23 (100)				
	Em2	20	20 (100)				
11	Em1	18	16 (89)				
	Em2	18	14 (78)				
12	Em1	25	20 (80)				
	Em2	17	9 (53)				
13	Em1	26	22 (85)				
	Em2	26	20 (77)				
14	Em1	21	19 (90)				
	Em2	19	11 (58)				
15	Em1	18	5 (28)				
	Em2	23	23 (100)				
16	Em1	22	22 (100)				
	Em2	17	17 (100)				
17	Em1	17	12 (71)				
	Em2	17	16 (94)				
18	Em1	14	13 (93)				
	Em2	20	18 (90)				
19	Em1	25	23 (92)				
	Em2	26	12 (46)				
20	Em1	21	16 (76)				
	Em2	20	20 (100)				
Mean	**Em (Em1 and Em2)**	**19.6**	**15.2 (77)**	**Mean**	**Ec El**	**19.5 22.2**	**14 (72) 16.5 (74)**

Em1, Em2: two electrophoretic forms of the isolate of Mali; Ec, El: isolates originating from Madagascar and Egypt, respectively (see text).

[a] This study.

[b] Trouvé et al. (1996).

the transmission of genes and results from the doubling of parent-offspring relatedness under selfing compared to outcrossing. In this context, alleles have a 50% transmission advantage (Charlesworth 1980). On the other hand, the homozygosity resulting from selfing uncovers a genetic load by the expression of recessive deleterious mutations, which should promote random mating (Charlesworth and Charlesworth 1987). A mixed mating system can be selected as a consequence of biparental inbreeding (Ronfort and Couvet 1995; Uyenoyama 1986), a phenomenon that is expected in these organisms because the asexual reproduction at the larval stage may give rise to clonal individuals in the same final host. Such a mixed mating system has already been identified in pulmonate snails (Städler et al. 1993, 1995; Doums et al. 1996; Coutellec-Vreto et al. 1997; Viard et al. 1997) as well as in numerous plant species [Charlesworth and Charlesworth (1987) for a review; Karron et al. 1995].

Mate Choice

The choice of mate was analyzed by performing concurrent infections of mice with one Ek, one Em1, and one Em2 worm. The estimation of genetic exchanges was done following the same procedure as described above. For each replicate, the intraisolate outcrossing rate was compared with the interisolate one. Since these two datasets are not independent, we performed a sign test using Statistica 4.1 for Macintosh.

Three reproductive modes have been used by the Em individuals (Em1 and Em2): selfing, outcrossing with a related individual (intraisolate outcrossing), and outcrossing with an unrelated individual (interisolate outcrossing) (Table 2). However, Em individuals produced significantly more offspring through intraisolate outcrosses than via interisolate outcrosses ($p < .01$). This result is clearly illustrated by comparing the mean proportion of intra-outcrossed offspring (83%) with the mean of interoutcrossed offspring (7%) for Em individuals. This clearly highlights that individuals originating from the same isolate (Em) preferentially outcrossed with each other.

These results can be explained in view of the long process of two evolutionary factors: first, inbreeding depression is expected to decrease selfing rate and, second, hybrid breakdown likely reduces the interisolate outcrossing. These two factors act in synergy and seem to account for the high intra-cross-fertilization observed. Whereas this result on mate choice could be interpreted as postzygotic incompatibility between the two isolates, we should note that if such an incompatibility exists, it would have induced a decrease of the interisolate outcrossing rate in the first experiment with double infection. On the contrary, we did not observe a significant difference between intra- and interisolate outcrossing rates (Table 1). Although the mate choice experiment involved a small number of replicates, the results are highly significant and clearly confirm the hypothesis proposed in a previous article (Trouvé et al. 1996), namely, that there is mate preference between related individuals.

This preferential intraisolate outcrossing may result from two phenomena: (1) a preferential mating between related individuals, that is, assortative mating. Such a phenomenon has already been pointed out in a number of species, for example, *Drosophila melanogaster (*Wu et al. 1995), *Tribolium confusum (*Wade et al. 1995), as well as in parasites, on schistosomes (Tchuem Tchuenté et al. 1993), or (2) a postcopulatory prezygotic isolation, that is, a gametic selection which would operate in the female genital tract (sperm competition or cryptic female choice; Eberhard 1996). These postcopulatory isolation mechanisms have been observed in a wide range of taxa [for reviews see Bishop (1996), Bishop et al. (1996), Gomendio and Roldan (1993), Parker (1990), and Wirtz (1997)]; however, no study has been carried out in this context for parasites.

Table 2. Selfing and outcrossing rates exhibited by each parent of a group of three worms: Em1 + Em2 + Ek

Trio no.	Parent	Number of offspring analyzed	Number (%) of eggs fertilized by the sperm of		
			Em1	Em2	Ek
1	Em1	14	0 (0)s	14 (100)	0 (0)
	Em2	16	12 (75)	3 (19)s	1 (6)
	Ek	17	9 (53)	8 (47)	0 (0)s
2	Em1	23	6 (26)s	14 (61)	3 (13)
	Em2	19	19 (100)	0 (0)s	0 (0)
	Ek	17	16 (94)	0 (0)	1 (6)s
3	Em1	24	0 (0)s	24 (100)	0 (0)
	Em2	20	14 (70)	2 (10)s	4 (20)
	Ek	17	2 (12)	15 (88)	0 (0)s
4	Em1	20	6 (30)s	14 (70)	0 (0)
	Em2	19	16 (84)	0 (0)s	3 (16)
	Ek	21	10 (48)	11 (52)	0 (0)s
5	Em1	21	0 (0)s	21 (100)	0 (0)
	Em2	21	16 (76)	3 (14)s	2 (10)
	Ek	19	5 (26)	12 (63)	2 (11)s
Mean	**Em1**	**20.4**	**2.4 (12)s**	**17.4 (85)**	**0.6 (3)**
	Em2	**19**	**15.4 (81)**	**1.6 (8)s**	**2 (11)**
	Ek	**18.2**	**8.4 (46)**	**9.2 (51)**	**0.6 (3)s**

Em1, Em2: two electrophoretic forms of the isolate of Mali; Ek: isolate originating from Cameroon (see text); s = selfing rate.

Hence further work is needed to distinguish between these hypotheses.

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Quantitative Trait Loci Influencing Honeybee Alarm Pheromone Levels

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Quantitative trait loci (QTL) mapping procedures were used to identify loci that influence the levels of alarm pheromones found in the stinging apparatus of worker honeybees. An F_1 queen was produced from a cross between a queen of European origin and a drone descended from an African subspecies. Haploid drones from the hybrid queen were individually backcrossed to European queens to produce 172 colonies. Samples of stings were taken from backcross workers of these colonies. Alarm pheromone levels were determined by gas chromatography. RAPD markers were scored from the haploid drone fathers of these colonies. The multiple-QTL model (MQM) of MapQTL was used to identify QTLs that influence the levels of four alarm pheromone components. Seven independent, potential QTLs were identified with LOD scores greater than two, and one at LOD 1.88. We identified one QTL for n-decyl acetate,

three for n-octanol, four for isopentyl acetate, and one for hexyl acetate. One region of linkage group XI shows a strong influence on body size and the levels of three alarm pheromone components. This locus explained 40% of the variance for the amount of n-decyl acetate (LOD 6.57). In general, the QTLs influencing alarm pheromone levels were independent of previously identified loci that influenced the stinging behavior of these colonies. The only exception was a potential locus influencing levels of n-octanol, which was inversely correlated with stinging behavior.

The honeybee (*Apis mellifera* L.) could become an important model organism for the study of behavioral genetics. Compared to most other insects, much is known about the complex behavior of this social Hymenopteran and the pheromones that assist in mediating the interactions of nest mates. Genetic analyses are facilitated in honeybees because the males (drones) are haploid. The major difficulty confronting honeybee geneticists is that of producing and maintaining sufficient numbers of colonies, especially of selected, inbred stocks. Many economically important traits, such as honey production, depend on the social interaction of many individuals in a colony. A colony normally consists of about 20,000–60,000 individuals and has worker bees (nonreproductive females) from 10 to 20 subfamilies. Workers in different subfamilies have the same queen mother but different haploid drone fathers. Male haploidy in the honeybee has been exploited to evaluate modes of inheritance (Guzmán-Novoa and Page 1994; Rothenbuhler 1964; Tucker 1986), estimate heritabilities (Collins et al. 1987a; Moritz et al. 1987; Oldroyd et al. 1991), compare the activities of genetically different subfamilies (Page and Robinson 1991), and to generate a linkage map to study various traits (Hunt and Page 1995).

Colony defense is a complex behavior of worker bees. Workers are alerted by a disturbance, they extrude their stingers and release alarm pheromone. Alarm pheromone is a mix of many compounds that serve to alert and recruit other workers to defend the nest (Blum et al. 1978). Alarm pheromone stimulates workers to fly out, strike the intruder, and perhaps sting. In the process of planting a sting, the intruder is marked with the pheromone, which can attract other nearby bees to sting.

An African subspecies of the honeybee (*A. m. scutellata*) was imported to Brazil in 1956 and has become the predominant race in South and Central America (Rinderer and Hellmich 1991; Spivak 1991; Winston 1992). This "Africanized" bee is much more likely to sting and pursue people and animals (Collins et al. 1982; Stort 1975a–c; Villa 1988). Differences in the amounts of alarm pheromone components in honeybee stings has been demonstrated between some European honeybees (EHB) and Africanized honeybees (AHB; Collins et al. 1989). Of the 11 principal sting alarm pheromone components (Blum et al. 1978; Collins and Blum 1983), 7 components were present in higher amounts in AHB. Hexyl acetate was 7.4 times higher in AHB than EHB, but isoamyl alcohol and isopentyl acetate were higher in EHB. The difference was hypothesized to be a contributing factor in the excessive levels of defensive behavior exhibited by AHB (Collins et al. 1982). Collins et al. (1987a) also determined that the relative concentrations of many of these alarm pheromones were heritable.

The pronounced behavioral differences between the Africanized and European honeybee populations were used to identify quantitative trait loci (QTL) that influence honeybee defensive behavior (Hunt et al. 1998). Colonies in Mexico that had been verified as to European or African type by morphometrics (Sylvester and Rinderer 1987), mitochondrial DNA (Ebert PR, Page RE Jr, Nielsen DI, Hunt GJ, and Guzmán-Novoa E, unpublished data), and behavior (Guzmán-Novoa and Page 1993) were used to select behavioral extremes. Individuals from these extremes were crossed to produce an F_1 queen. Previous work indicated genetic dominance for the highly defensive behavior of Africanized bees (Guzmán-Novoa and Page 1994). Therefore colonies for testing were obtained by using the haploid drones from the F_1 queen in backcrosses to European queens by single-drone inseminations of the queens. Five loci with potential effects on stinging behavior were identified, as were seven loci that appear to influence body size of drones, workers, or both. The following report extends this work by mapping loci that influence levels of alarm pheromone components.

Materials and Methods

The strategy for identifying QTLs was to obtain data on molecular markers from haploid drones to correlate with phenotypic data on alarm pheromone levels of their worker progeny. Therefore the map was based on haploid drones, but potential QTLs were identified by progeny testing in colonies that they fathered. We assumed that phenotypic variability that we observed was caused by the genotypes of the drone fathers so that we could treat each colony as one backcross "individual." The reasons for using this method were that RAPD marker analyses are more efficient in haploids and drones contribute the same haploid genome to all of their progeny.

Breeding and Sample Collection

One hundred seventy-two colonies were produced by using haploid drones from an F_1 queen in single-pair matings to European queens (Hunt et al. 1998). Each of these European queens were sisters and shared the same haploid father. The colonies were also rated for stinging behavior. Workers were taken from each colony and kept on ice in a plastic bag. Three samples of 10 bees were taken from each colony and the entire sting apparatus (the stinger, venom sack, and adhering tissue) was removed with forceps from each bee and 10 stingers were placed in a vial with 1 ml of methylene chloride and a small amount of the drying agent sodium sulfate. We used 10 samples from each colony to control for sampling error and any variability between the workers that resulted from genetic variation in the queen. Vials were sealed and kept at −20°C until they were analyzed.

Biochemical Analyses

The sting samples were analyzed for the relative amounts of alarm pheromone components following the technique of Blum et al. (1978). One to three samples (usually three) consisting of 10 bees each from 89 colonies were analyzed. Originally all the colonies were sampled, but some samples were lost due to leakage. Analyses were performed with a Hewlett Packard 5890 gas chromatograph (GC), with a flame ionization detector and HP5 (DB5) column. An aliquot of each sample was analyzed using a heating cycle of 50°C–250°C, at a rate of 5°C/min with helium as the carrier gas. Standards for 2-methyl-1-butanol, butyl acetate, hexyl alcohol, isopentyl acetate, 2-heptanol, hexyl acetate, 1-octanol, 2-nonanol, and octyl acetate at 0.1 μg/L in methylene chloride were run prior to and following each day's GC runs. Identities of peaks were determined based on comparison of relative retention times with those of the standards run the same day. The relative amount of each compound was calculated by comparing the area under

the curve from the GC output with the area under the curve of the standard. Several peaks from unknown compounds in the same range of retention times as the standards were also used in statistical analyses, using just the area under the peaks. These unknown compounds were subsequently determined to be n-decyl acetate and hexyl acetate with a Finnigan GCQ gas chromatograph/mass spectrophotometer with a cycle of 35°C–150°C at 5°C/min, using helium as the carrier gas. The column was a Restek Corporation Rtx-5MS.

Linkage Mapping

The linkage map was based on segregation of RAPD markers in 179 haploid drones which were progeny of the F_1 queen. These drones were the fathers of the colonies that were sampled for alarm pheromones. Methods for generating RAPD markers and linkage mapping were as described previously (Hunt et al. 1998; Hunt and Page 1995; Williams et al. 1990). Primers for generating RAPD markers were obtained from Operon Technologies (Alameda, CA) and the University of British Columbia Biotechnology Laboratory (Vancouver, BC). All linkage group designations refer to a previously published map (Hunt and Page 1995). Linkage group identity was confirmed by markers that were segregating in more than one mapping population. In some cases these were sequence-tagged sites (STSs). RAPD markers were generated in polymerase chain reactions (PCRs) with 10-nucleotide DNA primers of arbitrary sequence and separated in gels consisting of agarose and Synergel (Diversified Biotech, Boston, MA). MapMaker software was used for linkage mapping and the Kosambi mapping function was used to convert recombination frequencies into map distance.

QTL Mapping

RAPD markers were scored in the haploid drone progeny of the F_1 queen, but phenotypic data on alarm pheromones were obtained from their backcross worker progeny. QTL analyses were accomplished with the software package MapQTL (Van Ooijen and Maliepaard 1996). First, standard interval mapping procedures were used to identify potential QTLs (Lander and Botstein 1989). Then we used the multiple-QTL model (MQM) of MapQTL to fit more than one QTL at a time. This analysis uses the marker that is closest to the LOD score peak as a cofactor to account for a large proportion of the variance attributable to that QTL. MQM analysis can increase the precision and power of QTL mapping (Jansen 1993; Jansen and Stam 1994; Zeng 1993, 1994). We considered any LOD peak exceeding 1.5 to be suggestive of a linked gene influencing an alarm pheromone component. We used LOD 1.5 as a threshold because we wished to indicate all potential QTLs for alarm pheromone production. Therefore we used all of markers near a LOD peak that exceeded 1.5 in the analyses. If the multifactor analysis decreased the LOD score associated with a particular cofactor of less than 1.5, that marker was dropped from subsequent analyses as a cofactor. If any other LOD score peak increased above the threshold, the associated marker was added as a cofactor. Then the analysis was repeated in this iterative fashion until there was no change in the cofactor list (one to four iterations). Using our threshold of LOD 1.5, an individual QTL would need to explain at least 7–8% of the phenotypic variance to be detected in this study. A LOD of about 3.0 would be needed to limit the experiment-wise type I error to 0.05 in a genome the size of the honeybee (Lander and Kruglyak 1995).

Results

n-Decyl Acetate

The highest LOD score in this study (6.57) was for a QTL on linkage group XI influencing the amount of this compound (Figure 1A, Table 1). This one locus explained 40% of the phenotypic variance for the amount of n-decyl acetate. LOD score peaks were also observed for n-octanol (4.67) and isopentyl acetate (2.51) in this chromosomal region. The European alleles were associated with increased levels of all these pheromones at this locus. Of interest, a previous study indicated a major QTL for body size (as measured by wing length) at this same locus (Figure 1A). The only other possible QTL identified for this compound was at marker 268-.59 (LOD 1.83, not shown in figures).

n-Octanol

Besides the locus on linkage group XI (described above), two other potential QTLs were identified that influenced the amount of n-octanol. One locus influencing this compound lies on linkage group XIII (LOD 3.22; Figure 1B); the second is shown in Figure 1C (LOD 1.88). The LOD score associated with this latter locus is the lowest that we show in this study, and should only be considered suggestive. But this locus coincides with a QTL that influences several aspects of stinging behavior that were assessed in this same array of colonies (Hunt et al. 1998). Although the alleles inherited from the Africanized parent previously were associated with an increased number of stings in defensive behavior assays for this locus, in this study European alleles were associated with increased levels of n-octanol.

Isopentyl Acetate

For the principal active component of honeybee alarm pheromone, isopentyl acetate, only one possible QTL was identified by simple interval mapping on group IX (LOD 1.67; Figure 1D). However, MQM analysis fitted four cofactors with LODs greater than 1.5, causing the LOD score on group IX to increase to 3.34. The LOD score for a QTL on group I increased to 3.77 (Figure 1E, Table 1), the LOD score shown in Figure 1A increased to 2.51, and the LOD score shown in Figure 1B increased from 0.47 to 2.72. The African alleles for the QTLs on groups IX and I were associated with increased levels of isopentyl acetate.

Hexyl Acetate

One QTL was identified and only one cofactor was used in the analysis. The QTL lies on group IX (Figure 1D; LOD 2.35). The European allele was associated with higher levels of hexyl acetate.

Discussion

Previously simulations of genomes in which no QTLs were present have shown that the cumulative distributions for the maximum values of the test statistics (LODs) are nearly identical for interval mapping (IM) and MQM mapping, indicating that the same thresholds for controlling experiment-wise error rates can be used for both techniques. The same thresholds can also be used in situations where multiple QTLs are segregating because the cofactors used in MQM mapping eliminate their effects from all chromosomes except the one currently being scanned for presence of a QTL, as long as there are sufficient degrees of freedom to estimate residual variance. The number of cofactors should be less than two times the square root of the number of observations (Jansen 1994). Only one to four cofactors were identified for each trait in this study. Therefore the approximate threshold of LOD 3.0 that is needed to control experiment-wise error to $\alpha = 0.05$ in the

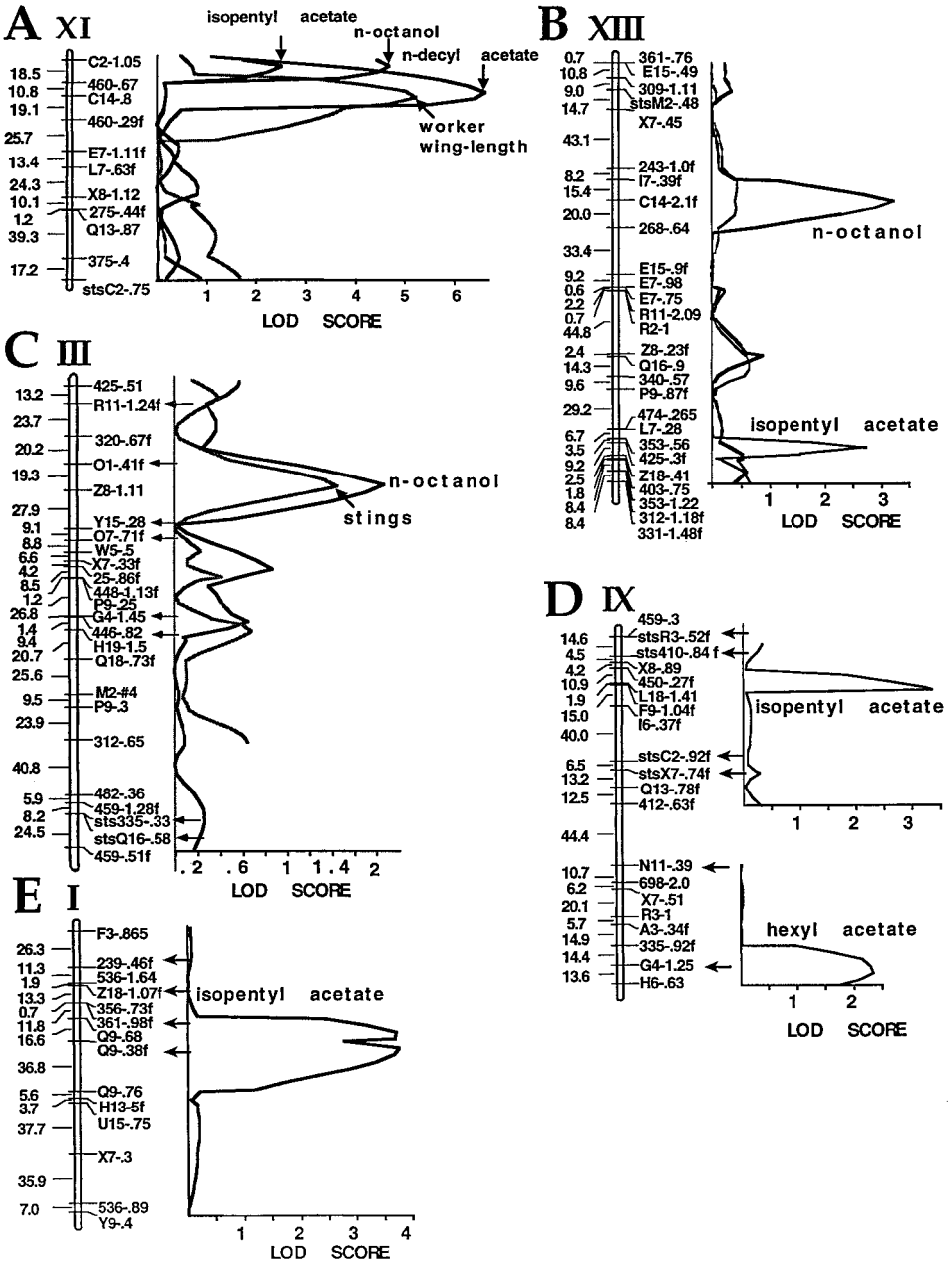

Figure 1. Possible quantitative trait loci influencing honeybee alarm pheromone production. **(A)** LOD score peaks for isopentyl acetate, n-octanol, and n-decyl acetate on linkage group XI. This region also influences body size (LOD score for wing length = 5.15; Hunt et al. 1998). **(B)** QTLs for n-octanol and isopentyl acetate on linkage group XIII. **(C)** The LOD score peak for n-octanol on group III is linked to a potential QTL influencing the number of stings in a defensive behavior assay (data from Hunt et al. 1998). **(D)** The LOD score peaks for isopentyl acetate and hexyl acetate on group IX. **(E)** A LOD score peak for isopentyl acetate on group I. Linkage group numbers are from Hunt and Page (1995). RAPD markers are designated by the primer names followed by the approximate size of the marker fragment. Markers indicated by arrows were common to both linkage maps. Sequences for the sequence-tagged sites are designated by the "sts" prefix. Results shown are the output of MQM mapping.

honeybee genome applies to the results presented here (Lander and Kruglyak 1995).

Fitting multiple QTLs was very useful for identifying the locations of genes that influence pheromone levels. For example, MQM mapping greatly increased the LOD scores of putative loci that affected the level of isopentyl acetate, which is considered to be the principle active component of the alarm pheromone blend. With MQM mapping the LOD scores of the three loci associated with isopentyl acetate levels increased over those obtained from IM. The three respective LOD scores were 1.67, 1.24, and 1.34 with IM compared to 3.34, 3.77, and 2.51 with MQM mapping (see Table 1). In the present study, MQM analyses always increased the LOD scores associated with putative QTLs, except for the sole QTL influencing hexyl acetate. For hexyl acetate, the LOD score remained unchanged because only the cofactor linked to this QTL exceeded the threshold (1.5) that we set as a criterion for using a marker in the analyses. But for this locus and most of the others, the precision of MQM mapping appeared to be better than simple IM because the LOD score peak was narrower in the output from MQM mapping (not shown).

Some loci had pleiotropic effects on the amounts of several components of sting alarm pheromone. Three compounds, isopentyl acetate, n-octanol, and n-decyl acetate mapped to the same location on linkage group XI, suggesting that the same gene may be influencing levels of all three. This locus coincides with a highly significant QTL for body size. Larger bees produced more of these three compounds because the same marker alleles associated with increased body size were associated with greater concentrations of these three compounds. We also observed a coincidence of peaks for n-octanol and isopentyl acetate on group XIII. The African parental allele decreased levels of both these compounds (Table 1), indicating that the genes at these loci may be influencing common metabolic pathways. It was unexpected to find that African alleles at these loci, as well as three others, decreased the amount of the alarm pheromone components. Alleles from the Africanized parent were associated with increased levels of pheromone for only three of the eight loci that we identified. Our results contrast with a previous study involving many colonies with naturally mated queens that reported higher levels of most alarm pheromone components in Africanized bees, even when controlling for age and environment (Collins et al. 1989). Apparently there is significant genetic variation for alarm pheromone production in both European and Africanized honeybees.

Collins et al. (1989) found a positive correlation between the amount of specific alarm pheromone components and increased stinging behavior. However, there has been no study until the present one that used segregating families from a single hybrid queen, so the previous association was not a genotypic correlation. In the present study, very little correlation between alarm pheromone levels and defensive behavior was found. Potential loci that influenced the amount of alarm pheromone components were unlinked to loci that influenced stinging behavior in the

same colonies. The only exception was a possible QTL for n-octanol on group III (Figure 1C). The QTL influencing n-octanol coincides with the LOD score peak for a potential QTL influencing the number of stings observed in this same array of colonies. This locus appeared to have an influence on the number of stings observed in a colony defensive behavior assay (LOD 1.44) and on other behaviors frequently found in highly defensive bees, such as the tendency for the bees to fly up ($p < .0001$) and the tendency to sting the beekeeper during colony manipulations ($p < .01$; Hunt et al. 1998). The marker alleles from the more defensive Africanized parent were associated with increased stinging, but the European alleles were associated with increased levels of n-octanol. This suggests that a gene(s) that increases the level of n-octanol may reduce defensive behavior, or that the hybrid queen had genes linked in *trans* that elevated these two traits. During earlier assays of biological activity, Collins and Blum (1983) found that isopentyl acetate and other alarm pheromone components alerted caged bees. But no such response was found for n-octanol. Perhaps production of n-octanol can reduce defensive behavior in some way. It is also possible that this is a spurious QTL because it had the lowest LOD score (1.88) that we report in this study.

In general, this study suggests that the major genes influencing the amount of alarm pheromones are not directly affecting the stinging behavior in honeybee colonies. Previously assays of both caged bees and whole colonies demonstrated that Africanized honeybees respond more strongly to the application of isopentyl acetate than do European honeybees (Collins et al. 1987a,b). Africanized bees also react to visual stimuli more rapidly and deposit many more stings in the target (Villa 1988). The present study offers additional evidence that the intense defensive response of Africanized bees involves primarily a lower threshold of response to stimuli that elicit defensive behavior, rather than increased levels of alarm pheromone.

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Table 1. LOD scores and the nearest RAPD marker for putative QTLs influencing the levels of alarm pheromones in the honeybee sting apparatus

Pheromone component	Nearest marker	Interval map[a]	MQM map[b]	African allele[c]
n-Decyl-acetate	460-.67	4.97	6.57	Decreases
n-Octanol	C2-1.05	2.92	4.67	Decreases
	C14-2.1f	1.89	3.22	Increases
	O1-.41f	1.18	1.88	Decreases
Isopentyl acetate	C2-1.05	1.34	2.51	Decreases
	L18-1.41	1.67	3.34	Increases
	425-.3f	0.47	2.72	Decreases
	Q9-.38f	1.24	3.77	Increases
Hexyl acetate	G4-1.25	2.35	2.35	Decreases

[a] Simple IM with MapQTL software.

[b] MQM of MapQTL software.

[c] The effect of substituting an African allele at the QTL on the amount of each compound extracted from the sting apparatus.

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