

# Allochronic speciation, secondary contact, and reproductive character displacement in periodical cicadas (Hemiptera: *Magicicada* spp.): genetic, morphological, and behavioural evidence

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## Abstract

Periodical cicadas have proven useful in testing a variety of ecological and evolutionary hypotheses because of their unusual life history, extraordinary abundance, and wide geographical range. Periodical cicadas provide the best examples of synchronous periodicity and predator satiation in the animal kingdom, and are excellent illustrations of habitat partitioning (by the three morphologically distinct species groups), incipient species (the year classes or broods), and cryptic species (a newly discovered 13-year species, *Magicicada neotredecim*). They are particularly useful for exploring questions regarding speciation via temporal isolation, or allochronic speciation. Recently, data were presented that provided strong support for an instance of allochronic speciation by life-cycle switching. This speciation event resulted in the formation of a new 13-year species from a 17-year species and led to secondary contact between two formerly separated lineages, one represented by the new 13-year cicadas (and their 17-year ancestors), and the other represented by the pre-existing 13-year cicadas. Allozyme frequency data, mitochondrial DNA (mtDNA), and abdominal colour were shown to be correlated genetic markers supporting the life-cycle switching/allochronic speciation hypothesis. In addition, a striking pattern of reproductive character displacement in male call pitch and female pitch preference between the two 13-year species was discovered. In this paper we report a strong association between calling song pitch and mtDNA haplotype for 101 individuals from a single locality within the *M. tredecim*/*M. neotredecim* contact zone and a strong association between abdomen colour and mtDNA haplotype. We conclude by reviewing proposed mechanisms for allochronic speciation and reproductive character displacement.

*Keywords:* allochronic speciation, hybridization, *Magicicada*, reproductive character displacement, secondary contact, speciation

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## Introduction

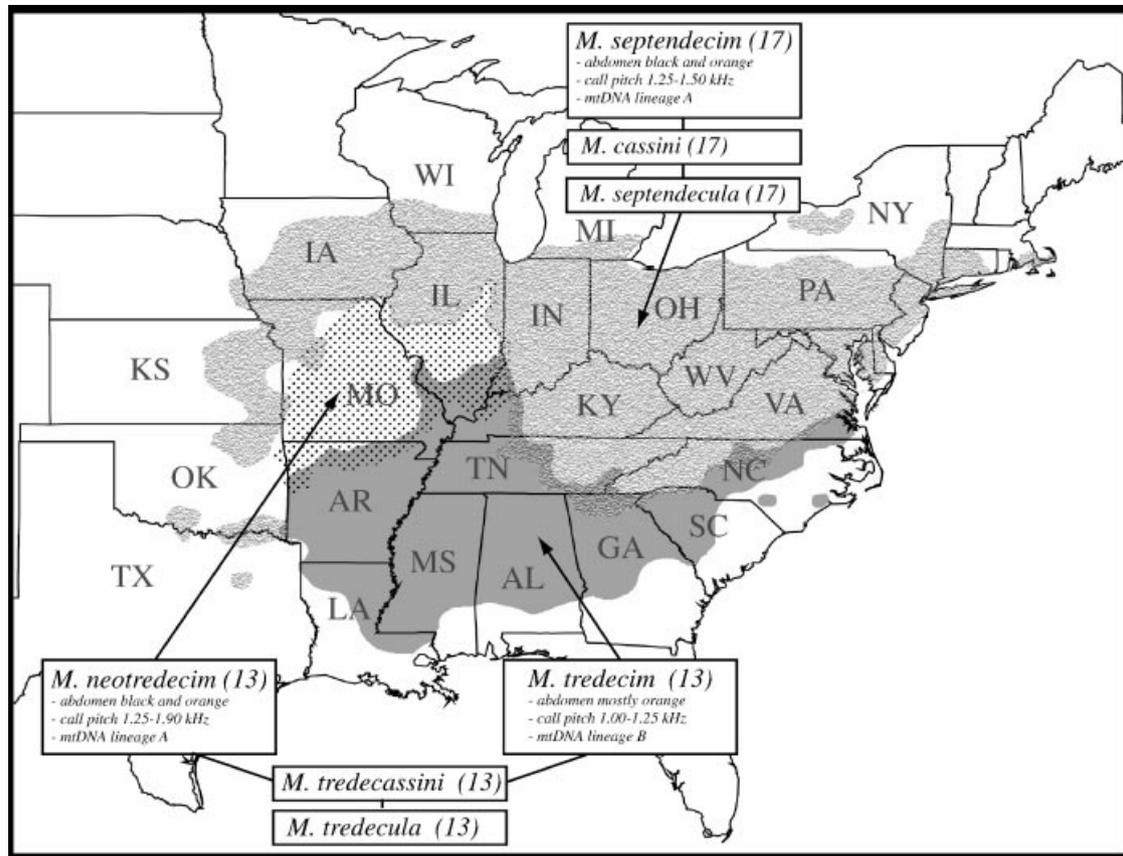
Secondary contact between isolates can lead to complex species interactions that challenge our understanding of species and the speciation process (Harrison 1993; Noor 1999; Jiggins & Mallet 2000). Within the periodical cicadas (*Magicicada* spp.) of eastern North America, the *M. -decim* cognate species (described below) provide an example of extrinsic isolation, divergence, and secondary contact,

with little evidence of hybridization. We present a review of our research relating to speciation and secondary contact in periodical cicadas and present new data on the species-specificity of genetic, behavioural, and morphological characters in the zone of contact between two sister species. We conclude with some observations concerning allochronic speciation in *Magicicada*.

## *Magicicada*: definitions and species

Three sympatric, morphologically distinct, synchronized *Magicicada* species with 17-year life cycles coemerge in the

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**Fig. 1** Distribution of the seven periodical cicada (*Magicicada*) species, summarized from county-level maps in Simon (1988) and from 1993 to 1998 field surveys. The 17-year species are sympatric except in peripheral populations: *M. cassini* alone inhabits Oklahoma and Texas, while only *M. septendecim* is found in some northern populations (Dybas & Lloyd 1974). Two 13-year species, *M. tredecim* and *M. neotredicim*, overlap with each other only in the central U.S. The other 13-year species, *M. tredecassini* and *M. tredecula*, are sympatric with each other and overlap with the former species in the south and the latter species in the north.

northern and plains states of the U.S. (*M. septendecim*, *M. cassini*, and *M. septendecula*); each corresponds to at least one morphologically and behaviourally similar 'cognate' species inhabiting the midwestern and/or south-eastern states (*M. tredecim*, *M. neotredicim*, *M. tredecassini*, and *M. tredecula*; Fig. 1). The cognate groups are abbreviated M. -decim, M. -cassini, and M. -decula for convenience. On the basis of shared morphology, behaviour, and genetic markers (reviewed in Williams & Simon 1995; Simon *et al.* 2000; Marshall & Cooley 2000), each species appears most closely related to a cognate with the alternative life cycle, a pattern best explained by multiple allochronic speciation events.

#### Brood and species formation

Superimposed on the species relationships of *Magicicada* is the complex biogeography of the 'broods', which are largely parapatric populations of the same life cycle that

emerge in different years and are thus temporally isolated (Marlatt 1907; Alexander & Moore 1962; Lloyd & Dybas 1966; Simon 1988). By convention, 17-year brood year-classes are numbered sequentially from I to XVII, and 13-year brood year-classes are numbered from XVIII to XXX, although there are only 12 known 17-year broods and three known 13-year broods (Simon 1988). With the exception of Brood VII (at the extreme northern edge of *Magicicada*'s range), each brood of a given life cycle contains species belonging to all three species groups, although some populations of each brood are lacking one or more species.

Brood formation appears to involve temporary life-cycle anomalies that cause different populations to become unsynchronized. Most temporal migrants are doomed, because periodical cicadas reproduce successfully only when densities are sufficient to 'satiating' local predators (Marlatt 1907; Beamer 1931; Alexander & Moore 1962; Dybas 1969; Karban 1982; Williams *et al.* 1993). Therefore, the establishment of a new brood by temporal migrants

must be a rare event, involving large numbers of cicadas. Most of the modern *Magicicada* broods were probably formed since the last glacial maximum, and their biogeography has been hypothesized to reflect climatic processes likely involved in their formation (Alexander & Moore 1962; Lloyd & Dybas 1966). Some evidence suggests that temporary accelerations of one or four years may be most important in brood formation (Lloyd & Dybas 1966; Lloyd & White 1976).

While brood formation appears to result from temporary life-cycle fluctuations, the existence of cognate species with different life cycles indicates that permanent four-year life-cycle alteration is one mechanism of species formation in *Magicicada* (Alexander & Moore 1962; Lloyd & Dybas 1966; Lloyd & White 1976; Lloyd *et al.* 1983). Most *Magicicada* species of the same life-cycle type share nearly coincident ranges, and most species likely predate the last glacial cycle; thus biogeographic clues as to their origins have been erased. One exception involves the 13-year -decim species, one of which, *M. neotreddecim*, appears to have originated recently via allochronic speciation (Marshall & Cooley 2000; Simon *et al.* 2000).

#### *The discovery of M. neotreddecim*

The discovery of *M. neotreddecim* is the result of long-term research. The first step was the discovery of two distinct genetic lineages in the M. -decim species. The existence of two different abdominal colouration morphs, both ventrally orange, one lighter-coloured with few abdominal markings and the other with dark transverse bands has been recognized within the M. -decim species at least since 1962 (Alexander & Moore 1962; R. D. Alexander, personal communication). Because the lighter coloured phenotype is most common in 13-year M. -decim, individuals of this type were deliberately chosen as the neotype and neoallo-type for *M. treddecim* (R. D. Alexander, personal communication). Martin & Simon (1988, 1990) surveyed three genetic markers for the M. -decim species from the largest brood of 13-year cicadas (Brood XIX), which occupies parts of the Midwest and South, and the largest brood of 17-year cicadas (Brood X), which extends from the Midwest into the north-eastern states. They discovered that 13-year M. -decim cicadas from the northern part of Brood XIX were indistinguishable in mitochondrial genotype, phosphoglucomutase allozyme frequency, and mean abdominal colour from 17-year Brood X *M. septendecim*. This shared genetic lineage was termed 'lineage A.' In contrast, the southern Brood XIX M. -decim differed from the lineage A cicadas in abdomen colour, mitochondrial DNA (mtDNA) haplotype, and phosphoglucomutase allozyme frequency. This unique genetic lineage was termed 'lineage B.' Martin & Simon (1988, 1990) suggested that the northern 13-year cicadas were recently derived from 17-year cicadas by

life-cycle switching, perhaps by the deletion of a postulated four-year dormancy period in the second nymphal instar (White & Lloyd 1975). Biogeographic evidence suggested that the northern 13-year cicadas were derived from 17-year ancestors rather than vice versa; otherwise rapid and implausible range changes would be required to explain the relative distributions of *M. septendecim* (widespread) and northern 13-year cicadas (confined to an area bounded on the west, north, and east by *M. septendecim*; Fig. 1).

#### *Secondary contact between the lineages*

Martin and Simon's discoveries were unexpected, and their sampling scheme had not been designed to pinpoint the genetic boundary within 13-year M. -decim or to locate populations in which both genotypes existed. They postulated that if these lineages existed in secondary contact, random mating would reunite gene pools that had been separated for more than a million years (Martin & Simon 1988, 1990; Williams & Simon 1995). Mating between 13- and 17-year cognate species had been experimentally induced and no behavioural barriers to gene flow were known (Lloyd & Dybas 1966; Alexander 1968). The next opportunity to resample Brood XIX was in 1998.

Another large brood of 13-year cicadas, Brood XXIII, inhabits the Mississippi Valley and has a range similar to that of Brood XIX, though slightly smaller. Intensive sampling within Brood XXIII revealed individuals of both the A and B lineages (Simon *et al.* 2000). Simon *et al.* (2000) also found that: (i) lineage A cicadas of Brood XXIII were indistinguishable in abdominal colour, mitochondrial haplotype and geographical distribution from the 17-year Brood X and northern 13-year Brood XIX lineage A individuals; (ii) cicadas of both lineages coexist in a zone of overlap within Brood XXIII; and (iii) genetic intermediates were absent in the overlap zone, suggesting assortative mating by cicadas of the two lineages. Intensive resampling of Brood XIX in 1998 (Marshall & Cooley 2000; C. Simon *et al.*, unpublished data) identified mixed populations in this brood, within a narrow zone of overlap geographically similar to that in Brood XXIII.

#### *Newly discovered differences in mating behaviour*

In 2000, 13-year mtDNA lineage A was named a new species, *M. neotreddecim*, on the basis of newly discovered behavioural differences restricting gene flow between lineage A (*M. septendecim*/*M. neotreddecim*) and lineage B (*M. treddecim*; Marshall & Cooley 2000). Within mixed *M. neotreddecim*/*M. treddecim* choruses, male *M. neotreddecim* produce calling songs with high dominant pitch (mean 1.71 kHz), while *M. treddecim* have calling songs with low dominant pitch (mean 1.11 kHz; Marshall & Cooley 2000;

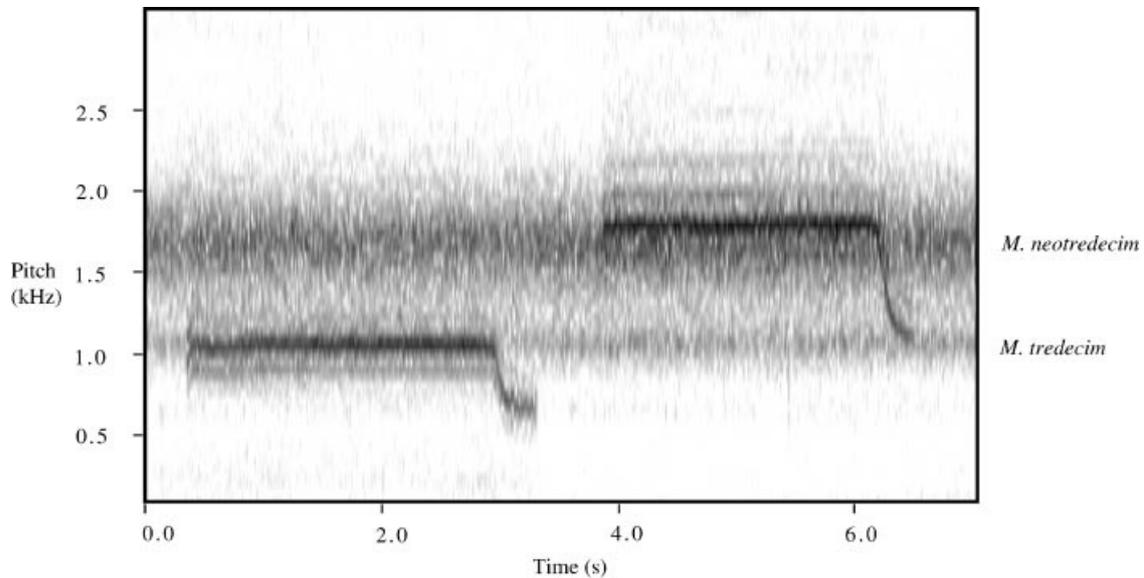


Fig. 2 Spectrogram (power spectrum vs. time) showing a two-banded, *Magicicada tredicim*/*M. neotredicim* chorus of male calls with one call of each species standing out against the background chorus. Individual calls end with a downslur. Comparatively faint slurs of background chorus males overlap and are not visible. Intervening time between calls has been removed.

Fig. 2). Courtship songs of the two species show similar pitch differences. Female call pitch preferences, as measured by female 'wing flick' signal responses to male calls (Cooley 1999; J. R. Cooley & D. C. Marshall, submitted), are species-specific (Marshall & Cooley 2000). Females collected from mixed choruses of *M. neotredicim*/*M. tredicim* fall into a bimodal distribution of pitch preference, males exhibit a bimodal distribution of call pitch, and mixed choruses produce little sound at pitches intermediate to those of the two species. These observations provide further evidence that assortative mating is taking place (Cooley 1999; Marshall & Cooley 2000). Analysis of archived recordings (University of Michigan Museum of Zoology) indicated that the same song pitch types exist within Brood XXIII (D. C. Marshall & J. R. Cooley, unpublished data). The importance of song pitch to the *Magicicada* mating system is underscored by recent discoveries (Fonseca *et al.* 2000) that the neural architecture of cicadas allows fine-scale pitch discrimination.

#### Reproductive character displacement

Geographic variation in male song pitch and female pitch preference within *M. neotredicim* exhibits a striking pattern of reproductive character displacement (Brown & Wilson 1956; Howard 1993; Fig. 3). Where *M. tredicim* overlaps *M. neotredicim*, *M. neotredicim* male calling songs and female mating preferences are centred around 1.7 kHz, while those of allopatric populations range between 1.3 and 1.5 kHz (Marshall & Cooley 2000). Extremely displaced

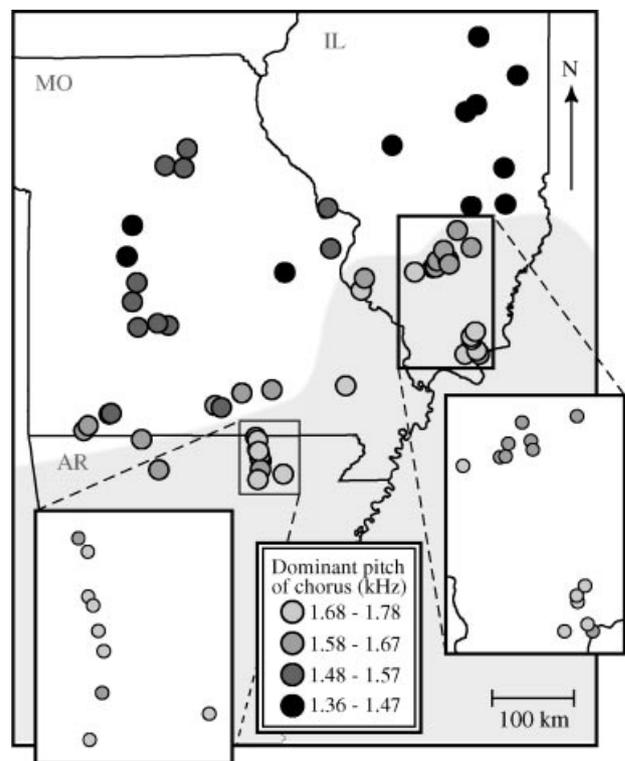
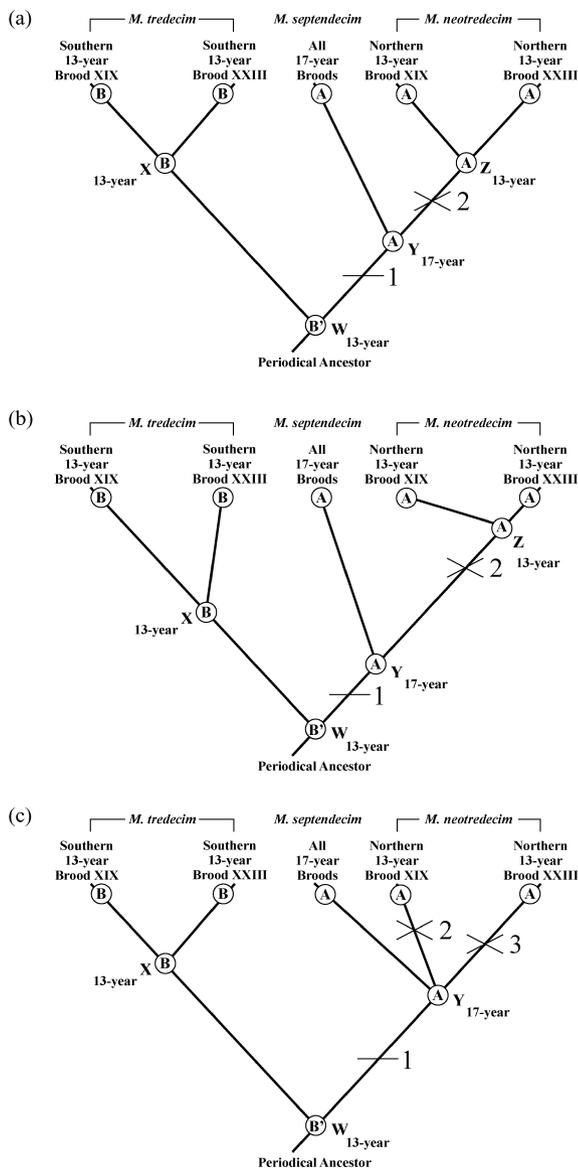


Fig. 3 Geographic variation in dominant chorus pitch of *Magicicada neotredicim*, showing higher-pitch calls in and near the region of overlap with *M. tredicim*. Lighter shaded circles indicate higher-pitch calls. Shaded region is approximate *M. tredicim* range. Reproduced with permission from Marshall & Cooley (2000).



Order of events

X, Z

2

Y

1

W

Z

2

X

Y

1

W

2, 3

X

Y

1

W

**Fig. 4** Hypotheses of the formation of 13- and 17-year *M. -decim* lineages. The split between mtDNA lineages A and B occurred first (node W), followed by the *Magicicada septendecim*/*M. neotreddecim* split (node Y). Numbered lines on tree branches mark the addition or subtraction of four years to/from the life cycle. The circled letters A and B at the tips and nodes indicate lineage type. (a) The evolution of *M. neotreddecim* involving one permanent life cycle change followed by the subdivision of a brood. (b) The evolution of *M. neotreddecim* in one brood by a permanent life cycle change followed by its migration to another brood. (c) The evolution of *M. neotreddecim* involving two separate permanent life cycle changes. See text for details.

*M. neotreddecim* song pitches are found exclusively within and adjacent to the zone of contact. A weaker reciprocal pattern in calling song pitch variation may exist in *M. treddecim*, but this is based on less complete sampling (Marshall & Cooley 2000). The pattern of character displacement in *M. neotreddecim* song pitch lends strength to the argument that *M. neotreddecim* originated in the time since the last glacial maximum, because the near-perfect correlation of song displacement and sympatry and the biogeographic relationships of *M. septendecim*, *M. neotreddecim* and *M. treddecim* would likely not have persisted through range changes during Pleistocene glacial cycles (Marshall & Cooley 2000).

#### Derivation of *M. neotreddecim* and its presence in two broods

The presence of *M. neotreddecim* or similar cicadas in both major 13-year broods (XIX and XXIII; Simon *et al.* 2000) could be explained in three ways (Fig. 4). In all schemes, the ancestor is a periodical cicada with a 13-year life cycle (node W, lineage B'), a hypothesis supported by the fact that among- and within-population genetic variation is an order of magnitude larger in present-day *M. treddecim* than in *M. septendecim*. This ancestor gave rise to a 13-year proto-Brood XIX + XXIII (node X, lineage B) that later split into the current 13-year broods XIX and XXIII. The ancestor at

node W also gave rise via a permanent four year life-cycle extension (event 1) to *M. septendecim* (node Y; lineage A). Between nodes W and X, and W and Y, some amount of evolution took place so that node X evolved into lineage B and node Y evolved into lineage A. In scenario one (Fig. 4a), *M. neotreddecim* evolved from *M. septendecim* via a single four year life-cycle reversion (event 2, forming node Z lineage A) which caused it to join and gain predator protection from existing 13-year proto brood XIX + XXIII. Later, temporary life-cycle anomalies caused this combined lineage A + B proto-brood to split into the current Broods XIX and XXIII with *M. neotreddecim* already in place in both. In this scheme, nodes X and Z are of the same age. In scenario two (Fig. 4b), node X predates the formation of *M. neotreddecim* (event 2). *M. neotreddecim* formed (event 2) from a single four year reversion event from a 17-year ancestor and joined either Brood XIX or XXIII. Then, via a temporary life-cycle anomaly, a subset of *M. neotreddecim* individuals became synchronized with the other 13-year brood. Under this hypothesis, node Z is more recent than node X. In scenario three (Fig. 4c), *M. septendecim* (node Y) contributed separate *M. neotreddecim*-like species to preexisting Broods XIX and XXIII; this would have involved two independent 17- to 13-year life-cycle reversions (events 2 and 3). In scenario three the order of origination of *M. neotreddecim* in XIX and XXIII is uncertain, and node X predates events 2 and 3.

#### *The contact zone is not a hybrid zone*

The pattern of reproductive character displacement indicates that, subsequent to secondary contact, selection has acted against heterospecific sexual interactions of *M. neotreddecim* and *M. treddecim*. Instead of forming a stable hybrid zone with at least limited introgression of genetic material, these species have evidently evolved to reduce opportunities for introgression. The mechanism driving the species' divergence could have taken different forms, depending on current or past opportunities for gene flow. Gene flow might have occurred in the past, if interspecific mating occurred and hybrids were at least partially fertile; however, current discussions of evolution following secondary contact emphasize the difficulty of enhancing assortative mating when significant gene flow occurs between the species (e.g. Butlin 1987; Rice & Hostert 1993; Noor 1999). Alternatively, the 13-year *M. -decim* species may have been genetically isolated upon first contact by incompatible sexual signals or hybrid sterility, in which case the observed signal divergence would be explained not by selection against hybrids *per se*, but by selection against other forms of wasteful heterospecific sexual interactions (e.g. signal interference or time wasted in ineffectual courtships).

Present-day hybridization between *M. neotreddecim* and *M. treddecim* may be rare or nonexistent within the contact zone. Evidence from playback experiments suggests that females in the overlap zone today rarely respond to the calling songs of heterospecific males (Marshall & Cooley 2000). Additional information on the existence of present-day gene flow in the overlap zone could be sought by examining the relationship between song pitch and abdomen colour within *M. neotreddecim* or *M. treddecim*. If present-day introgression is occurring, then lower song pitch should tend to be associated with lighter abdomen colour within the species *M. neotreddecim*, and vice versa for individuals of *M. treddecim*. If signal divergence of *M. neotreddecim* and *M. treddecim* long ago eliminated opportunities for hybridization, then stabilizing selection within the species may have removed introgressed alleles affecting the species-specific song pitch. However, evidence of past hybridization could be observed in mtDNA haplotype or abdomen colour variation, if, within the overlap zone, the alternative abdomen colours and mtDNA haplotypes are 'selectively equivalent' (cf. Hewitt 1993) in the genetic backgrounds of both species. Under these conditions selection would not tend to remove alleles affecting such traits unless they were linked to song pitch.

Even if selection reinforcing reproductive character differences later decreased levels of hybridization, historical introgression would have eroded the correlations between: (i) abdominal colour morphs; (ii) mtDNA haplotypes; and (iii) calling-song pitch or pitch preference types. Previous work established associations between: (i) abdomen colour and mtDNA haplotype (Martin & Simon 1988, 1990); and (ii) abdomen colour and male song pitch/female song pitch preference (Marshall & Cooley 2000) but did not examine correlations between song pitch and mitochondrial genotype. In this paper, we examine correlations among mtDNA haplotype, calling song pitch, and abdominal colour for individuals from a population within the zone of overlap and find little evidence for introgression.

#### **Materials and methods**

During the 1998 emergence of 13-year Brood XIX, we collected 150 males from a mixed *M. treddecim*/*M. neotreddecim* chorus for analysis of song pitch, abdomen colour, and mtDNA haplotype. The collection site was a privately owned wood 0.25 miles south of County Road 62 on County Road 51 at a powerline right-of-way, just outside the north-west boundary of the Harold E. Alexander Wildlife Management Area, Sharp Co., AR. We scored the ventral abdomen colour of each individually recorded male using a method similar to that of Martin & Simon (1988), assigning each male a whole-number value from 1 (dark transverse marking present) to 4 (transverse markings absent). An abdominal-sternite-colour chart can

be viewed at <http://www.eeb.uconn.edu/collections/cicadacentral/magi/abcolor.htm>. We recorded the calling song of each male using a portable cassette recorder with a microphone and parabola, or a videocassette recorder with built-in microphone, and we analysed dominant call pitch using Canary 1.2.4 (Cornell Bioacoustics Laboratory, Ithaca, NY) (detailed methods in Marshall & Cooley 2000). Specimens were then stored in 95% ethanol for later DNA analysis.

For 101 males from the Sharp Co., AR, population, whole-genome DNA was isolated from a small sample of diced leg tissue from each cicada using a Qiagen DnEasy™ tissue kit (Qiagen Corp.). An approximately 330 bp portion of domain III of the SSU (12s) rRNA gene was amplified using primers SR-J-14233 and SR-N-14588 (Simon *et al.* 1994). These primers were chosen because they flank a known 12s *Bgl*III restriction polymorphism found to be a consistent discriminator between the A and B mitochondrial lineages sampled over a broad geographical area (Martin & Simon 1990). Polymerase chain reactions (PCRs) were carried out in 25 µL volumes consisting of 1 µL template DNA, 1.25 µL each primer (10 mM), 2.5 µL of 10× PCR reaction buffer, 2.5 µL 8 mM dNTP's, 0.125 µL Takara X-Taq™ (Takara Shuzo Co, LTD.), *Thermus aquaticus* DNA polymerase (5 units/µL), and 16.375 µL ddH<sub>2</sub>O. DNA was denatured initially at 94 °C for 2 min, then 30 cycles of amplification were carried out under the following conditions: 92 °C denaturation for 45 s, 55 °C annealing for 45 s, and 72 °C extension for 75 s. Five microliters of PCR product and negative control were electrophoresed on a 1% agarose gel stained with ethidium bromide to verify product size and purity. The PCR products were cleaned with a Qiagen PCR purification kit (Qiagen Corp.), then cycle sequenced using the ABI cycle sequencing Big Dye™ kit (Perkin-Elmer Biosystems). Sequencing reactions were carried out in 10 µL volumes using 2.5 µL template DNA, 1 µL primer, 4.0 µL Big Dye (PE Biosystems) mix, and 2.5 µL ddH<sub>2</sub>O, for 25 cycles (96 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min). After cycle sequencing, the DNA was cleaned using 0.5 g hydrated Sephadex™ (DNA Grade, G-50 Fine; Pharmacia) in spin columns. All samples were run for 9 h on an ABI Prism™ 377 automated sequencer. All PCR fragments were sequenced in both directions, and all sequences were aligned using Sequencher™ (Gene Codes Corp.) after each chromatogram had been inspected. The alignments were inspected by eye, but needed no adjusting. For comparison and to aid in alignment, five *M. septendecim* individuals from three broods were also sequenced (see Collection localities below).

We divided our male sample into two groups, above and below an intermediate pitch of 1.3 kHz, because song pitch is bimodal and species-diagnostic (Marshall & Cooley 2000), with no intermediate phenotypes. We used Mann-Whitney U-tests to evaluate any relationships between call

pitch and abdomen colour or genotype in the 101-male sample. For the entire 150-male sample, statistical association of abdomen colour class and male song pitch within *M. neotreddecim* and within *M. treddecim* was measured using a Kruskal-Wallis test. All statistical analyses were conducted using SYSTAT Version 5.2.1 for the Macintosh (Systat, Inc.).

#### Collection localities

*M. septendecim*. Brood III: Henderson County, IL 0.9 miles east of County Road 164, 3 miles south of right angle bend (9 June 1980, CS, JA); Mahaska County, IA, Lake Keoma State Park (9 June 1997, CS, WS, JZ). Brood X: Owen County, IN, Hoot Woods 5 miles off Highway 231 on Hoot Road (21 May 1987, CS, AM). Brood XIII: Scott County, IA, Scott County Park (16 June 1990, CS, WS); Peoria County, IL, Jubilee College State Park (16 June 1990, CS, WS).

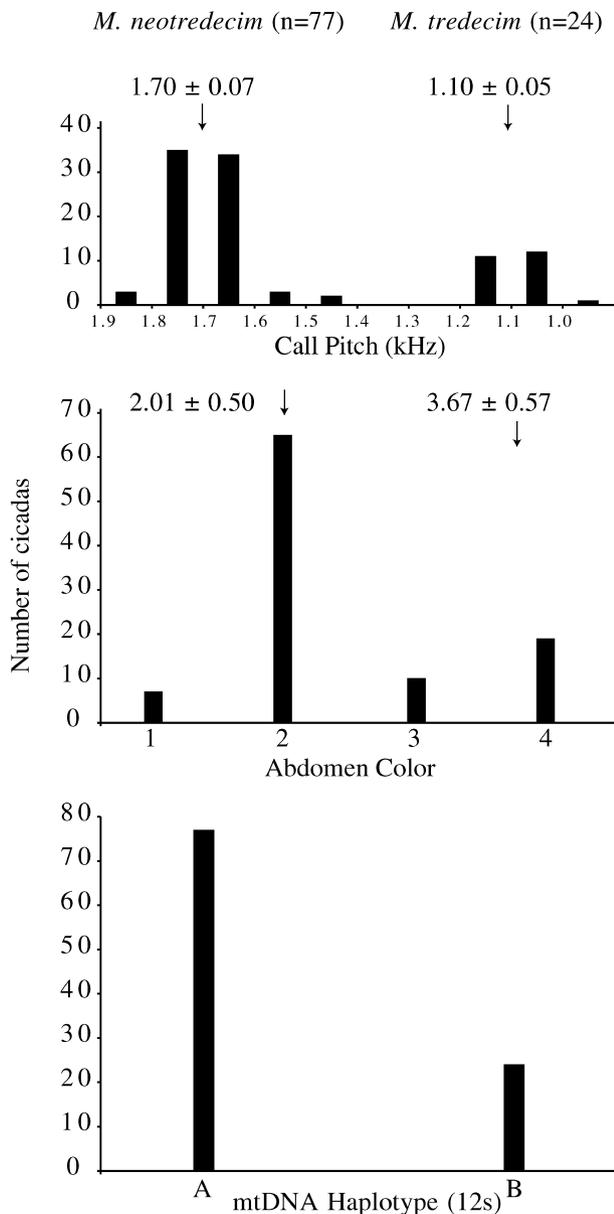
*M. treddecim*, *M. neotreddecim*. Brood XIX: Sharp County, AR, privately owned woods 0.25 miles South of County Road 62 on County Road 51 at a powerline right-of-way, just outside the north-west boundary of the Harold E. Alexander Wildlife Management Area (June 1998, JC, DM).

#### Results

Within the region sequenced, including the restriction site polymorphism, there are 6 bp differences between the two mtDNA lineages, all confined to unpaired regions of 12s rRNA secondary structure (see Hickson *et al.* 1996). In all individuals examined, these six differences do not vary independently and are consistent within the lineage. Calling song pitch and mtDNA haplotype were always congruent; thus mtDNA haplotype was species-specific (Table 1, Fig. 5). Abdominal colour was strongly congruent with calling song and mtDNA haplotype, but was not absolutely species-specific. Seven *Magicicada neotreddecim*

**Table 1** A Kruskal-Wallis test, with call pitch as dependent variable, indicates overall relationship between pitch and abdomen colouration in a mixed sample of 150 *Magicicada neotreddecim* and *M. treddecim* (test statistic = 45.969,  $P < 0.001$ ); the break between the two species occurs within abdomen colour class 3

Abdomen colour		Call	
Class	Count	Pitch (mean ± SD)	Rank-Sum
1	11	1.73 ± 0.09	1138.5
2	103	1.70 ± 0.08	8863.5
3	15	1.46 ± 0.33	880
4	21	1.16 ± 0.20	443



**Fig. 5** Call pitch, abdomen colour and mtDNA haplotype in *Magicicada neotredicim* and *M. tredicim* at the Sharp County AR site. Species were identified on the basis of call pitch, which is bimodal. Average abdomen colour differs significantly between species (Mann–Whitney  $U = 62.65$ ;  $P < 0.001$ ). The distribution of mtDNA haplotypes between the species is significantly nonrandom (Fisher's Exact Test  $P < 0.001$ ); all *M. neotredicim* were lineage A, and all *M. tredicim* were lineage B. Arrows indicate mean values of call pitch and abdomen colour for each species.

were classified in abdominal colour category 1, 64 in category 2, four in category 3, and two in category 4. No *M. tredicim* were classified in category 1, one in category 2, six in category 3, and 17 in category 4. Within each species, we found no association between calling-song pitch and abdomen colour (Table 2).

**Table 2** Kruskal–Wallis tests show no association between male song pitch and abdomen colour class within (a) *Magicicada neotredicim* ( $n = 125$ ) or (b) *M. tredicim* ( $n = 26$ ) from Sharp County, AR, within the zone of overlap of the two species

(a) *M. neotredicim*

Abdomen	Mean Pitch (kHz)	Count	Rank-Sum
1	1.73	11	862.5
2	1.70	103	6296.5
3	1.71	9	567.5
4	1.72	2	148.5

Kruskal–Wallis test statistic = 2.470;  $P = 0.481$

(b) *M. tredicim*

Abdomen	Mean Pitch (kHz)	Count	Rank-Sum
1	n/a	n/a	n/a
2	1.13	1	20.5
3	1.09	6	86.0
4	1.10	19	244.5

Kruskal–Wallis test statistic = 1.049;  $P = 0.592$

## Discussion

Abdomen colour does not perfectly distinguish *Magicicada neotredicim* and *M. tredicim*. However, the lack of correlation between abdomen colour and calling-song pitch within each species suggests that hybridization is not the reason for this lack of species-specificity. The perfect correspondence between mtDNA haplotype and call pitch further supports the conclusion that gene exchange is not occurring between these species. Even the strong pattern of reproductive character displacement does not necessarily imply gene flow, past or present, between *M. neotredicim* and *M. tredicim*. Gene flow requires that hybrid offspring reproduce successfully, but reproductive character displacement can occur in response to heterospecific sexual interactions other than mating or the production of hybrid offspring. Time and effort wasted in interspecific courtship alone could lead to selection favouring elaboration of differences between species' sexual signals, especially if the species already differed at first contact. The calling song pitches of *M. neotredicim* and *M. tredicim* recorded away from the zone of contact differ by approximately 300 Hz. If these allopatric populations accurately reflect the precontact conditions for these species, then even before any character displacement occurred the *M. -decim* within 13-year mixed-species choruses would have had some tendency to mate assortatively, although their preferences would likely not have been as exclusive as those

of present-day displaced populations (Marshall & Cooley 2000).

Our data, however, do not eliminate the possibility of past gene flow. In another study, *M. septendecim* with especially light-coloured abdomens were found to characterize Brood X populations near its boundary with Brood XIX (A. Paradis & C. Simon, unpublished data). These unusual *M. septendecim* could be evidence that: (i) some *M. tredecim* abdomen-colour alleles are leaking across the *M. tredecim*/*M. septendecim* species boundary; (ii) abdomen colour is affected by environmental variation in a manner that causes *M. septendecim* to converge on *M. tredecim* in the southern part of its range; or (iii) other alleles leading to light abdominal colour have appeared independently in some populations of *M. septendecim*. The pattern of abdomen-colour variation in Brood X supports the first hypothesis; light-coloured *M. septendecim* are restricted to populations adjacent to Brood XIX populations of *M. tredecim*. The second hypothesis is diminished and the third hypothesis is supported by the existence of a limited population of surprisingly light-coloured *M. neotredecim* in Brood XIX far from any populations of *M. tredecim* (Allerton Park, Piatt County, IL; D. Marshall and J. Cooley, unpublished data) and surprisingly light-coloured abdomens in Brood XIV on Cape Cod, Massachusetts (A. Paradis & C. Simon, unpublished data) supporting the interpretation that abdomen colour, is in some cases, a homoplasious character. Further evaluation of variation in abdominal colour and other nuclear markers may reveal the extent of gene flow between *M. tredecim* and *M. neotredecim* at first contact.

#### Models of *Magicicada* speciation

Given that only seven *Magicicada* species are known, the conditions promoting species formation and/or persistence must be limited. Understanding speciation involves determining the mechanisms that isolate populations sufficiently to allow their continuing evolutionary divergence. Two mechanisms might foster allochronic isolation of *Magicicada* populations: (i) genetic changes that influence life-cycle length, thereby isolating mutant founders (Alexander & Moore 1962; Lloyd & Dybas 1966; Marshall & Cooley 2000; Simon *et al.* 2000); or (ii) environmental cueing of latent phenotypic plasticity in life-cycle length, later made more permanent by genetic changes selected under the new environmental regime (Lloyd & Dybas 1966; Marshall & Cooley 2000). These alternatives (mutation and plasticity) are general and should apply to other forms of phenotypically mediated isolation, such as host-plant specificity (e.g. Bush 1992). Speciation models involving only a few temporal founders are implausible in *Magicicada*, because periodical cicadas are dependent on high population densities for predator satiation and successful reproduction (Marlatt

1907; Beamer 1931; Alexander & Moore 1962; Dybas 1969; Karban 1982; Williams *et al.* 1993); this is perhaps a greater problem for the mutation-based mechanism (i) above. However, Marshall & Cooley (2000) and Simon *et al.* (2000) note that rare life-cycle mutants might escape predation and establish an incipient species if they happen to coemerge with an overlapping 'nurse-brood' of the same life-cycle length. The 'nurse-brood' mechanism could also facilitate survival of founders isolated by mechanism (ii); however, environmental cueing of life-cycle plasticity has the potential to isolate large numbers of individuals at once, given an extrinsic cue of sufficient magnitude.

#### Species interactions at secondary contact

If secondary contact of populations leads to fitness losses through hybrid sexual interactions, then selection favours changes that reduce these losses. One outcome of secondary contact is the formation of a hybrid zone, in which selection tends to reduce the impact of hybrid sexual interactions by removing factors leading to hybrid unfitness. For example, two subspecies of *Chorthippus* grasshoppers that diverged in allopatric glacial refugia came into contact and formed a hybrid zone along the crest of the Pyrenees after deglaciation permitted reinvasion of territories uninhabitable during the last glacial maximum (Cooper & Hewitt 1993; Hewitt 1993). In *Chorthippus*, gene flow and recombination resulting from incomplete hybrid sterility appear to be overwhelming selection for assortative mating (see Hewitt 1993; Butlin 1998), leading to a local erosion of barriers to interbreeding and the formation of a hybrid zone.

Another outcome of secondary contact is reproductive character displacement, in which selection reduces the possibility of hybrid sexual interactions. In *Magicicada*, selection for assortative mating has apparently prevailed, perhaps because high levels of hybrid sterility or slightly incompatible mating signals already existed at the time of contact. *Magicicada* and *Chorthippus* illustrate two alternatives facing species achieving secondary contact. The path taken will be determined by the degree to which gene flow is possible and the degree to which members of each species are sexually attractive to members of the other at contact.

#### Concluding remarks

*M. neotredecim* inhabits midwestern habitat that will likely be unsuitable for *Magicicada* during the next glacial maximum (see Webb *et al.* 1993). What does the future hold for this species? For all but the most mobile species, the populations surviving in glacial refuges are most likely founded from the edge of the earlier distribution located farthest from the glacial boundary, while populations near

the glacial boundary simply go extinct (Hewitt 1996). Thus, the most southern, song-displaced *M. neotreddecim* have the greatest likelihood of colonizing a refugium during the next glacial cycle. If this scenario is realistic, then after the next glacial retreat all undisplaced *M. neotreddecim* will have gone extinct, and the pattern of character displacement linking *M. neotreddecim* to an allochronic speciation event in the *M. septendecim* lineage will have been erased. Repeated glacial cycles may cause *M. neotreddecim* to become even more distinct by creating new contexts for signal evolution through the elimination of intermediates or new instances of secondary contact between species that have diverged while in glacial refugia. Perhaps such a pattern of existence in glacial refugia, allochronic speciation, evolution in response to secondary contact, and extinction of intermediates explains the existence of the distinct *M. -decim*, *M. -cassini*, and *M. -decula* cognate groups.

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