

ALL-MALE ASEXUALITY: ORIGIN AND MAINTENANCE OF ANDROGENESIS IN THE ASIAN CLAM *CORBICULA*

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Androgenesis is a rare form of asexual male reproduction found in disparate taxa across the Tree of Life. Phylogenetic analyses of mitochondrial genes suggest that androgenesis has arisen repeatedly in the Asian clam genus *Corbicula*. Two of these androgenetic species have been introduced to North America. Multiple lines of genetic evidence suggest that although nuclear recombination between these two species is rare, mitochondrial genome capture is a frequent consequence of androgenetic parasitism of heterospecific eggs. Egg parasitism may also rarely result in partial nuclear genome capture between closely related species of *Corbicula*, which provides a mechanism for the otherwise clonal species to avoid the deleterious effects of asexuality. Egg parasitism among congeners may explain why androgenesis has been maintained in *Corbicula* after fixation and has not yet led to population extinction. This mechanism also provides an explanation for the apparent multiple origins of androgenesis in *Corbicula* as seen on the mitochondrial DNA phylogeny. We suggest that a single androgenetic lineage may have repeatedly captured mitochondrial genomes (as well as portions of nuclear genomes) from various sexual species, resulting in several distinct androgenetic species with distantly related mtDNA genomes and divergent morphologies.

KEY WORDS: Androgenesis, asexuality, *Corbicula*, genome capture, Muller's Ratchet, phylogeny.

Androgenesis occurs when offspring carry nuclear chromosomes from only the male parent. Androgenetic males parasitize maternal resources—eggs and/or nutrition during development—to produce offspring that do not incorporate maternal DNA. This gives a substantial fitness advantage to the androgenetic individual over sexual conspecifics, and will lead to the rapid spread

of androgenesis within a population under most simulation conditions (McKone and Halpern 2003). However, the maintenance of androgenesis after fixation is hard to explain, because most simulated scenarios lead to population extinction (McKone and Halpern 2003).

Androgenesis has been identified as the main form of reproduction in very few species, in part because androgenesis can lead to population extinction, particularly in dioecious species (McKone and Halpern 2003), and in part because androgenesis

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is difficult to detect without extensive genetic analysis. However, artificially induced androgenesis is commonly used as a tool in a variety of research programs (e.g., Hasimoto 1934; Surani et al. 1984; Datta 2005; Grunina and Recoubratsky et al. 2005; Rapacz et al. 2005; Brown et al. 2006). Spontaneous androgenesis has occurred in laboratory stocks of the fruit fly *Drosophila melanogaster* (Komma and Endow 1995), in inbred stocks of some plants (e.g., Chen and Heneen 1989), and in hybrid complexes of the stick insect genus *Bacillus* (Mantovani and Scali 1992). Only a few divergent eukaryotic lineages appear to reproduce exclusively through androgenesis: the Saharan cypress tree, *Cupressus dupreziana* (Pichot et al. 2001), haploid drone lineages of the little fire ant, *Wasmannia auropunctata* (Fournier et al. 2005), and four species in the clam genus *Corbicula*: *C. leana* (Komaru et al. 1998), *C. fluminea* (Ishibashi et al. 2003), *C. australis* (Byrne et al. 2000), and *C. fluminalis* (Korniushin 2004). This surprising species diversity of androgenetic *Corbicula* and the widespread, overlapping ranges of these species make this clam genus an excellent system to investigate the spread and maintenance of androgenetic reproduction in natural populations of *Corbicula*.

Study System

The genus *Corbicula* contains sexually reproducing species with two separate sexes (Okamoto and Arimoto 1986; Glaubrecht et al. 2003) as well as hermaphrodites, at least some of which reproduce through androgenesis (Komaru et al. 1997; Komaru and Konishi 1999; Byrne et al. 2000; Qiu et al. 2001; Korniushin 2004). *Corbicula* have a worldwide distribution in fresh and brackish waters in Asia, Africa, the Middle East, and Australia, and in the past century have invaded freshwater rivers in the Americas and Europe (reviewed in McMahon 1982). Since its first known appearance on Vancouver Island in British Columbia in 1924 (Counts 1981), *Corbicula* has spread throughout the United States, where it can reach densities of well over 2000 individuals per square meter (McMahon 1999). Its North American distribution seems primarily limited by winter freezing in the north (Counts 1986). Although the precise effects on native fauna and flora have not been determined, at a minimum *Corbicula* prevents recolonization of disturbed areas by native endangered unionid mollusks (Fuller and Imlay 1976) and can reduce algal density in rivers (Cohen et al. 1984). *Corbicula* are also a significant biofouling nuisance species for industry (for review, see Isom 1986).

There are three *Corbicula* morphotypes that have invaded the Americas, and all of these have the biflagellate sperm recognized as a diagnostic marker of androgenesis (Konishi et al. 1998; Byrne et al. 2000; Lee et al. 2005). Clonal reproduction is further suggested by little or no genetic variation within populations of a morphotype, as documented by both allozyme loci (Smith et al. 1979; Hillis and Patton 1982; McLeod 1986) and mitochondrial

and nuclear DNA sequences (Siripattawan et al. 2000; Lee et al. 2005; this study). The two North American invaders ("form A" and "form B" of Britton and Morton 1986, or the "white morph" and "purple morph" of Hillis and Patton 1982) have significantly different shell morphologies (Hillis and Patton 1982), are fixed for different allozyme loci between morphotypes (Hillis and Patton 1982), and have unique mitochondrial DNA sequences associated with each morphotype (Siripattawan et al. 2000; Lee et al. 2005). They are easily distinguishable in the field based on color and shape of exterior shell. We therefore consider these different species. However, there is presently no consensus on the exact species identification for any of the invasive *Corbicula*.

The mechanism of androgenetic reproduction is well characterized in *Corbicula*. After fertilization by a biflagellate sperm that contains a full complement of nuclear chromosomes, the oocyte ejects the entire maternal nuclear genome as two polar bodies (Komaru et al. 1998, 2000; Ishibashi et al. 2003) but retains the mitochondria. Developing embryos are brooded within the hermaphrodite mother's gills, where they probably receive nutritional benefits (Kraemer et al. 1986). Embryos are found within the gametogenic follicles before gamete release, suggesting that self-fertilization is a common mode of reproduction (Kraemer 1978). However, mucosal strands containing sperm have been observed connecting siphons of two clams (Kraemer et al. 1986). Thus, if androgenetic sperm can successfully parasitize the eggs of another individual, then androgenesis could confer a substantial fitness benefit compared to sexually reproducing individuals in *Corbicula*, assuming all else is equal (McKone and Halpern 2003).

Egg Parasitism in Androgenetic Species

Androgenetic pollen of the Saharan cypress can parasitize the ovules of a closely related congener to propagate its own clonal offspring (Pichot et al. 2001). This suggests that in some systems, androgenetic individuals may not only have an advantage over sexually reproducing members of their own species, but over closely related species as well. There is some evidence that androgenetic *Corbicula* may also have the ability to parasitize the eggs of closely related species. In the Río Grande, where both form A and form B *Corbicula* are sympatric, form B mitochondrial sequence has been found in clams with the form A morphotype (Lee et al. 2005). This could be explained by either typical hybridization between species, or by mitochondrial genome capture after a form A sperm parasitized the egg of a form B clam. However, current evidence for hybridization is somewhat contradictory: McLeod (1986) observed several polymorphic allozyme loci in a population of form B that was sympatric with form A, and suggested that nuclear gene exchange between forms A and B was a possible explanation. In contrast, Hillis and Patton (1982)

found no evidence for gene exchange in sympatric populations of forms A and B, as the allozyme loci they examined were completely fixed and diagnostic between the two species, and they observed no clams with intermediate morphologies. Furthermore, Lee and coworkers analyzed DNA sequences of the large ribosomal subunit (28S) gene from 15 individuals of each morphotype at one sympatric locality, but found no evidence of shared alleles, as would be expected if these species were regularly hybridizing (Lee et al. 2005). However, the very limited number of sympatric populations used in these previous studies of North American *Corbicula* leaves open the possibility of gene exchange between the species.

In this article, we collected data on mitochondrial and/or nuclear genes from 23 localities of *Corbicula* (including 10 sympatric localities for forms A and B) to test for either nuclear recombination or egg parasitism between the two species. If androgenesis does not serve as a barrier to frequent nuclear genetic exchange, we predict that nuclear markers will be shared between the two species. Alternatively, if there is mitochondrial genome capture in the absence of nuclear recombination, we expect nuclear markers to be diagnostic between species, whereas some mitochondrial genomes will be shared. Finally, we built a mitochondrial phylogeny using mitochondrial sequences from across the global range of *Corbicula* to test whether the observed androgenetic species diversity within the Americas is the result of diversification from a common androgenetic ancestor, or whether mitochondrial lineages represent separate evolutionary lineages.

Methods

SAMPLE COLLECTION

Clams were collected from 23 localities in Argentina, Korea, Mexico, The Netherlands, and the United States (Table 1). Forms A and B were classified in the field by exterior shell morphology. Ten of these localities included sympatric populations of forms A and B, 11 localities had allopatric populations of form A only, one locality included sympatric populations of forms A and C (a third species introduced into South America), and one locality had the sexual species *C. japonica* only. Specimens from Argentina were obtained from C. Ituarte; specimens from the River Waal and the River Lek in The Netherlands were obtained from A. de Vaate; specimens from Georgia, USA, were obtained from J. Williams and R. Butler; and specimens from Arizona, USA were obtained from M. Sredl. Ethanol-preserved specimens of *C. japonica* from a Korean fish market were obtained from J. Bickham, and the original locality for these specimens is unknown. DNA for sequencing was extracted from tissues using Qiagen DNeasy tissue extraction kit (Qiagen Inc., Hilden, Germany); DNA for restriction site analysis was extracted using protocol 1 from Hillis et al. (1996). In most cases tissue was taken from the foot or main body

of the clam; for one form B sample from the Colorado River we used gonadal tissue.

RESTRICTION SITE ANALYSIS

To examine the possibility of hybridization events between the various putative species of *Corbicula*, we examined the nuclear ribosomal RNA gene regions (rDNA arrays) from 17 populations (165 individuals) of form A and seven populations (70 individuals) of form B (Table 1). These samples represent populations introduced throughout the United States, as well as into Argentina, The Netherlands, and Mexico, and included seven localities where forms A and B are sympatric. Specimens were individually digested with five restriction enzymes: *Eco* RI (recognition sequence: G/AATTC), *Bgl* II (A/GATCT), *Bst* EII (G/GTNACC), *Nco* I (C/CATGG), and *Pvu* II (CAG/CTG). Restriction maps of rDNA arrays were obtained by double-digestion with pairs of restriction enzymes, using the conserved *Eco* RI restriction sites in the 18S and 28S genes as reference sites (Figs. 1 and 2). Restriction fragments were separated on 0.8% agarose gels (5 V/cm for 15 h), along with a one kb-plus ladder as a size standard. DNA was transferred to nylon membranes using the protocol of Southern (1975). Southern blots were then probed using either a series of radioactively labeled oligonucleotides that were located on each side of the conserved *Eco* RI reference sites (primers 28aa, 28w, 18d, and 18e of Hillis and Dixon 1991), or (for the single digests) radioactively labeled clones of the 18S and 28S genes (pI19 and p2546; Arnheim 1979).

IN SITU HYBRIDIZATION

To examine possible polyploidy or other chromosomal anomalies, we performed in situ hybridization of the nucleolar organizing regions (NORs) regions on three individuals of each form from Georgetown, San Gabriel River, Texas, USA (Table 1). We used the pI19 rDNA fragment cloned and described by Arnheim (1979) as a probe. The plasmid was biotin-labeled, and in situ hybridization followed the procedure described by Moyzis et al. (1987). We examined 25 cells per form.

GENE AMPLIFICATION AND SEQUENCING

To examine nuclear sequence diversity at a finer scale than our restriction digests, we amplified and sequenced the first internal transcribed spacer (ITS-1) and a portion of the large ribosomal subunit (28S) of nuclear rDNA from four sympatric populations (Table 1). We used universal primers 18dd and 5.8S (Hillis and Dixon 1991) to get an initial ITS-1 sequence. As portions of the 5.8S ribosomal gene appear to have been duplicated and reversed within the ITS regions, we designed an alternative, bivalve-specific primer using sequences from scallops and unionids (GenBank accession nos. AY294561, AY319383-5, AY313964, AJ534981, AJ428407-9), 5.8Ssh3:

Table 1. Sampling localities of *Corbicula* species used in this study, and number of clams from each sampling location used in a particular laboratory procedure (see *Methods*).

Population location	Number of clams per procedure				
	COI locus sequenced	ITS-1 locus sequenced	28S locus sequenced	Restriction map of rDNA	In situ hybridization of NOR
Argentina: Río de la Plata					
Ensenada					
Form A	7			10	
Form C	10	1 (7 clones)			
Atalaya					
Form A				10	
Korea: Fish market					
<i>C. japonica</i>	4				
Mexico: Río San Juan					
Castillos					
Form A				10	
The Netherlands: River Lek					
Form A	2			10	
The Netherlands: River Waal					
Form C	2				
USA: Arizona: Drainage canal					
Phoenix					
Form A	10			10	
Form B	10			10	
USA: Georgia: Savannah River					
1.6 km upstream of Georgia					
Hwy 119					
Form A				7	
USA: Illinois: Lake Michigan					
Chicago					
Form A				10	
USA: Texas: Blanco River					
Near mouth at San Marcos River					
Form A				10	
USA: Texas: Colorado River					
Webberville					
Form A	25			10	
Form B	25	1 (7 clones)	1 (8 clones)	10	
Hornsby					
Form A	10				
Form B	10				
Longhorn Dam					
Form A	10			10	
Form B	10			10	
USA: Texas: Little Brazos River, (Brazos River drainage)					
Crossing of Texas Hwy. 21					
Form A	9			10	
Form B	10			10	

Continued

Table 1. Continued.

Population location	Number of clams per procedure				
	COI locus sequenced	ITS-1 locus sequenced	28S locus sequenced	Restriction map of rDNA	In situ hybridization of NOR
USA: Texas: Llano River (Colorado River drainage)					
Roosevelt (N. Llano River)					
Form A				10	
Headsprings (S. Llano River)					
Form A				10	
USA: Texas: Pinto Creek (Río Grande drainage)					
Crossing of US Hwy 90					
Form A	9			10	
Form B	9			10	
Crossing of RR 1008					
Form A				10	
Form B				10	
USA: Texas: Pecos River (Río Grande drainage)					
Pandale					
Form A	10	1 (7 clones)	1 (8 clones)		
Form B	7	1 (6 clones)	1 (6 clones)		
USA: Texas: San Gabriel River (Brazos River drainage)					
Georgetown					
Form A	20	1 (8 clones)	1 (8 clones)		3
Form B	20				3
Crossing of Texas Hwy 29					
Form A	11			10	
Form B	11			10	
USA: San Saba River (Colorado River drainage)					
Crossing at Hwy 864					
Form A				8	
USA: Texas: Spindletop Branch					
Drainage ditch near Winnie					
Form A	10				
Total	261	5	4	235	6

5'ATTACATTAATTCACGCACCTG3'. To amplify within the 28S gene, we used primers D23F and D4RB (Lee et al. 2005). Reaction conditions were: 2.5 μ l Thermopol 10 \times buffer with MgCl₂ (NEB), 2.5 μ l 25 μ M dNTPs, 0.75 μ l each 10 mM primer, 0.2 μ l NEB *Taq* polymerase, and 1–3 μ l DNA extract, brought to a total volume of 25 μ l with double-distilled water. PCR conditions were 94°C 1 min 30 sec, and 35 cycles 94°C 1 min 60°C (ITS-1)/55°C (28S) 1 min 72°C 2 min, 72°C 5 min. To sequence individual alleles, PCR products were cloned using the Invitrogen TOPO TA Cloning[®] kit with pCR[®] 2.1-TOPO[®] vector (Invitrogen Life Technologies, Grand Island, NY). For PCR of clones we used the reaction conditions and primers provided by the kit. We sequenced between six and eight clones per individual to examine within-individual variation in the rDNA arrays (Table 1). These sequences were deposited into GenBank (accession nos ITS-1: EU090360-95; 28S: EU090400-29).

To compare nuclear and mitochondrial diversity within North American *Corbicula*, primers HCO and LCO (Folmer et al. 1994) were used to amplify a 710-bp fragment of COI for 261 clams from 14 sampling locations (Table 1) under the following PCR conditions: 1 μ l DNA extract, 1.5 μ l 10 \times buffer without MgCl₂, 1.5 μ l 25 μ M dNTPs, 1 μ l 24 μ M MgCl₂, 1 μ l each 10 mM primer, 1 μ l *Taq* polymerase, brought to a volume of 25 μ l with double-distilled water. PCR cycles were run on an Applied Biosystems Gene Amp 2700 thermocycler, with an initial starting temperature of 94°C for 1 min 30 sec, followed by 35 cycles of 94°C 1 min, 46°C 1 min, 72°C 2 min, and a final extension of 72°C 7 min. Sequences were run on either an Applied Biosystems 377 or an Applied Biosystems 3100 automated sequencer. Four Korean sequences representing two haplotypes were deposited into GenBank (accession nos. EU90396-9). All of our American and European mitochondrial

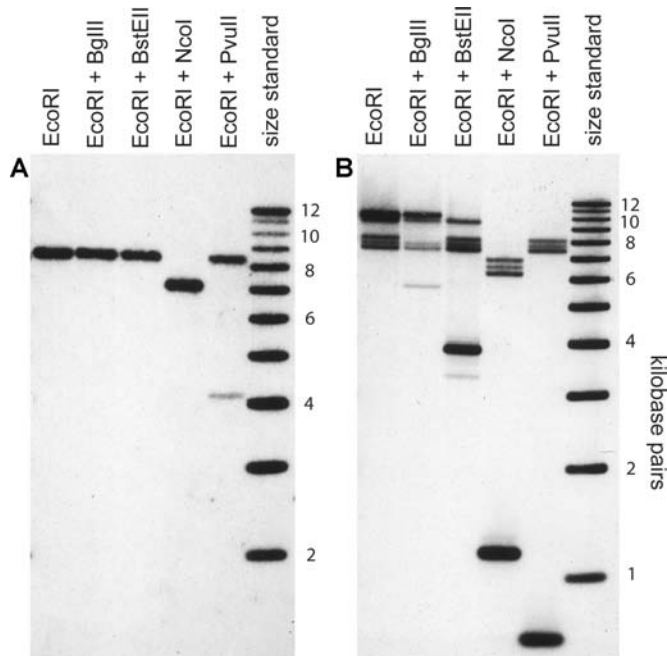


Figure 1. Example autoradiograms of Southern blots from restriction digests of (A) form A and (B) form B *Corbicula* genomic DNA, hybridized to radioactively labeled 28aa primer. In (A), the 8.3-kb fragment seen in lanes A1, A2, A3, and A5 corresponds to the fragment bounded by the conserved *Eco* RI site near the end of the 28S gene, through the intergenic spacer, to the conserved *Eco* RI site near the end of the 18S gene in the adjacent repeat (see Fig. 2). The 4.2-kb low-copy-number fragment seen in lane A5 is the result of the polymorphic *Pvu* II site seen in some copies of the IGS of form A *Corbicula*. The 7.1-kb fragment in lane A4 corresponds to the *Eco* RI to *Nco* I fragment from the 28S gene into the IGS of form A. In contrast to the uniformity of the rDNA array length seen in form A, individuals of form B *Corbicula* (seen in autoradiograph B) show five distinct array lengths (the *Eco* RI to *Eco* RI fragments of the five arrays are 7.4, 7.6, 8.1, 9.9, and 10.1 kb in length). In comparing lanes B1 and B2, note that the *Bgl* II site in the IGS is confined to just one of the five rDNA arrays (the 8.1-kb fragment is the only one cut). Similarly, *Bst* EII sites are only present in the IGS of the 10.1 fragment (see lane B3, and note the low-frequency polymorphism present in this array). The two Type 2 arrays share an *Nco* I site in the IGS (lane B4: note the high-frequency 1.2-kb fragment), and all the arrays have a conserved *Nco* I site in the 18S gene (which produces fragments of 6.2, 6.4, and 6.9 kb in the three Type 1 arrays). Finally, the two Type 2 arrays in form B share a *Pvu* II site in the 28S gene, which produces the ~0.8-kb fragment in lane B5.

sequences matched existing GenBank sequences from previous studies on *Corbicula* (Table 2; haplotypes H1, H2, and H4).

PHYLOGENETIC ANALYSES: NUCLEAR PHYLOGENIES

To explore evidence for nuclear genetic exchange between North American *Corbicula* species, we built phylogenies of both ITS-

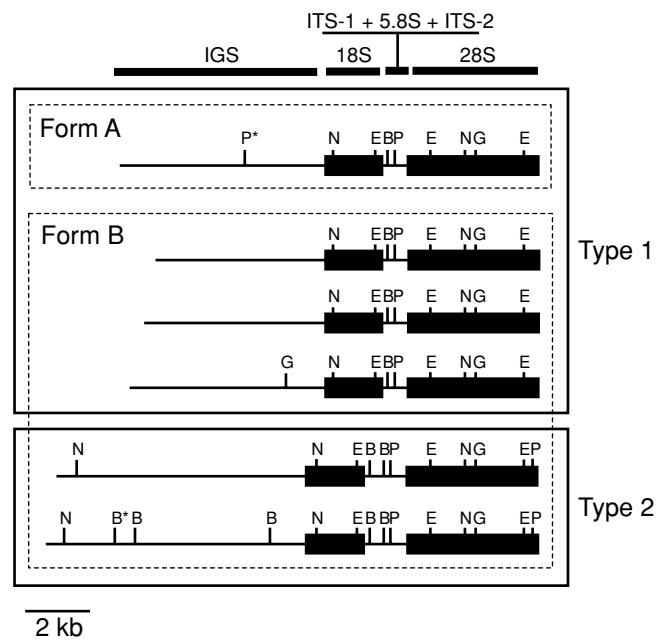


Figure 2. Restriction-site maps of ribosomal DNA repeat regions in North American *Corbicula* form A and form B. Restriction sites for a given enzyme are noted (P: *Pvu* II, N: *Nco* I, E: *Eco* RI, B: *Bst* EII, G: *Bgl* II). The diversity of array types in form B clams represents diversity within individual clams. IGS, intergenic spacer; ITS-1, internal transcribed spacer 1; ITS-2, internal transcribed spacer 2. The asterisk indicates a polymorphic restriction site (present in some copies of the array but not in others). These arrays are repeated in tandem in the genome, so hundreds of copies of each array are present in a given individual.

1 and 28S sequences. Previously published 28S sequences for both North American invasive *Corbicula* (forms A and B) and the South American morph (form C) were obtained from GenBank (AF519526-8) and added to our dataset. Sequences were similar enough that there were relatively few areas of alignment ambiguity, so all sequences were aligned by hand using MacClade 4.06 (Maddison and Maddison 2000). Alignments are available on the TreeBase website, (<http://www.treebase.org>, study accession no. S1894). Ends were trimmed to reduce the amount of missing data. In the ITS-1 alignment, all gaps, including a 161-bp indel found in three sequences, and any regions not easily aligned were removed before phylogenetic analyses (278 bp total removed, 590 bp remaining). The best-fit model of evolution was determined using the Bayesian information criterion (BIC) implemented in the program ModelTest version 3.7 (Posada and Crandall 1998). The maximum-likelihood estimate of the phylogeny was determined using the program GARLI version 0.952b2.r171 (Zwickl 2006: <http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html>). The default settings were used for all searches. A Bayesian analysis was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). We

Table 2. GenBank numbers and localities for each haplotype designation. Species designation indicated when found within its native range; species designations for *Corbicula* found outside of its native range is not given as invasive species have not yet been conclusively identified. Haplotypes are based on 564 bp of the mitochondrial COI gene. Some sequences were trimmed to fit this length. Sperm morphology is indicated when known for at least one species within the haplotype group. Biflagellate sperm are considered a marker for androgenesis, whereas monoflagellate sperm indicates sexual reproduction (Glaubrecht et al. 2003).

Haplotype	Country	GenBank accession No.	Sperm morphology
H1	China, France, Germany Japan (<i>C. leana</i>), Korea, The Netherlands (this study), Thailand (<i>C. fluminea</i>), USA (form A; this study)	AF519495-507, AF196268, AF196280-81, AF269090, AF269092-3, AY097263-75, AY943243, DQ285577	biflagellate ^{1,2}
H2	China, France, Korea, Japan, USA (form B)	AF120666, AF196278-9, AF196269, AF519509-11, AY097300, AY097308-11	biflagellate ²
H3	Germany	AY097276	
H4	Argentina (Form C; this study), France, Germany, The Netherlands (this study)	AF269095, AF519508, AY097277-81	biflagellate ²
H5	China, France, Indonesia (<i>C. javanica</i> , <i>C. subplanata</i> , <i>C. linduensis</i>), The Netherlands	AF269096-8, AY275668, DQ285600-3, DQ285579	
H6	Germany	AY097283	
H7	Germany	AY097284	
H8	Germany	AY097285	
H9	Germany	AY097286	
H10	Germany	AY097287	
H11	Germany	AY097288	
H12	Germany	AY097289	
H13	Germany	AY097290	
H14	Germany	AY097291	
H15	China	AY097292	
H16	China	AY097293	
H17	China	AY097294	
H18	Israel	AY097295-8	
H19	Israel	AY097299	
H20	Taiwan	AF457991	
H21	China	AF457990	
H22	China	AF457989	
H23	Korea	AF457992	
H24	Germany	AY097301	
H25	France	AY097302	
H26	France	AY097303	
H27	France	AY097304	
H28	France	AY097305	
H29	France	AY097306	
H30	France	AY097307	
H31	Indonesia	AF457993	
H32	Japan	AY097312	
H33	Japan	AY097313	
H34	Japan	AY097314	
H35	Japan	AY097315	
H36	Japan (<i>C. japonica</i>)	AF196271	monoflagellate ³
H37	Germany	AY097262	
H38	Germany	AY097282	
H39	Argentina	AF519512	
H40	Indonesia (<i>C. loehensis</i>)	AY275666, DQ285580	monoflagellate ⁴
H41	Indonesia (<i>C. loehensis</i>)	AY275667, DQ285581	monoflagellate ⁴
H42	Indonesia (<i>C. matannensis</i>)	AY275663	monoflagellate ⁴
H43	Indonesia (<i>C. matannensis</i>)	AY275664	monoflagellate ⁴

Continued

Table 2. Continued.

Haplotype	Country	GenBank accession No.	Sperm morphology
H44	Indonesia (<i>C. matannensis</i>)	AY275665	monoflagellate ⁴
H45	Indonesia (<i>C. possoensis</i>)	AY275661	monoflagellate ⁴
H46	Indonesia (<i>C. possoensis</i>)	AY275662	monoflagellate ⁴
H47	Indonesia (<i>C. moltkiana</i>)	AY275657	monoflagellate ⁴
H48	Indonesia (<i>C. moltkiana</i>)	AY275658	monoflagellate ⁴
H49	Indonesia (<i>C. moltkiana</i>)	AY275659	monoflagellate ⁴
H50	Indonesia (<i>C. moltkiana</i>)	AY275660	monoflagellate ⁴
H51	Japan (<i>C. japonica</i>)	AF367440	monoflagellate ³
H52	Japan (<i>C. japonica</i>)	AF367441	monoflagellate ³
H53	Madagascar (<i>C. madagascariensis</i>)	AF196275	
H54	The Netherlands	AF269091	
H55	France	AF269094	
H56	Australia (<i>C. australis</i>)	AF196274	biflagellate ⁵
H57	Japan (<i>C. sandai</i>)	AF196273	monoflagellate ⁶
H58	Japan (<i>C. sandai</i>)	AF196272	monoflagellate ⁶
H59	Thailand (<i>C. fluminea</i>)	AF196270	
H60	USA	U47647	biflagellate ²
H61	Vietnam	AF468018	
H62	Vietnam	AF468017	
H63	China	AF457999	
H64	China (<i>C. fluminalis</i> / <i>C. cf japonica</i> ⁷)	AF457998	
H65	China (<i>C. fluminalis</i> / <i>C. cf japonica</i> ⁷)	AF457997	
H66	China (<i>C. fluminalis</i> / <i>C. cf japonica</i> ⁷)	AF457996	
H67	China (<i>C. fluminalis</i> / <i>C. cf japonica</i> ⁷)	AF457995	
H68	China	AF457994	
H69	Korea (fish market; this study)	EU090397	
H70	Korea (fish market; this study)	EU090396-7, EU090399	
H71	Indonesia (<i>C. anomioides</i>)	DQ285604	
H72	Indonesia (<i>C. anomioides</i>)	DQ285605	
H73	Indonesia (<i>C. possoensis</i>)	DQ285596	monoflagellate ⁴
H74	Indonesia (<i>C. possoensis</i>)	DQ285597	monoflagellate ⁴
H75	Indonesia (<i>C. possoensis</i>)	DQ285598	monoflagellate ⁴
H76	Indonesia (<i>C. possoensis</i>)	DQ285599	monoflagellate ⁴
H77	Indonesia (<i>C. matannensis</i>)	DQ285591	monoflagellate ⁴
H78	Indonesia (<i>C. matannensis</i>)	DQ285592	monoflagellate ⁴
H79	Indonesia (<i>C. matannensis</i>)	DQ285593	monoflagellate ⁴
H80	Indonesia (<i>C. matannensis</i>)	DQ285594	monoflagellate ⁴
H81	Indonesia (<i>C. matannensis</i>)	DQ285595	monoflagellate ⁴
H82	Indonesia (<i>C. matannensis</i>)	DQ295587, DQ285590	monoflagellate ⁴
H83	Indonesia (<i>C. matannensis</i>)	DQ285586	monoflagellate ⁴
H84	Indonesia (<i>C. matannensis</i>)	DQ285588	monoflagellate ⁴
H85	Indonesia (<i>C. matannensis</i>)	DQ285589	monoflagellate ⁴
H86	Indonesia (<i>C. matannensis</i>)	DQ285583, DQ285585	monoflagellate ⁴
H87	Indonesia (<i>C. matannensis</i>)	DQ285582	monoflagellate ⁴
H88	Indonesia (<i>C. matannensis</i>)	DQ285584	monoflagellate ⁴
H89	Thailand (<i>C. lamarckiana</i>)	DQ285578	
H90	Unknown (“ <i>C. fluminea</i> ”)	DQ264393	
H91	Unknown (“ <i>C. fluminea</i> ”)	AY874525	
<i>Neocorbicula limosa</i>	Argentina	AF196277	monoflagellate ⁴
<i>Polymesoda caroliniana</i>	USA	AF196276	monoflagellate ⁴

¹Konishi et al. 1998; ²Lee et al. 2005; ³Komaru et al. 1997; ⁴Glaubrecht et al. 2003; ⁵Byrne et al. 2001; ⁶Hachiri and Higashi 1970 (as cited in Konishi et al. 1998); ⁷Korniushin (2004) finds that specimens labeled *C. fluminalis* from China are significantly different from those from the *C. fluminalis* type locality, and suggests they might instead group with *C. japonica*.

ran four independent MCMC analyses (each using four chains) for five million generations, and trees and parameters were sampled every 100 generations. MrConverge (as described in Brown and Lemmon 2007: <http://www.evotutor.org/MrConverge/>) was used to determine when convergence between runs had been reached, the posterior probability of bipartitions, and the post burn-in Bayesian consensus tree with branch lengths.

PHYLOGENETIC ANALYSES: MITOCHONDRIAL PHYLOGENY

To place our North American mitochondrial sequences into the historical context of the genus *Corbicula*, we constructed a molecular phylogeny of *Corbicula* clams using mitochondrial cytochrome oxidase I (COI) sequences, incorporating GenBank data from previous studies into our dataset (Table 2). Sequences were aligned manually using MacClade 4.06 (Maddison and Maddison 2000) and trimmed to 584 bp to minimize missing data at either end of the sequence. For analysis, each haplotype was given a number, with identical sequences collapsed to one haplotype (Table 2). Alignment is available on the TreeBase website (<http://www.treebase.org>, study accession no. S1894). The program ModelTest version 3.7 (Posada and Crandall 1998) was used to determine the appropriate model of evolution under the Akaike criterion. The program GARLI (Zwickl 2006) was used to find the maximum-likelihood estimate of the phylogeny. The default settings were used for all searches. We selected bivalves *Neocorbicula limosa* and *Polymesoda caroliniana* to root the tree (following Glaubrecht et al. 2003). The Bayesian consensus topology was obtained as described above for ITS-1 and 28S.

We evaluated hypotheses on the maximum number of independent androgenetic lineages by comparing the Bayesian posterior probabilities of alternative tree topologies. These posterior probabilities were determined by filtering post burn-in trees that matched a given topology (a backbone constraint tree) using PAUP* version 4b (Swofford 2002), and dividing the number of trees sampled consistent with that topology by the total number of sampled trees. We filtered topologies using a series of backbone constraints (Table 4), allowing for different modes of reproduction in some of the unstudied taxa in our analysis. We tested the monophyly of androgenetic taxa with respect to known sexual taxa (Table 2: androgenetic haplotypes H1, H2, H4, and H56), which is our expectation if androgenesis had evolved once with no reversions to sexuality. To test support for two independent, monophyletic clades of androgenetic *Corbicula*, we filtered the Bayesian post-burn-in tree sample for all possible combinations of two clades of androgenetic taxa (Table 4), and then filtered those trees to remove any which were also consistent with complete monophyly (i.e., that would place the two clades as sister taxa). Finally, we tested for support for three separate clades of androgenetic taxa, again using succes-

sive filters to remove trees consistent with both of the other two hypotheses.

Results

DIVERSITY OF RIBOSOMAL RNA ARRAYS

Restriction-site maps of the nuclear ribosomal DNA repeats (rDNA arrays) for each form are shown in Figure 2. Across 140 individuals (70 form A, 70 form B) from seven sympatric North American localities (Table 1), both species of *Corbicula* are fixed for mutually diagnostic sets of rDNA arrays (Fig. 2). Each individual within a species shares a set of restriction sites and rDNA length variants (to the level of resolution of the restriction maps). In form A, we found one polymorphic restriction site in the rDNA arrays (Fig. 2, Type 1). In contrast, each form B individual showed multiple restriction-site patterns represented by five different restriction-site maps (Fig. 2). Three of the form B rDNA array types are similar to the form A pattern in length and restriction sites (Fig. 2, Type 1), but the remainder of form B restriction map sites are restricted to form B individuals (Fig. 2, Type 2).

Hybridization of ribosomal markers to DNA in interphase cells revealed a modal number (in at least 80% of observed cells) of three NORs per cell in form A individuals, but seven NORs per cell in form B individuals (Fig. 3). Occasional counts were plus or minus one NOR, possibly due to visual overlap of NORs in individual cells. The greater than twofold increase of visible NORs in form B compared to form A is consistent across all cells.

NUCLEAR PHYLOGENIES

Sequences cloned from individual clams did not form monophyletic clades in either the ITS-1 or 28S nuclear phylogenies (Fig. 4). Several form B cloned ITS-1 sequences contain an insertion of 161 bp, which was expected based on the mapped rDNA array variants (Fig. 2). Phylogenetic analysis of this locus (in which the insert was deleted from the analysis; see *Methods*) places the alleles with the insertion in a separate clade with high support (BPP = 1.0), regardless of whether the clam had the form A or form B mitochondrial COI haplotype (Fig. 4, Type 2). The remainder of form B alleles are found in clades with form A and form C (Fig. 4, Type 1). The 28S tree had a similar topology to the ITS-1 tree; although we cannot link any individual sequence unequivocally to our restriction-site map data, there is a strongly supported clade of form B clones similar to the ITS-1 Type 2 clade (BPP = 1.0).

POPULATION-LEVEL MITOCHONDRIAL ANALYSES

In the American invasive *Corbicula*, a given cytochrome oxidase I (COI) mitochondrial haplotype is usually species-specific. However, in three of four river drainages sampled (seven of nine

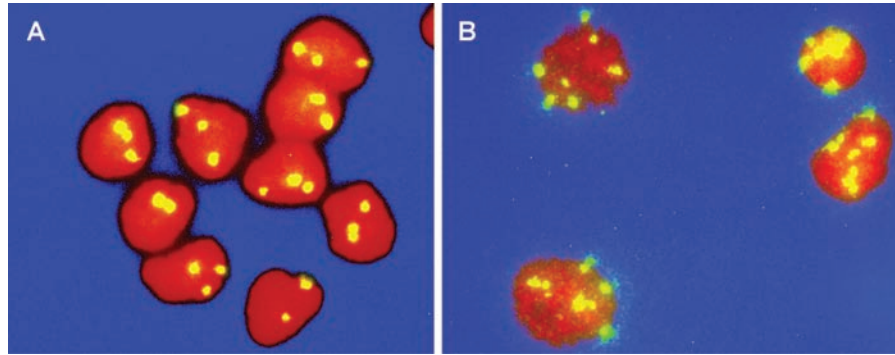


Figure 3. In situ hybridization of ribosomal RNA repeats in invasive form A and form B collected from San Gabriel River, Texas, USA. Cells fluoresce red; light areas indicate where RNA probes have attached to chromosomes at the nucleolar organizing regions (NORs). Representative cells from each form are shown. (A) Form A *Corbicula* show three distinct NORs per cell. (B) Form B *Corbicula* show seven to nine distinct NORs per cell.

sampling locations) where form A and form B are sympatric, some North American form B individuals showed the form A mitochondrial haplotype, and one form A individual showed the mitochondrial haplotype of form B (Table 2). Form A was fixed

for the form A haplotype both in the allopatric population sampled (Spindle Top Branch; Table 1) and where it occurs in sympatry with form C (Table 3). Form C individuals were fixed for a third unique mitochondrial haplotype.

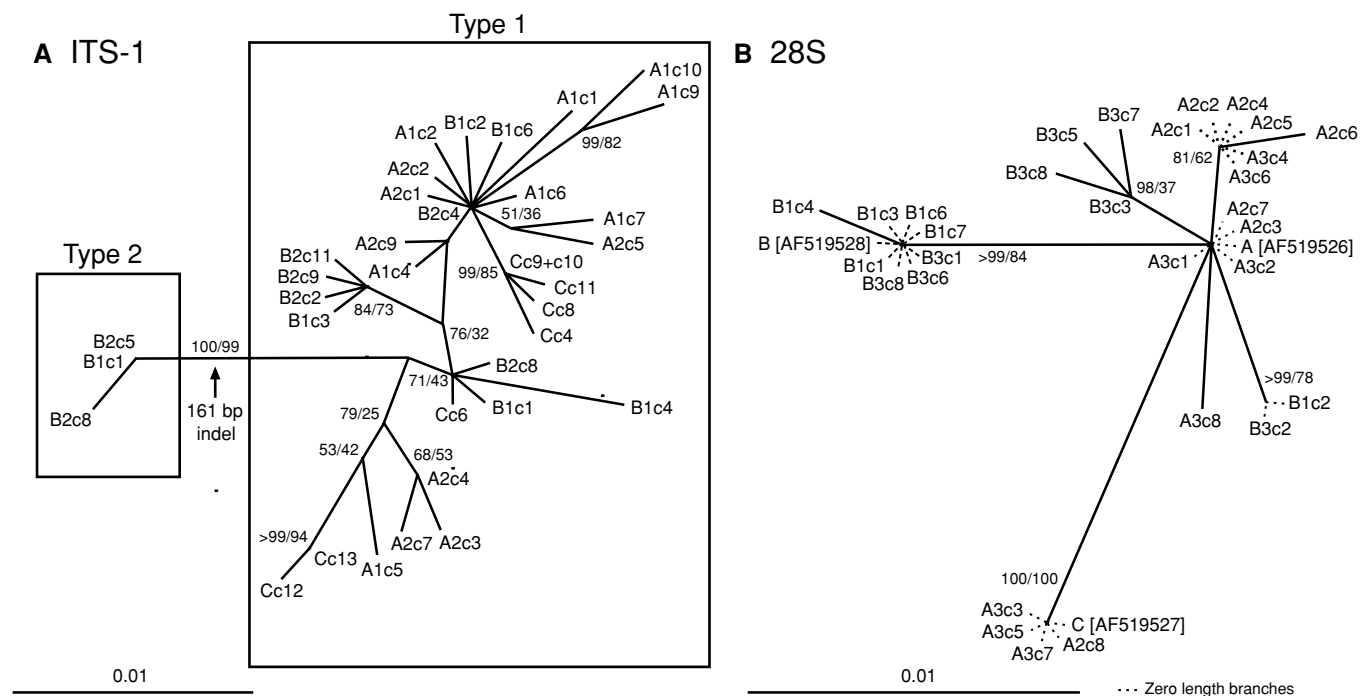


Figure 4. Bayesian consensus phylogenies for North American *Corbicula* populations, with branch lengths optimized under maximum likelihood. Numbers at nodes are Bayesian posterior probabilities and maximum-likelihood bootstrap proportions (BPP/MLBP). A1, ITS-1 clones from form A with form A COI haplotype (haplotype H1 from Table 2) from San Gabriel River, Texas, USA; A2, ITS-1 and 28S clones from form A with form B COI haplotype (H2 from Table 2) from Pecos River, Texas, USA; A3, 28S clones from form A with form A COI haplotype (H1) from San Gabriel River, Texas, USA; B1, ITS-1 and 28S clones from form B with form B COI haplotype (H2) from Pecos River, Texas, USA; B2, ITS-1 clones from form B with form A COI haplotype (H1) from Colorado River, Texas, USA; B3, 28S clones from form B with form B COI haplotype (H2) from San Gabriel River, Texas, USA; C, ITS-1 clones from form C from Río de la Plata, Ensenada, Argentina (H4). (A) Phylogeny based on sequences of the first internal transcribed spacer of the ribosomal DNA (ITS-1). Types correspond to rDNA restriction array types in Figure 2. (B) Phylogeny based on sequences of the large ribosomal subunit (28S); includes GenBank sequences for each form (the results of direct sequencing and not cloning of individual alleles; Lee et al. 2005).

Table 3. Data on mismatch between mitochondrial haplotype and species identification for North and South American *Corbicula*. Number of individuals of each species with the mitochondrial marker (mtDNA) of the other species compared to the total of each species collected.

River drainage	Form B with form A mtDNA/ total collected	Form A with form B mtDNA/ total collected	Form C with form A mtDNA/ total collected	Form A with form C mtDNA/ total collected
Río de la Plata, Argentina (1 location)			0/10	0/7
Phoenix, Arizona, USA (1 location)	0/10	0/10		
Brazos River drainage, Texas, USA (3 locations)	11/44	0/42		
Río Grande drainage, Texas, USA (2 locations)	1/16	1/19		
Colorado River drainage, Texas, USA (3 locations)	38/45	0/45		
Total	50/115	1/116	0/10	0/7

ANDROGENETIC *CORBICULA* ARE NOT MONOPHYLETIC ON mtDNA TREES

Our maximum likelihood and Bayesian phylogenetic analyses of mitochondrial DNA across the various species of *Corbicula* did not support a single clade of mitochondrial lineages from androgenetic species. On our mitochondrial tree, androgenesis is polyphyletic with respect to diploid sexually reproducing species (Fig. 5). Our analyses identified four androgenetic clades, and each of the three American mitochondrial lineages was nested within a separate clade (haplotypes H1: form A, H2: form B, H4: form C; Fig. 5). Hypothesis testing using our mitochondrial data showed low support for a single androgenetic clade (Bayesian posterior probability, BPP = 0.034726) or for two androgenetic clades (BPP, 0.044208) (Table 4). Three androgenetic clades were found in nearly half of the sampled mitochondrial trees (BPP = 0.539508), primarily because H4 (form C) and H56 (*C. australis*) form a clade in about half the trees (BPP = 0.5109). Four separate clades were found in only about one-third of the trees sampled (BPP = 0.381558). Overall, the hypotheses of three or four androgenetic clades received considerably higher support (53.9% and 38.1% of trees, respectively) than the hypotheses of one or two clades (3.4% and 4.4% of trees, respectively).

Discussion

In this study, we used a variety of molecular techniques to examine possible genetic interactions between sympatric species of androgenetic lineages in the clam genus *Corbicula*. We analyzed genetic data from two species that have been introduced to North America to assess past or present interspecific nuclear gene exchange, and to test for egg parasitism with mitochondrial genome-capture between species. These data provide insights into the evolutionary spread and maintenance of androgenesis.

HISTORIC NUCLEAR EXCHANGE BETWEEN *CORBICULA*

On the basis of our restriction-site maps of nuclear rDNA arrays (Fig. 2) and sequence-based phylogenies (Fig. 4), we propose

that form B originated from nuclear genome capture of a second species of *Corbicula* by a form A-like androgenetic ancestor. This genome capture event created a new androgenetic species that incorporated both the form A-like genome as well as part or all of the genome of the second species. The nuclear rDNA arrays of form A show the usual pattern of homogenization (Hillis and Dixon 1991), with a uniform repeat length and a single polymorphic site in the intergenic spacer (Figs. 1 and 2). However, there are five distinct rDNA arrays in all individuals of form B; these differ in the lengths of the intergenic and ITSs, as well as in the number and type of restriction sites (which will correspond to base pair differences between array types). Although three of these patterns are quite similar to the rDNA repeats found in form A individuals (Fig. 2, Type 1), two patterns (Fig. 2, Type 2) are highly divergent from both form A and the other form B rDNA repeats. The presence of two distinct patterns within each individual clam suggests that the nuclear content of form B individuals may have origins in two separate evolutionary lineages.

Our phylogenetic analyses of two sequenced markers within the rDNA repeats (Fig. 4), the ITS-1 and a portion of the large ribosomal subunit (28S), also demonstrate nuclear diversity within form B. Previous sequence analysis of this 28S locus showed no diversity within form B clams (Lee et al. 2005). However, Lee et al. (2005) directly sequenced PCR product without the additional step of separating individual alleles prior to sequencing. If certain ribosomal alleles are preferentially amplified due to PCR reaction conditions (e.g., Buckler et al. 1997; Keller et al. 2006) only one sequence could be detected in the organism when more than one allele is actually present. In contrast, because we cloned individual sequence fragments, we were able to capture nuclear diversity within North American *Corbicula*. In particular, both ITS-1 and 28S phylogenies show at least one divergent, well-supported (BPP = 1.0) form B clade (Fig. 4). Based on the presence of a 161-bp insert in the ITS-1 sequence, this form B clade (Fig. 4) corresponds to the Type 2 ribosomal restriction patterns found only within form B (Fig. 2). The presence of multiple clades per individual is further evidence that form B clams contain nuclear

Table 4. Bayesian posterior probabilities of alternative tree topology hypotheses, given a post-burn-in sample of 181,912 trees. Haplotypes for androgenetic *Corbicula* are H1 (form A), H2 (form B), H4 (form C), and H56 (*C. australis*) (see Table 2). Haplotypes for sexual *Corbicula* and the outgroup taxa are not specified in this table for simplicity, but can be found in Table 2.

Hypothesis	BPP
One androgenetic clade	
((H1,H2,H4,H56), sexual))	0.034726
Two androgenetic clades	
((H1,H2,H4),H56,sexual))	0.000225
((H1,H2,H56),H4,sexual))	0.001946
((H1,H4,H56),H2,sexual))	0.039459
((H2,H4,H56),H1,sexual))	0.002067
((H1,H2),(H4,H56),sexual))	0.000005
((H1,H4),(H2,H56),sexual))	0.000066
((H1,H56),(H2,H4),sexual))	0.000044
Total	0.044208
Three androgenetic clades	
((H1,H2),H4,H56,sexual))	0.000632
((H1,H4),H2,H56,sexual))	0.020318
((H1,H56),H2,H4,sexual))	0.049612
((H2,H4),H1,H56,sexual))	0.000698
((H2,H56),H1,H4,sexual))	0.000885
((H4,H56),H1,H2,sexual))	0.467363
Total	0.539508

DNA that originated from separate nuclear lineages. The remainder of form B nuclear sequences group with form A sequences in our phylogenies. Although the Type I rDNA arrays are similar between forms A and B and suggest a recent common ancestor for this portion of their genomes, they can be distinguished in the restriction-site analyses, and we can see no evidence of any ongoing nuclear recombination between the two species in the rDNA restriction-site data. The lack of observed heterozygotes at diagnostic allozyme loci at the sympatric localities sampled by Hillis and Patton (1982) and McLeod (1986) is also consistent with the absence or rarity of regular nuclear exchange between forms A and B.

The intragenomic sequence diversity detected in North American *Corbicula* compared to the lack of mitochondrial diversity is not unexpected. PCR-mediated error in replication of sequences (Tindall and Kunkel 1988; Bradley and Hillis 1997; Kobayashi et al. 1999) is likely in our dataset, and will account for some of the variation around individual nodes in our nuclear phylogenies (Fig. 4). We cannot distinguish this in vitro error from natural variation, which can be observed between the hundreds of copies of the rDNA genes, particularly in asexuals (e.g., Gandolfi et al. 2001; Feliner et al. 2004; Keller et al. 2006). However, this minor variation does not detract from our argument that major differentiated rDNA clades exist within form B, particularly as these

are consistent with our RFLP analysis. Highly divergent rDNA genes can indicate two divergent ancestral species (as in Hugall et al. 1999; Lim et al. 2000; Muir et al. 2001), and the reduction of concerted evolution and gene conversion (as in Gandolfi et al. 2001; Mes and Cornelissen 2004; Keller et al. 2006). These multiple ribosomal haplotypes can be maintained in a genome over periods spanning millions of years (e.g., Muir et al. 2001; Keller et al. 2006).

The nucleolar organizer regions (NORs) are the chromosomal locations of the rDNA tandem repeats. Concerted evolution of the tandem repeats occurs through a combination of gene conversion and unequal crossing over during a recombination between homologous chromosomes (Hillis and Dixon 1991). Form A is triploid, and our in situ hybridization of interphase cells with an rDNA probe revealed three NORs, consistent with one NOR per haploid chromosome set (Fig. 3). Previous chromosome counts of form B suggest it is triploid (Lee et al. 2005), and although we were unable to successfully karyotype form B from our sampling localities, chromosomal smears show approximately 54 chromosomes (R. Baker and S. Hedtke, unpubl. data), the number expected for a triploid in this genus (Okamoto and Arimoto 1986). However, in situ hybridization of form B cells show seven visible NORs in each cell rather than only three. Therefore, form B likely has multiple NORs per chromosome set. In situ hybridization of a chromosome spread also shows seven distinct NORs in form B (R. Baker, unpubl. data). This apparent discrepancy between ploidy and the number of NORs could be resolved if the homologous chromosomes of two ancestral genomes have recombined in form B, resulting in chromosomes that have duplications of the NORs with rDNA arrays present from both ancestral genomes. These multiple chromosomal locations of NORs per haploid chromosome set in form B could explain why homogenization between these sets of rDNA arrays (especially via unequal crossing over) is limited (Hillis and Dixon 1991; Copenhaver and Pikaard 1996; Parkin and Butlin 2004), and why polymorphisms in the ribosomal sequence are observed within all form B individuals.

The greater diversity of rDNA arrays, the presence of multiple clades of rDNA sequences, and the incongruence between ploidy and NOR number in form B clams compared to form A clams could all be the consequence of hybrid origins for form B, which caused greater genetic and chromosomal diversity. The clonal nature of androgenesis appears to have resulted in fixation of this diversity across individuals of form B.

NUCLEAR RECOMBINATION BETWEEN ANDROGENETIC *CORBICULA* IS RARE

Restriction-site maps of nuclear ribosomal RNA genes and their associated spacer regions (rDNA) are uniform within each morphotype among all localities, but consistently distinct between forms A and B at sympatric as well as allopatric localities (Fig. 2).

The distinct rDNA arrays of the two morphotypes at all sympatric localities means they are acting like distinct species, with no regular, ongoing nuclear recombination evident. The uniformity of the diagnostic nuclear rDNA arrays across the introduced range of the two forms, together with the diagnostic allozymic and morphological characteristics reported by Hillis and Patton (1982) and McLeod (1986), indicates that two distinct species of androgenetic *Corbicula* have been introduced to North America. However, the presence of some polymorphic allozyme loci in populations of form B where it is sympatric with form A (McLeod 1986) suggest that there is a possibility for rare incorporation of genes from one species to the other. Nonetheless, given that other allozyme loci in the same sympatric population examined by McLeod (1986) are fixed for different alleles in the two forms, the observed polymorphism cannot be the result of ongoing typical hybridization.

MITOCHONDRIAL-MORPHOTYPE MISMATCH COMMON IN NORTH AMERICA

In North American invasive *Corbicula*, most individuals of the same morphotype share a mitochondrial haplotype (Lee et al. 2005; this study). However, we detected mitochondrial lineages discordant with morphology in three of four sampled river drainages with sympatric populations of forms A and B (Table 3). Because little or no recent nuclear genetic exchange seems to have occurred between morphotypes, this mitochondrial mismatch has three possible explanations: (1) heteroplasmic mitochondrial DNA; (2) population polymorphism in the ancestor of both species; or (3) egg parasitism between species. Egg parasitism would occur after a sperm from one species extruded nuclear DNA from an egg of the other species (Lee et al. 2005), replacing the nuclear DNA of the mother species with that of the father species. The mitochondrial DNA of offspring would come from the egg and have the same sequence as the maternal lineage.

We do not believe that our results can be explained by the presence of heteroplasmic mtDNA. There is no evidence in animals for solely paternal inheritance of mitochondria, although paternal leakage can occur rarely (e.g., humans: Bromham et al. 2003; fruit flies: Satta et al. 1988; Kondo et al. 1990; mice: Gyllenstein et al. 1991). However, double uniparental inheritance (DUI) has been detected in two bivalve families: Unionidae (Liu et al. 1996) and Mytilidae (Zouros et al. 1992). In these groups, the male gametic tissue retains mitochondria from both sperm and egg, whereas male somatic tissue and females retain mitochondria only from the egg (Fisher and Skibinski 1990). Male and female mitochondrial sequences can become quite divergent (Mizi et al. 2005). Both DUI and paternal leakage is typically detected by PCR amplification of more than one divergent sequence within an individual (Fisher and Skibinski 1990; Zouros et al. 1992). We ran multiple PCR amplifications of the gonadal tissue of a form B clam with form A mitochondrial sequence, and found that this

discordant sequence was the only allele amplified (similar results in Stepien et al. 1999). Thus, DUI or paternal leakage leading to heteroplasmic mtDNA is unlikely to be the source of nuclear and mitochondrial discordance in North American *Corbicula*.

Retention of mitochondrial alleles from a common polymorphic ancestral population seems unlikely given our phylogenetic analysis (Fig. 5). The two mitochondrial lineages found in form A (haplotype H1) and form B (haplotype H2) are distinct lineages, separated by unique mitochondrial sequences found in sexual species and other androgenetic taxa (Fig. 5). In addition, any ancestral polymorphism would have to be retained through many bottleneck events, often likely involving single individuals, as these introduced species invaded drainages across North America. This makes retained polymorphism an extremely unlikely explanation.

Ongoing egg parasitism (the genetic capture of eggs through androgenesis) between sympatric North American *Corbicula* species could explain the observed mitochondrial discordance (Lee et al. 2005, Table 3). Our results suggest that egg parasitism can occur spontaneously between species in *Corbicula*. This ability to parasitize the eggs of another species may result in more than just the capture of mitochondrial genomes. Within a population of conspecifics, egg parasitism can cause rare partial nuclear genome capture: incomplete extrusion of the maternal genome after penetration of nonreductional, androgenetic sperm has been observed in laboratory populations of *C. leana* and *C. fluminea*, causing a rise in ploidy level (Komaru et al. 2001, 2006). If this were to happen between an androgenetic clam and one of its close sexual or androgenetic relatives, chromosomes from both would contribute to offspring, which would in turn carry alleles for androgenesis and potentially could reproduce clonally. This type of nuclear genome capture between species could explain our observed data in form B clams. An androgenetic ancestor of form A could have combined genomes through egg parasitism with another androgenetic or a sexual species, such that the resulting form B contained nuclear chromosomes from multiple species, but the mitochondrial DNA of the second ancestor. If form B is indeed triploid, as the preliminary karyotypic data suggest, then the homologous chromosomes of the two ancestral species may have recombined but retained some duplications (consistent with our rDNA data). Given that forms A and B occur in both Asia as well as in introduced populations in North America, the origin of these species must predate the introduction of forms A and B to the New World.

McLeod (1986) observed extremely low average heterozygosity (frequency of 0.0025) in a population of form B that was sympatric with form A, despite a relatively high proportion of polymorphic loci in the population (0.227). At that time, self-fertilization was considered to be an explanation for the low levels of heterozygosity. Observed low heterozygosity can be an

indication of high selfing rates in sexually reproducing taxa. In the case of androgenetic *Corbicula*, chromosomes from the sperm replace those of the egg, so selfing in the usual sense (sexual recombination of two genomes of the same individual) does not occur. However, this genetic pattern would also be observed if nonreductional sperm were generated by premeiotic doubling of chromosomes prior to spermatozoa formation (analogous to the mechanism proposed for oocytes, e.g., Haccou and Schneider 2004). Meiosis and recombination proceed after doubling, so gametes retain the same number of chromosomes as the somatic cell, but alleles have been shuffled due to recombination and segregation. Sperm production following premeiotic doubling would decrease heterozygosity at individual loci: random assortment of alleles in a heterozygous father would produce both homozygous and heterozygous spermatozoa, but a homozygote father would only produce homozygotes (similar to automixis in parthenogens; Maynard Smith 1978). Over time, heterozygosity is lost, but the population may retain multiple alleles per locus until they are lost through drift or selection. We interpret the increased proportion of polymorphic loci in sympatry (but with very low levels of individual heterozygosity) to be a likely reflection of rare capture of portions of the maternal genome through recombination of the two genomes before the maternal genome is extruded from the eggs.

McLeod's (1986) data and our observations are both compatible with this explanation. If nuclear recombination rarely occurs between maternal and paternal genomes prior to maternal chromosome ejection, or after maternal genome capture, novel genes could be incorporated into the genome, increasing population polymorphism at some nuclear loci. In the case of forms A and B, this must involve only small portions of the maternal genome, rather than the complete genome, because many loci remain diagnostically distinct between the two species (Hillis and Patton 1982; McLeod 1986; this study). If premeiotic doubling occurs in spermatogenesis, homozygosity would increase over time at polymorphic loci. In the case of rDNA arrays, however, multiple distinct arrays appear to be maintained in form B *Corbicula*, and we suggest that Type I and Type II rDNA arrays (Fig. 2) originated in separate evolutionary lineages. Unfortunately, the actual mechanism of spermatogenesis in *Corbicula* is not known to date, but the other two potential mechanisms of nonreductional sperm production—abortive cytokinesis of meiosis (Komaru et al. 1997) or apomictic sperm production (analogous to apomictic egg production, Maynard Smith 1978)—would not explain the observed data.

THE ORIGIN OF ANDROGENESIS

If rare genome capture occurs between species in *Corbicula*, our mitochondrial phylogeny may not be an accurate reflection of

organismal history. There are at least four species of *Corbicula* identified as androgenetic. These androgenetic lineages are unexpectedly polyphyletic with respect to sexually reproducing species on our mitochondrial phylogeny (Fig. 5), with Bayesian posterior probabilities suggesting as many as three or four separate clades (Table 4). However, because the mitochondrial phylogeny tracks the maternal lineage alone, what appears to be multiple origins of androgenesis may be a consequence of using mitochondrial data to create the phylogeny. There may be only one, relatively recent origin of androgenesis, followed by egg parasitism and resulting mitochondrial genome capture of related sexual species. To unequivocally distinguish between this and potential alternative explanations for multiple androgenetic lineages of *Corbicula*, nuclear data from multiple genes across the global distribution of *Corbicula* will be necessary. In particular, if androgenesis has a single origin, followed by egg parasitism and resulting mitochondrial genome capture from multiple species, all androgenetic clams should share a common set of nuclear chromosomes. Phylogenetic analysis of nuclear sequence data should therefore show a clade of closely related alleles found in all androgenetic clams. If nuclear genome capture between species occurs (as we have suggested for form B *Corbicula*) there may be additional distinct nuclear alleles in different androgenetic species, and the topology of these alleles would match that of the mitochondrial, or maternal, topology. We believe that this is the explanation for the two sets of highly divergent rDNA arrays in form B *Corbicula*.

We would recommend using single-copy genes to create a nuclear gene phylogeny of *Corbicula*. The rRNA genes used for this study are relatively well conserved across eukaryotes; this and their high copy number within a genome makes them easy to amplify, clone, and identify (Hillis and Dixon 1991), even in organisms such as *Corbicula* for which little sequencing has been done. Here we have explored rDNA variability to examine possible recent genetic interactions between species. Nonetheless, we recognize that concerted evolution can cause the reduction of historical signal over time. Because concerted evolution occurs even in nonrecombining asexuals (e.g., Hillis et al. 1991), the signature of hybrid origins or historic introgression can become lost when ancestral polymorphism becomes homogenized. In addition, true allelic diversity in this large gene family cannot easily be distinguished from PCR artifacts, and therefore obscures whether allelic diversity has been fully captured by a given sequencing effort. Single-copy genes, however, are less likely to be affected by concerted evolution, and all alleles can be identified if ploidy is known. Although incomplete lineage sorting (Maddison 1997) and recombination between alleles (Posada et al. 2002) could still affect our ability to accurately infer phylogeny, the use of multiple single-copy genes could help decipher the origins of androgenesis in *Corbicula* in future studies.

MAINTENANCE OF ANDROGENESIS

The potential for egg parasitism implies there may be an advantage not only to androgenetic over sexually reproducing members within a population (as discussed in McKone and Halpern 2003), but that androgenetic species may additionally benefit by being able to steal eggs from other, closely related species. Androgenetic alleles have been likened to selfish genetic elements such as sex chromosomes with meiotic drive and cytoplasmic male sterility (McKone and Halpern 2003), all of which can spread within a population at the expense of the fitness of the species as a whole (McKone and Halpern 2003). However, we propose that androgenetic alleles in *Corbicula* cannot only be viewed as selfish genetic elements, but as elements that have potentially reduced the probability of extinction for these asexual lineages.

Androgenesis is expected to lead to selection for reduced female function in hermaphrodites, which in turn decreases overall population fitness and can lead to extinction (McKone and Halpern 2003). Androgenetic *Corbicula* have a high rate of selfing (Kraemer 1978), and frequent selfing can by itself lessen selection pressure for reduced female function (McKone and Halpern 2003). We propose an additional mechanism essential to the future maintenance of androgenesis within the genus *Corbicula*: the ability of androgenetic sperm to parasitize the oocytes of closely related species. In addition to the direct reproductive benefits of egg capture, androgenesis could benefit from infrequent chromosomal rescue, when deleterious mutations are masked by rare nuclear genome capture, polyploidization, and/or recombination with the maternal genome. This would allow usually clonal androgenetic species to avoid the deleterious effects of Muller's Ratchet (the accumulation of deleterious alleles due to the lack of genetic recombination; Muller 1964; Felsenstein 1974). As has been suggested for other asexual systems, such as hybridogenetic fish (Vrijenhoek 1994), ostracods (Butlin et al. 1998), water fleas (Paland et al. 2005), and unisexual salamanders (Mable 2007), androgenesis could continue to persist in *Corbicula* due to rare genetic recombination. In the case of *Corbicula*, this would occur after egg parasitism of another lineage, with or without polyploidization due to incomplete maternal genome extrusion. The consequences of egg parasitism must be incorporated into future population and simulation studies to fully understand how these androgenetic alleles spread and are maintained in natural populations.

TAXONOMY OF NORTH AMERICAN CORBICULA

The morphological, allozymic, restriction site, and sequence data all support the presence of two distinct species of *Corbicula* in North America. Although sympatric populations of these two species do not appear to exchange nuclear genes on a regular basis, mitochondrial genomes of these species are sometimes exchanged through androgenetic parasitism of eggs. However, this occasional capture of interspecific mitochondrial genomes does

not affect the distinctiveness of either species in any of their other characteristics. In addition, rare nuclear genome captures may increase genetic diversity in one or both species, but do not result in the regular exchange of genes. Given that these two species are distinct morphologically (Hillis and Patton 1982), genetically (Hillis and Patton 1982; McLeod 1986; Siripattawan et al. 2000; Lee et al. 2005; this study), and ecologically (Britton and Morton 1986), and are not even each other's closest relatives, the current practice of referring to both form A and form B in North America as *C. fluminea* is not supported. Populations of form A appear to be derived from populations of *C. leana* in Japan, whereas populations of form B appear to have been derived from mainland Asia (Korea and/or China), where the name *C. fluminea* is applicable (Fig. 5). Therefore, we recommend that these two names be used in reference to the two species in North America, pending a thorough systematic review of Asian *Corbicula*.

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