J. N. Am. Benthol. Soc., 2006, 25(2):417–429

Taxonomy and genetics of the parthenogenetic mayfly *Centroptilum triangulifer* and its sexual sister *Centroptilum alamance* (Ephemeroptera:Baetidae)

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Abstract. Allozymes were used to examine population genetic structure and species boundaries in the clonal, obligately parthenogenetic *Centroptilum triangulifer* and its sexual sister species *Centroptilum alamance* from 3 sites in Pennsylvania and 1 site in North Carolina, USA. Genotype frequencies in field populations of *C. triangulifer* showed numerous departures from Hardy–Weinberg expectations (mostly heterozygote excesses) and significant linkage disequilibrium at most testable locus combinations, as expected for a clonal parthenogen. A total of 51 distinct clones of *C. triangulifer* was identified, some of which were found at multiple sites (separated by >700 km in one case). Each stream contained from 7 to 25 clones and clonal frequencies at a given site varied greatly over time. Allelic patterns in laboratory hybrids suggest that parthenogenesis in *C. triangulifer* is diploid and automictic, and that crossing over is suppressed. In contrast, *C. alamance* populations had only a few Hardy–Weinberg departures (all heterzygote deficiences) and little or no linkage disequilibrium. No published work provides characters to distinguish the 2 species, but our data support retention of specific status for *C. triangulifer* and *C. alamance* because: 1) consistent, fixed allelic differences were found over a broad geographic area (>700 km) with no evidence of introgression despite co-occurrence (sympatry) in the same stream, 2) laboratory hybridization experiments demonstrated the existence of postzygotic barriers to gene flow between the species, and 3) measures of both interspecific and intraspecific genetic similarity were comparable to those found between other mayfly species. Phylogenetic analysis of the genetic data suggests speciation preceded the transition to obligate parthenogenetic reproduction in *C. triangulifer*. Morphological examination of genetically identified specimens enabled us to describe subtle but consistent differences that can be used to distinguish the species. Thus, what initially appeared to be single populations with skewed sex ratios (7, 11, and 24% male) at 3 of our study sites was, in fact, 2 populations at each site—1 exclusively female (*C. triangulifer*) and the other a normal, sexual (1:1 sex ratio) population (*C. alamance*). A similar approach, combining detailed genetic and breeding experiments with close morphological study, could help resolve taxonomic problems in some of the numerous other bisexual/parthenogenetic mayfly taxa.

Key words: aquatic insect, parthenogenesis, clonal diversity, allozyme, hybridization, species concept.

Centroptilum triangulifer (McDunnough) is a parthenogenetic mayfly commonly found in slow-flow areas of small- to medium-sized streams throughout eastern North America (Gibbs 1973, 1977). It has a polyvoltine life cycle, and generation time is strongly dependent on ambient conditions of temperature and food (Sweeney and Vannote 1984). It is an ideal laboratory bioassay animal because of its ease of culture and moderate-to-high pollution sensitivity (Sweeney et al. 1993). The species is known only from females and is *thelytokous* (a form of parthenogenesis whereby virgin females lay eggs that hatch into females) and clonal (Sweeney et al. 1993).

The present investigation was initiated following the sudden appearance in 1996 of males at a locality (Chillisquaque Creek, Pennsylvania) where we had previously found only female *C. triangulifer* (the apparent male:female ratio was 1:9). Preliminary genetic analysis (using allozymes) suggested that this collection, rather than representing a single population with a female-biased sex ratio, was in fact 2 populations, 1 parthenogenetic and all female, and the other a sexually reproducing population with a 1:1 sex ratio. We were able to distinguish the parthenogens from the sexuals only by using genetic markers, but it seemed likely that the sexual population represented what

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Traver (1932) described as *Centroptilum alamance* (from males and females collected in North Carolina). No published work provides morphological characters to separate *C. alamance* and *C. triangulifer*, so specific identity can be inferred only by their sexuality.

Several North American mayfly species are known from both sexual and parthenogenetic populations (e.g., *Acerpenna macdunnoughi*, *Diphetor hageni*, *Eurylophella funeralis*). It seemed the nominal *Centroptilum triangulifer* and *C. alamance* might represent a similar situation (i.e., a single species that exists in both sexual and parthenogenetic forms) because of their apparent morphological indistinguishability. We decided a closer study was warranted to resolve that taxonomic question, as well as to investigate differences in mode of reproduction and their significance to the biology of these populations.

The study was widened geographically to include populations from several localities in Pennsylvania, as the well as from the type locality of *C. alamance* in North Carolina. We used allozymes to characterize levels and patterns of genetic variability in both *C. triangulifer* and *C. alamance*, look for evidence of recent gene flow between them, and examine the phylogenetic relationship between the 2 species. We used hybridization experiments to test for postzygotic barriers to gene flow and provide insights into the mode of parthenogenesis in *C. triangulifer*. We evaluated the validity of maintaining *C. triangulifer* and *C. alamance* as distinct species within the framework of commonly recognized species concepts based on evidence of recent gene flow, the potential for hybridization, and demographic patterns of genetic variability. Last, we used genetically identified specimens to seek reliable morphological characters for distinguishing larvae and adults of the 2 species.

Historical perspective: taxonomy and parthenogenesis

Centroptilum triangulifer was described originally (as *Cloeon triangulifer*) by McDunnough (1931) from adult females collected in Ontario and Quebec. *Centroptilum alamance* was described (as *Neocloeon alamance*) by Traver (1932) from adult males and females and larvae from North Carolina. Ide (1937) described the larva of *C. triangulifer* from Ontario and noted its similarity to *C. alamance*. Both McDunnough (1931) and Ide (1937) noted the absence of males in *C. triangulifer*. Edmunds et al. (1976) included both species in *Cloeon* and McCafferty and Waltz (1990) transferred them to *Centroptilum*. The 2 species, along with *Centroptilum minor* (McDunnough), are distinct from other North American *Centroptilum* by their lack of hindwings. No formal action has yet been taken, but the high degree of morphological similarity between *C. triangulifer* and *C. alamance* has led some mayfly taxonomists to suggest the possibility of synonymy.

Mayfly parthenogenesis was first proposed by Morgan (1911) for *Ameletus ludens* to explain the absence of males in New York populations. Clemens (1922) later demonstrated parthenogenesis in *A. ludens* by hatching eggs taken from a virgin female collected in the same area. Using similar techniques, Degrange (1960) tested 51 species of bisexual mayflies in France and found parthenogenetic egg hatch (ranging from <1–90% hatch rate) in 26 species, suggesting a high propensity for parthenogenetic reproduction in mayflies. Since then, mayfly parthenogenesis has been reported many times (Gillies and Knowles 1990, Gibbs and Siebenmann 1996, Harker 1997, Ball 2001, 2002; see Sweeney and Vannote 1987 for a review of earlier reports).

Mayfly parthenogenesis is generally thelytokous. Male *C. triangulifer* have not been reported, and the species is an obligate thelytokous parthenogen (Gibbs 1973, 1977, Sweeney and Vannote 1984, Sweeney et al. 1993). *Deuterotoky* (unfertilized eggs giving rise to both males and females) has been proposed to explain the phenomenon of parthenogenetic egg development in mayflies from populations that contain males (Huff and McCafferty 1974, McCafferty and Huff 1974, Bergman and Hilsenhoff 1978, Mingo 1978), but direct evidence of deuterotoky has been presented only for *Centroptilum luteolum* (Degrange 1956) and *Caenis knowlesi* (Gillies and Knowles 1990). The possibility that female-biased sex ratios represent a mixture of bisexual and thelytokous taxa has not been explored prior to our study.

Methods

Specimens for our study were collected from the eastern USA at 3 sites in Pennsylvania (MES = Meshoppen Creek, WCC = White Clay Creek, CHI = Chillisquaque Creek) and 1 site in North Carolina (ALA = Big Alamance Creek, the type locality for *C. alamance*; Table 1). Larvae were reared in the laboratory at 20°C using standard methods (Sweeney and Vannote 1984). A mixed-diatom culture, which was provided as food to larvae, was grown on acrylic plates washed continuously with a thin film of fresh stream water pumped from WCC (Sweeney and Vannote 1984, Sweeney et al. 1993).

Adults (imagos) reared in the laboratory were stored at −80°C prior to electrophoresis. Morphological voucher material (i.e., the larval exuviae and adult abdomen for a given individual) was preserved in alcohol for most specimens that were electro-

TABLE 1. Collection data for *Centroptilum* species. *C. triangulifer*: n = the number of individuals examined electrophoretically, no. of clones = unique multilocus genotypes; *C. alamance*: n = no. of individuals. n/a = not applicable.

Locality	Population code	Geographical coordinates	Years collected	*C. triangulifer*		*C. alamance*	
				n	No. of clones	n	% male
Meshoppen Creek, Pennsylvania	MES	41°49′19″N 75°50′22″W	1993–1996	24	13	2	50
Chillisquaque Creek, Pennsylvania	CHI	41°04′50″N 76°40′09″W	1987–1996	51	20	21	48
White Clay Creek, Pennsylvania	WCC	39°51′32″N 75°47′02″W	1989–2003	92	26	0	n/a
Big Alamance Creek, North Carolina	ALA	35°56′42″N 79°40′26″W	2002–2003	28	7	21	52

phoresed. All voucher specimens are archived at the Stroud Water Research Center, Avondale, Pennsylvania. Allozymes were separated using horizontal starch gel electrophoresis (Sweeney et al. 1987, 1993). Only adults were electrophoresed to assure scoring of the maximum number of allozyme loci (Funk et al. 1988). Twenty-five allozyme loci were scored for most individuals, and 19 loci were polymorphic in one or both species (18 in *C. triangulifer*, 12 in *C. alamance*). The ability to resolve differences between taxa or clones using multilocus allozyme genotypes increases significantly with the number of polymorphic loci used to characterize them (Hebert et al. 1988). Hebert et al. (1989) found the number of detectable *Daphnia* clones approached an asymptote at around 11 loci. Thus, the 18 polymophic loci used to characterize *C. triangulifer* provided high resolution for distinguishing clones. Allele frequencies, observed (H_{obs}) and expected (H_{exp}) heterozygosities, and genetic distances (Nei 1978) were calculated using BIOSYS-1 (Swofford and Selander 1981). All scorable loci (including monomorphic loci) were used in heterozygosity and genetic-distance calculations. Tests for linkage disequilibrium and conformance to Hardy–Weinberg expectations of genotype frequencies were done using GENEPOP version 3.3 (Raymond and Rousset 1995).

Hybridization experiments between *C. triangulifer* and *C. alamance* involved inducing mating in the laboratory using the technique described by Huff and McCafferty (1974). Small museum jars (Wheaton 30-mL glass with snap cap) filled halfway with filtered (0.45-μm pore), sterilized stream water from WCC were used for incubation of eggs. Oviposition was induced by placing the adult female onto the water surface of a jar.

The presence of viable sperm in males was tested by using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain and epifluorescent illumination while viewing prepared tissue under a Zeiss Universal microscope.

Results

Genetic structure of C. triangulifer

Genotype frequencies based on the analysis of females from all 4 geographic populations of *C. triangulifer* showed significant departures from Hardy–Weinberg expectations, 9 out of 10 of which represented an excess of heterozygotes. Significant linkage disequilibrium was observed for many pairwise locus combinations for all geographic populations (32% of testable combinations at MES, 61% at CHI, 62% at ALA, 71% at WCC). Progeny from 19 individual females, each representing a unique genotype, were reared in the laboratory for ≥4 generations; offspring from one female were followed through 27 generations. All progeny tested had multilocus genotypes identical to their mothers. This result confirmed that parthenogenetic reproduction for this species is clonal. The 18 polymorphic loci enabled us to distinguish 51 clones (unique multilocus genotypes) among the 195 field-collected individuals tested from 4 geographic locations. A maximum of 26 *C. triangulifer* clones was identified at one site (WCC). Eight of the 51 clones were found at >1 site; 7 clones were found at 2 sites in Pennsylvania (CHI+WCC or CHI+MES), and 1 clone was found at 2 sites in Pennsylvania (WCC+CHI) and at the North Carolina site (ALA). The presence and relative abundance of clones varied considerably over time at the 2 sites (WCC and CHI) for which samples were collected in >1 y (Fig. 1A, B, respectively). The observed heterozygosity level, or proportion of loci heterozygous, among tested clones averaged 19% (range 8–36%).

We have reared many thousands of field-collected *C. triangulifer* larvae to adults from the eastern USA over the last 20 y, but we have seen only 2 gynandromorphs (adults exhibiting various degrees of both male and female morphological structures). Both of these individuals contained female gonads. In addition, of the 197 individuals reared from field-collected specimens in the present study, only 2 appeared to be entirely male (1 from CHI and 1 from MES). Neither specimen had a multilocus genotype matching a known *C.*

TABLE 2. Allele frequences for *Centroptilum* populations. Only 2 individuals from the MES population of *C. alamance* were examined, so only presence (+) or absence (–) is shown for that population. Monomorphic loci (MDH1, MDH2, ME, aGPDH, HEX, and EST1) are not shown. *n* (in parentheses) = number of specimens. Population codes are given in Table 1.

Species:		*C. triangulifer*				*C. alamance*		
Population:		MES	CHI	WCC	ALA	MES	CHI	ALA
Locus	Allele							
ADK	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	0.88	0.88	0.89	1.00	+	0.91	1.00
	B	0.13	0.12	0.11	–	–	0.10	–
ARK	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	1.00	1.00	0.96	1.00	+	1.00	1.00
	B	–	–	0.04	–	–	–	–
SOD	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	0.96	1.00	1.00	1.00	+	1.00	1.00
	B	0.04	–	–	–	–	–	–
GPI	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	1.00	1.00	1.00	1.00	–	–	0.02
	B	–	–	–	–	+	0.10	0.98
	C	–	–	–	–	–	0.74	–
	D	–	–	–	–	+	0.17	–
PGM	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	Y	–	–	–	–	–	0.05	–
	Z	0.02	–	0.01	–	+	0.36	0.62
	A	0.83	0.86	0.84	0.59	–	0.26	0.38
	B	0.15	0.14	0.15	0.41	–	–	–
	C	–	–	–	–	+	0.33	–
MPI	*n*	(24)	(43)	(76)	(28)	(2)	(12)	(21)
	A	0.15	0.15	0.16	0.02	–	–	–
	B	0.42	0.21	0.32	0.21	+	0.21	0.05
	C	0.44	0.64	0.52	0.77	+	–	0.67
	D	–	–	–	–	+	0.79	0.21
	E	–	–	–	–	–	–	0.02
	F	–	–	–	–	–	–	0.05
TPI	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	–	0.09	0.04	–	–	–	–
	B	1.00	0.91	0.96	1.00	+	1.00	1.00
ALD	*n*	(24)	(50)	(92)	(28)	(2)	(21)	(21)
	A	1.00	0.97	0.99	1.00	+	1.00	1.00
	B	–	0.03	0.01	–	–	–	–
G6PDH	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	–	0.05	–	–	–	–	–
	B	1.00	0.95	1.00	1.00	+	1.00	1.00
ISDH1	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	0.96	1.00	1.00	1.00	+	1.00	1.00
	B	0.04	–	–	–	–	–	–
ISDH2	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	0.88	0.82	0.90	1.00	+	1.00	1.00
	B	0.13	0.14	0.10	–	–	–	–
	C	–	0.04	–	–	–	–	–
G3PDH	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	Y	–	–	–	–	–	–	0.02
	Z	–	–	–	–	+	1.00	0.98
	A	1.00	1.00	1.00	1.00	–	–	–
GDA	*n*	(24)	(51)	(92)	(27)	(2)	(20)	(21)
	X	–	–	–	–	+	0.35	0.07
	Y	0.06	–	–	–	–	–	–
	Z	–	–	0.09	0.28	+	0.65	0.91
	A	0.40	0.55	0.41	0.50	–	–	0.02
	B	0.15	0.20	0.35	0.22	–	–	–
	C	0.33	0.26	0.14	–	–	–	–
	D	0.06	–	0.02	–	–	–	–

TABLE 2. Continued.

Species:		*C. triangulifer*				*C. alamance*		
Population:		MES	CHI	WCC	ALA	MES	CHI	ALA
Locus	Allele							
PRO	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	Z	–	–	–	–	–	0.10	0.14
	A	0.04	–	0.02	–	+	0.88	0.86
	B	0.85	0.99	0.98	0.98	–	0.02	–
	C	0.10	0.01	0.01	0.02	–	–	–
TRI	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	Z	–	–	–	–	+	–	0.17
	A	0.21	−0.06	0.13	–	+	0.55	0.74
	B	0.38	0.35	0.46	0.70	–	0.45	0.10
	C	0.21	0.16	0.17	0.07	–	–	–
	D	0.21	0.43	0.24	0.23	–	–	–
	E	–	–	–	–	+	–	–
DIP1	*n*	(24)	(51)	(92)	(27)	(2)	(21)	(21)
	A	0.15	0.18	0.15	0.04	–	0.12	0.10
	B	0.48	0.58	0.61	0.76	+	0.86	0.67
	C	0.25	0.08	0.19	0.20	–	0.02	0.19
	D	0.13	0.17	0.05	–	+	–	0.02
	E	–	–	–	–	–	–	0.02
DIP2	*n*	(24)	(50)	(92)	(28)	(2)	(21)	(21)
	Y	–	–	–	–	–	–	0.05
	Z	–	–	–	–	–	0.76	0.83
	A	0.02	0.09	–	0.07	+	0.24	0.12
	B	0.69	0.58	0.47	0.73	–	–	–
	C	0.29	0.33	0.52	0.20	–	–	–
	D	–	–	0.01	–	–	–	–
AAT1	*n*	(24)	(40)	(92)	(26)	(2)	(7)	(17)
	Z	–	–	–	–	–	–	0.15
	A	1.00	0.84	1.00	1.00	+	0.43	0.79
	B	–	0.16	–	–	–	0.57	0.06
AAT2	*n*	(24)	(51)	(92)	(28)	(2)	(21)	(21)
	A	0.13	0.09	0.06	–	–	0.10	0.19
	B	0.88	0.91	0.94	1.00	+	0.91	0.81

triangulifer clone, but allozyme genotypes contained only alleles commonly found in *C. triangulifer* and, therefore, did not indicate a hybrid origin (see below). Thus, these 2 males were electrophoretically distinct, but morphologically indistinguishable, from *C. alamance*. It may be that these 2 males represent extreme cases of gynandromorphism. Neither was used in a mating experiment or dissected for the presence of sperm, so their fertility is unknown.

Genetic structure of C. alamance *and comparison with* C. triangulifer

Forty-four adult *C. alamance* were reared from 3 sites for genetic analysis. Sex ratios were ~1:1 at all sites (Table 1). Expected heterozygosity (H_{exp}) was 14% ($H_{obs} = 14\%$) at site ALA, 15% ($H_{obs} = 16\%$) at CHI, and 16% ($H_{obs} = 15\%$) at MES. In contrast to *C. triangulifer*, departures from Hardy–Weinberg expectations for *C. alamance* were observed at only 2 loci (MPI and TRI) at site ALA and 1 locus (MPI) at site CHI, and these were all heterozygote deficiencies rather than excesses. Linkage disequilibrium was significant for only 3.7% (2 of 54) of the testable locus combinations at ALA and none at CHI.

Alleles common in *C. triangulifer* were absent or rare in *C. alamance* at 7 loci (GPI, MPI, G3PDH, GDA, PRO, TRI, and DIP2; Table 2). *Centroptilum triangulifer* and *C. alamance* exhibited fixed allelic differences at 1 locus (G3PDH) and nearly fixed differences at 4 others (GDA, GPI, PRO and DIP2; Table 2). All individuals could be unambiguously assigned to one or the other species by their multilocus genotypes. Nei's genetic distance averaged 0.097 (range 0.001–0.205; Fig. 2) among *C. triangulifer* clones, and 0.079 (range 0.072–0.090) among *C. alamance* populations. The genetic distance between *C. triangulifer* clones and *C. alamance* populations averaged 0.296 (range 0.229–0.357).

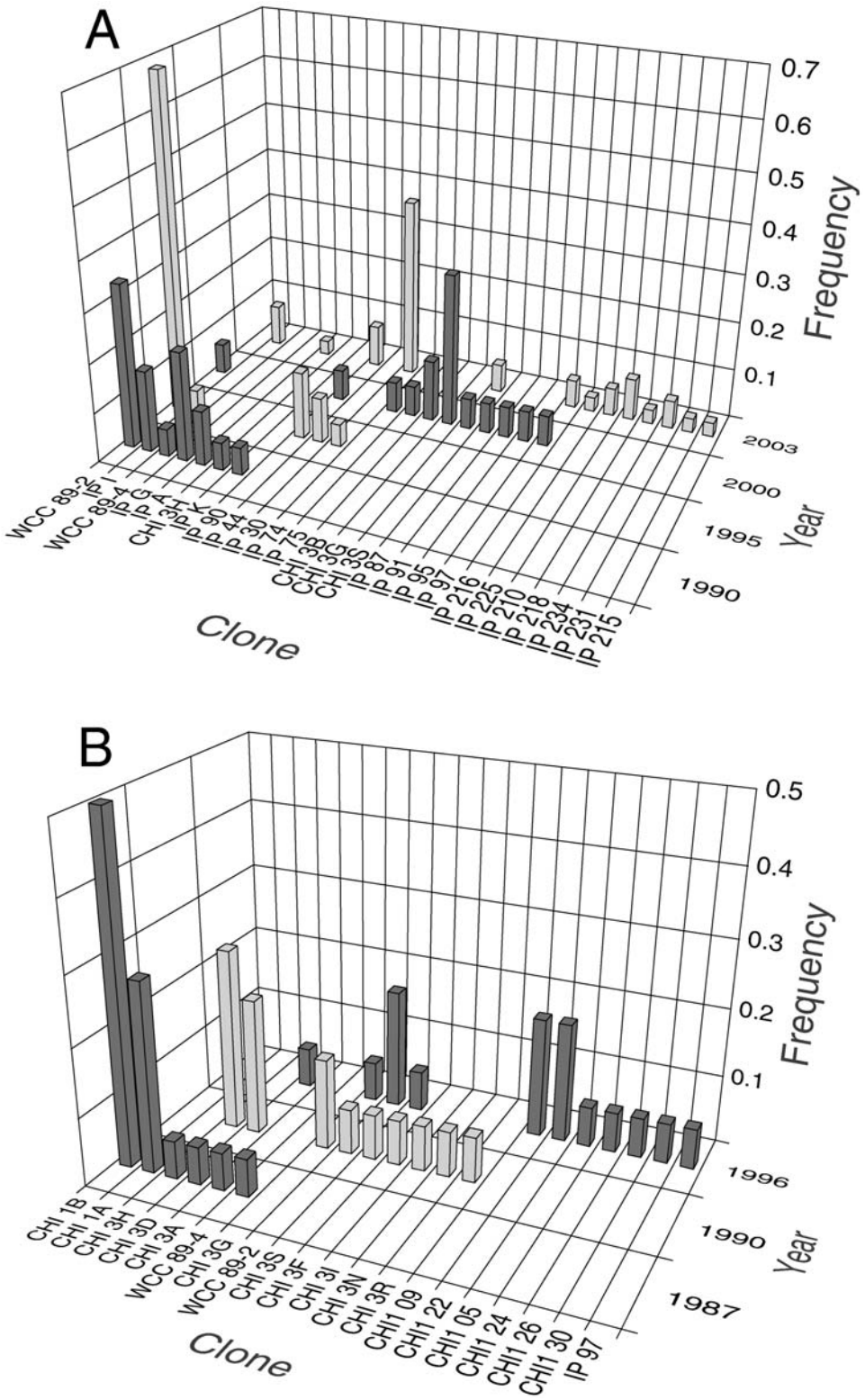

FIG. 1. Relative frequency of *Centroptilum triangulifer* clones found at sites WCC (A) and CHI (B) over several years of collection. Entries on the Clone axes are authors' inventory codes. Color of bars alternates to distinguish between years.

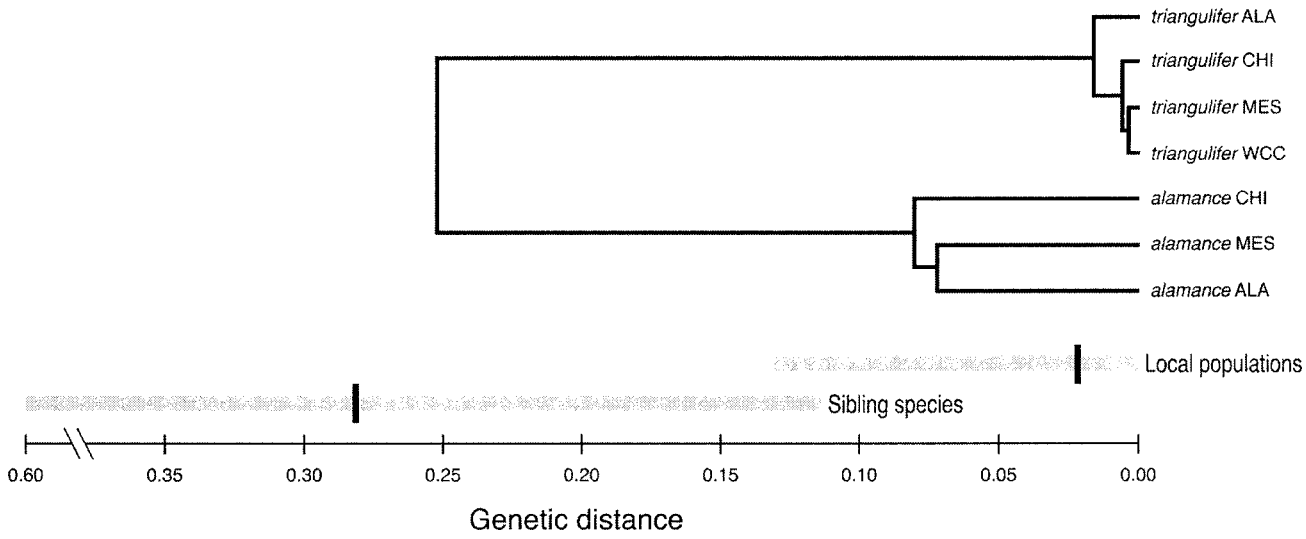

FIG. 2. Phenogram (Unweighted Pair-Group Method using Arithmetic Averages [UPGMA]) of 7 *Centroptilum* populations generated using Nei's (1978) genetic distance. Horizontal gray and vertical black bars represent the range and mean values, respectively, observed among local populations and sibling species of mayflies (from Funk and Sweeney 1990).

Mating and hybridization experiments

Copulation between male and female *C. alamance* was induced repeatedly in the laboratory, and eggs from 16 mated females were reared to the imago. Male progeny were present in 9 of the 16 clutches, and 4 males and 4 females were electrophoresed from each of the clutches that contained male progeny. For these 9 clutches, the presence of male progeny, as well as the presence of paternal alleles indicated successful fertilization and confirmed a Mendelian inheritance pattern. Progeny from the other 7 mothers were all female, indicating parthenogenetic development, presumably because of unsuccessful sperm transfer or fertilization. Unlike *C. triangulifer*, parthenogenesis in these females was not clonal (DHF, unpublished data).

Mating was induced between male *C. alamance* and 6 female *C. triangulifer*. Eggs from 1 of these mated females never hatched. Those from another mated female hatched, but none survived to adulthood. Two hundred forty-five adult progeny were reared from eggs taken from the remaining 4 mated females. Fifty-one adults (from 2 of the mothers) were analyzed electrophoretically. Thirty-four of these progeny had multilocus allozyme genotypes identical to their *C. triangulifer* mothers (i.e., were clones). Eggs laid by these females developed normally. In addition, 2 types of hybrids were detected among the 17 remaining electrophoresed progeny.

The 1st type of hybrid (full hybrid) was evident in 8 individuals that had expected hybrid allozyme genotypes at all 11 of the loci at which the parents differed. No evidence of polyploidy was observed. In particular, at locus TRI, for which the mother in 1 cross was heterozygous (genotype BC) and the father was homozygous (genotype AA), 2 genotypes were observed in the offspring, AB and AC, indicating hybrids were diploid and inheritance was Mendelian. It is noteworthy that all but one of the full hybrids were gynandromorphs, with varying degrees of maleness. Externally, all these individuals appeared to have male reproductive anatomy, but each had at least some visible female characteristics such as female-type maculae (see below) on one to several abdominal sternites. Only one full hybrid male exhibited copulatory behavior with a *C. alamance* female during laboratory mating experiments. Eggs from this female failed to hatch. DAPI stain failed to find sperm in male tissue dissected from 5 of the full hybrids with the most masculine appearance. Three of the full hybrids (2 gynandromorphs and 1 apparent female) contained eggs, but none oviposited when placed on water. Eggs were dissected from each of these individuals and incubated at 20°C for 90 d (typically hatch time for *C. triangulifer* is 9 to 15 d at this temperature; Sweeney and Vannote 1984). No eggs hatched or showed any sign of development. Thus, full hybrids appeared to be sterile.

The 2nd type of hybrid (partial hybrid) was evident in 9 individuals that had allozyme phenotypes indicating biparental inheritance at 7 loci, but only maternal alleles at 3 others (GPI, G3PDH, and TRI). All partial hybrids appeared to be female (although one had male forceps), and all oviposited when placed on water. Eggs from 3 of these females hatched (albeit at a much lower rate than their nonhybrid sisters), and

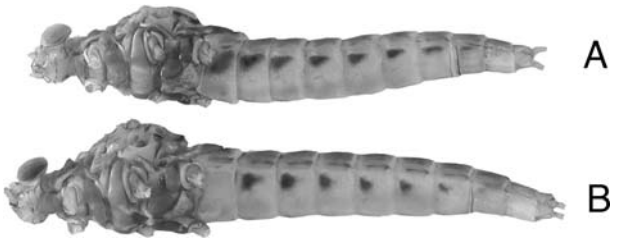

FIG. 3. Oblique ventral view of female imago of *Centroptilum triangulifer* (A) and *C. alamance* (B) illustrating differences in sternal maculation (see text for details). Legs, wings, and cerci removed.

rearing experiments and genetic tests with these progeny are ongoing.

Morphological differences between C. triangulifer *and* C. alamance

Distinguishing morphological characters were identified by examining voucher material from specimens whose identity was confirmed using electrophoresis. Adult *C. triangulifer* females have distinctive and well-defined, triangular-shaped, dark brown maculae on sternites 1 to 7 (absent in males) for which McDunnough (1931) named them (Fig. 3). On sternite 1, the macula is quite large and nearly quadrate. On the remaining segments these markings are in the shape of a right triangle. One side of the dark triangle, along the pleural edge of the sternite, extends nearly the length of the segment while the other side of the triangle extends mediad along the anterior margin of the sternite, and the hypotenuse is on the diagonal. On the 2nd segment, the sides of the dark triangle opposite the hypotenuse are subequal, but the side extending medially along the anterior margin of the sternite diminishes in length on posterior segments. In contrast, *C. alamance* females lack the triangular maculae on sternite 1. Sternites 2 to 7 have maculae, but these are in the form of a more diffuse cloud (rather than a distinct triangle) that is most intense and distinct near the anterolateral corner of the sternite. The size of the dark maculation diminishes on posterior segments, and the maculation does not extend the entire length of the segment along the lateral margin as it does in *C. triangulifer* (Fig. 3). Adult male *C. alamance* can be distinguished from all other North American *Centroptilum* species (except for *C. minor*) by their lack of hindwings and the presence of a large, truncate mesal process on the inner margin of the basal segment of the genital forceps (Traver 1932, 1935; Fig. 4), and from *C. minor* by their larger size, lack of paired submedian black dashes on sternites 2 to 6, and the shape of upper, turbinate portion of their

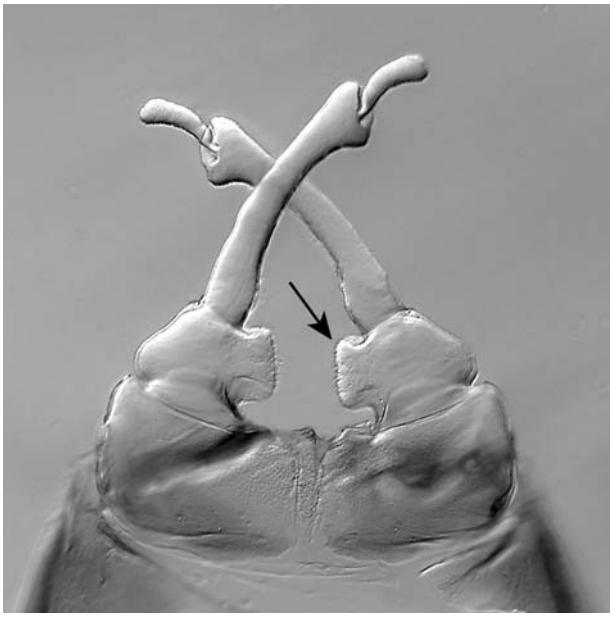

FIG. 4. Ventral view of male imago of *Centroptilum alamance* showing genital forceps (10th segment removed). Arrow indicates truncated mesal process on the basal segment of the forceps.

eyes. The 2 male *C. triangulifer* collected during the course of our study (see above) were indistinguishable from *C. alamance*. However, the rarity of male *C. triangulifer* makes our inability to distinguish them morphologically from male *C. alamance* unlikely to present a taxonomic problem. Most male *C. triangulifer* encountered actually represent gynandromorphs with varying degrees of masculinity, and as such, cannot be adequately accounted for in a taxonomic treatment.

In the larval stage, *C. triangulifer* have a distinct dark band on each femur at ~⅓ from the base (Fig. 5A). This band is missing or only faintly indicated in *C. alamance*. Gills on *C. triangulifer* have a white band ~⅔ distance from base, dividing the otherwise dark brown trachaeae (Fig. 5B). Gill trachaeation in *C. alamance* is dark brown throughout except for a small area at the base of the first 2 or 3 branches (Fig. 5C). Tails in both species have a speckled appearance, with a narrow dark band at the terminal margin of every 4th segment in the middle region. In addition, *C. triangulifer* larvae have narrow white bands midway between each dark band. These white bands are also present in *C. alamance* but are faint and difficult to see, and the 2 species are easily distinguished in the field using this character. The white bands disappear in preserved material, so this character is only useful for living or recently preserved material. Some differences in maculation of the larval tergites were observed, but were too variable to be useful taxonomically.

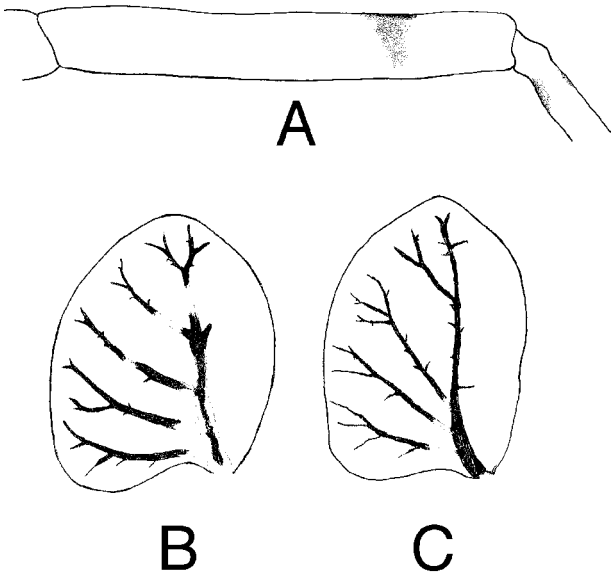

FIG. 5. A.—*Centroptilum triangulifer* femur. B.—*C. triangulifer* gill 4. C.—*C. alamance* gill 4.

Discussion

Population genetics, taxonomic status, and phylogenetic implications

Recent gene flow clearly has not occurred between natural populations of *C. triangulifer* and *C. alamance* given that several loci exhibited fixed or nearly fixed differences in allelic expression and the genetic distance measured between the taxa was large. Differences in sex ratio, heterozygote deficiency, and linkage disequilibrium appear to reflect life-history differences (i.e., parthenogenetic vs bisexual reproduction). *Centroptilium triangulifer* exhibited high levels of observed heterozygosity (in excess of expected levels at 9 of 10 polymorphic loci) and significant linkage disequilibrium, all of which are consistent with past studies of obligate parthenogens having clonal inheritance (Suomalainen et al. 1987). *Centroptilum alamance* also exhibited high levels of observed heterozygosity, but with little or no linkage disequilibrium or departures from Hardy–Weinberg expectations. The few departures from Hardy–Weinberg expectations were all heterozygote deficiencies rather than excesses. These patterns, and the 1:1 sex ratios observed in the field, suggest that little or no persistent parthenogenetic reproduction occurs in wild *C. alamance* populations. Heterozygosity levels in *C. triangulifer* and *C. alamance*, while typical of other baetid mayflies (e.g., Robinson et al. 1992, Monaghan et al. 2001, DHF and BWS, unpublished data), are somewhat higher than most nonbaetid mayfly species studied to date (Saura et al. 1979, Sweeney et al. 1986, 1987, Sweeney and Vannote 1987, Funk et al. 1988, Sweeney and Funk 1991, Studemann et al. 1994, Scillitani et al. 1996).

The question of what constitutes a species in the case of an obligate parthenogen has been the subject of some debate (Suomalainen et al. 1987). Species concepts based on reproductive isolation, such as the biological species concept of Mayr (1970) or Paterson's (1985) recognition concept, have limited applicability to organisms such as *C. triangulifer* that have abandoned sex. Templeton (1989) argued that his cohesion species concept accommodates asexuals more effectively than either the isolation or recognition concept. He considered 2 classes of cohesion mechanisms: genetic exchangeablity (i.e., the factors that define the limits of spread of new genetic variants through gene flow) and demographic exchangeability (i.e., the factors that define the fundamental niche and the limits of spread of new genetic variants through genetic drift and natural selection). Genetic exchangeability is applicable only to sexually reproducing populations and is addressed in isolation species concepts. Demographic exchangeability applies to both sexual and asexual species. According to Templeton (1989), "...every individual in a demographically exchangeable population is a potential common ancestor to the entire population at some point in the future".

Laboratory-induced copulations demonstrated that interbreeding of male *C. alamance* and female *C. triangulifer* resulted in progeny with hybrid genotypes, although these individuals proved to be mostly intersexes, and all were sterile. The opportunity for such hybridization certainly exists because both species were present at 3 of the 4 sites we sampled, but none of our field-collected material exhibited hybrid genotypes. Rather, we observed fixed allelic differences with no evidence of recent gene flow, a clear indication of reproductive isolation. Thus, *C. alamance* and *C. triangulifer* can be considered species under either of the isolation concepts. Fixed allelic differences also provide evidence of a lack of genetic exchangeability under the cohesion concept. However, the mere fact that parthenogenetic *C. triangulifer* clones are genetically incompatable with sexual *C. alamance* does not necessarily indicate that the collection of parthenogenetic clones we refer to as *C. triangulifer* constitutes a distinct evolutionary lineage. If the *C. triangulifer* clones observed at various localities arose spontaneously from the respective local *C. alamance* populations and their reproductive incompatability with the latter was caused only by factors such as a change in ploidy, then what we call *C. triangulifer* would not constitute a cohesive evolutionary lineage but rather a series of local phenomena (i.e., no

evidence of demographic exchangeability), and would, therefore, not be considered a species under the cohesion concept. However, *C. triangulifer* clones were consistently distinct from *C. alamance* in the populations we sampled, distinguishable from each other by the same suite of alleles in populations separated by >700 km. In fact, 8 of our *C. triangulifer* clones were found at >1 site, with 1 found at 3 sites (WCC, CHI, and ALA). Thus, *C. triangulifer* and *C. alamance* represent 2 distinct (demographically exchangeable) lineages, each worthy of specific status under the cohesion concept.

The genetic and morphological characters observed in our study provide some insight into the evolutionary relationship between these 2 species. Alleles at 7 loci (GPI, G3PDH, MPI, GDA, PRO, TRI, and DIP2; Table 2) were common in *C. triangulifer* clones yet absent in *C. alamance*. In particular, allele A for G3PDH and GPI was fixed in *C. triangulifer* but was absent (G3PDH) or known only from a single heterozygote (GPI) in *C. alamance*. This pattern suggests that the *C. alamance* and *C. triangulifer* lineages diverged from a common ancestor, and that this divergence occurred prior to the transition to obligate parthenogenetic reproduction in *C. triangulifer*. Thus, *C. alamance* and *C. triangulifer* are sister taxa rather than having a sexual parent/parthenogenetic daughter relationship. The loss of sexuality such as has apparently occurred in *C. triangulifer* is generally considered an evolutionary dead end, as evidenced by the fact that parthenogenetic taxa, almost without exception, represent the terminal branches of phylogenetic trees (i.e., there are few or no parthenogenetic taxa above the species group). Thus, transitions to asexuality are likely to have been geologically recent, and parthenogenetic taxa may, therefore, exhibit only minor genetic and morphological divergence from their sexual parents or siblings. This recent divergence seems to be the case for *C. triangulifer* and *C. alamance*. Morphological distinction of *C. triangulifer* and *C. alamance* is based on relatively minor color differences in larvae and female imagos (Figs 3, 5). Genetic differences for *C. triangulifer* and *C. alamance*, though small, are consistent and are of similar magnitude to those observed among other closely related mayfly species (e.g., Funk et al. 1988).

Parthenogenetic mode in mayflies, and C. triangulifer *in particular*

Most reports of parthenogenesis in mayflies are based on the ability of a small proportion (generally <10%) of eggs from unmated females of normally sexual species to hatch in the laboratory. This type of parthenogenesis is often referred to as occasional or accidental and the term *tychoparthenogensis* has been proposed. Normal parthenogenesis, on the other hand, can be either obligatory (where an egg always develops parthenogenetically) or facultative (where an egg may be either fertilized or develop parthenogenetically). Obligate thelytokous (all female) parthenogenesis has been reported for only a few species of mayflies. In North America, *Ameletus ludens* Needham (Ameletidae; Clemens 1922) and *C. triangulifer* (Baetidae; Gibbs 1977) are known only from females (see 2 apparent male *C. triangulifer* noted above, and Needham [1924] reported 2 males for *A. ludens*) and, thus, are considered obligate parthenogens. In the case of *C. triangulifer*, >85% of eggs from unmated females hatch. Other North American species that appear to be obligately thelytokous include the baetids *Baetis foemina* McDunnough and *B. hudsonicus* Ide (McDunnough 1936, Ide 1937).

Some mayfly species appear to have both bisexual and parthenogenetic populations (males and females at some sites and only females at others). Examples include the baetids *Baetis bicaudatus* Dodds (Dodds 1923), *Acerpenna macdunnoughi* (Ide), and *Diphetor hageni* (Eaton) (Bergman and Hilsenhoff 1978, McCafferty and Morihara 1979) and the ephemerellid *Eurylophella funeralis* (McDunnough) (Sweeney and Vannote 1987). The term facultative parthenogenesis has sometimes been used to characterize species such as these, but this term is best restricted to instances where an individual egg can either be fertilized or develop parthenogenetically, a phenomenon that has not been documented in mayflies.

Some mayfly species have female-biased sex ratios in certain populations. In the case of *Eurylophella funeralis*, Sweeney and Vannote (1987) suggested such populations consisted of a mixture of parthenogenetic and bisexual individuals. Ball (2001) showed that some *Stenonema femoratum* females from populations with female-biased sex ratios produced mixed broods of sexually and parthenogenetically produced offspring. Samples from several of our sites appeared to have female-biased sex ratios before we were able to distinguish *C. triangulifer* from *C. alamance*. However, once the 2 species could be distinguished, it was evident that 2 species were present, 1 thelytokous and the other with a 1:1 sex ratio. Closer examination may reveal that other apparently female-biased populations of mayflies consist of a similar mix.

Many (perhaps most) obligately thelytokous insect species are *apomictic* (no meiosis occurs, and the zygoid chromosome number is maintained throughout oogenesis), and polyploidy is common (Suomalainen et al. 1987). All known tychoparthenogens, on the

other hand, are *automictic* (meiosis is preserved in some form). The early stages of meiosis typically are normal (including bivalent formation and chromosome reduction) but, instead of fertilization by sperm, the zygoid phase is restored by fusion of 2 nuclei from the same individual. This process may be accomplished by a variety of cytological mechanisms (Lamb and Willey 1987, Suomalainen et al. 1987). The genetic consequences of the various parthenogenetic mechanisms differ markedly. Apomicts are clonal; thus, heterozygosity (which is usually high, especially in polyploids) is maintained, and new variation is acquired via mutation. In contrast, most automicts are not clonal. Homozygosity is enforced in individual lineages for some types of automictic parthenogenesis (e.g., gamete duplication). In other types, some degree of heterozygosity is maintained, but may be reduced as a result of crossing over.

We have not directly observed the cytological mechanism of parthenogenesis in *C. triangulifer*, but we have seen no electrophoretic evidence of polyploidy (e.g., we observed no 3-allele genotypes or evidence of dosage disparity among alleles in heterozygotes). If *C. triangulifer* were apomictic, fertilized eggs should be at least triploid. However, we have clear evidence of diploidy from allozyme genotypes at the TRI locus of full-hybrid progeny from one heterospecific mating. Thus, we suggest that *C. triangulifer* is a diploid automictic parthenogen. Unlike most automictic parthenogens, *C. triangulifer* is clonal. In fact, no changes in multilocus genotype or losses of heterozygosity have been observed over many clonal generations (27 in one isofemale line) in the laboratory. The conservation of heterozygosity suggests that meiosis occurs without crossing over and that diploidy is restored by fusion of the central 2 ootid nuclei (Suomalainen et al. 1987).

The above conclusion assumes a single mode of (parthenogenetic) egg development in *C. triangulifer*. Alternatively, >1 mode could be occurring in the same individual (as has been reported for at least 1 *Drosophila* species; Sprackling 1960). If this were the case in *C. triangulifer*, it might be that: 1) development in the 86% of eggs that normally hatch parthenogenetically (Gibbs 1977, Sweeney and Vannote 1984) is apomictic (or that ploidy is restored by a form of automixis that preserves heterozygosity, as described above), and that either these eggs cannot be fertilized or fertilization results in (possibly lethal) polyploidy or aneuploidy; and 2) the remaining 14% of eggs that do not normally hatch parthenogenetically undergo normal meiosis (without automixis) and can, therefore, be fertilized normally. Such a situation could explain why only 16% of the progeny from our heterospecific matings exhibited fully hybrid genotypes (resulting from fertilization of eggs from group 2 above) and suggests the possibility that our partial hybrids represent aneuploids, perhaps trisomic (i.e., a diploid with an extra chromosome of one type), resulting from the fertilization of an already diploid egg (from group 1 above).

Obligate parthenogenesis appears to be relatively rare in mayflies, but parthenogenesis in its various forms may be locally common. For example, 50 species of mayflies occur in White Clay Creek, Pennsylvania, and 7 (14%) of these are known locally only from females. Two of these are obligate parthenogens (*Ameletus ludens*, *C. triangulifer*) and the other 3 represent what are believed to be local parthenogenetic populations of species that exist bisexually elsewhere (*Eurylophella funeralis*, *Ephemera varia*, *Centroptilum minor*, *Acerpenna macdunnoughi*, *Diphetor hageni*). This phenomenon obviously is of taxomonic and evolutionary interest, and it is a complication in ecological studies that compare different taxa or sites. An approach that combines detailed genetic and breeding experiments with close morphological analyses similar to our study could help resolve taxonomic problems in other bisexual/parthenogenetic mayfly taxa. The precise cytological mechanisms and genetic consequences of parthenogenesis in mayflies remain largely unexplored. Are local parthenogenetic populations part of the same gene pool as their sexual relatives? Can these parthenogens revert to sexuality in the presence of males, or has the transition to asexuality been permanent? Answers to these questions can have important taxonomic and ecological implications.

Acknowledgements

R. M. Weber and D. I. Rebuck provided invaluable assistance in the field and laboratory. N. A. Wiersema and R. D. Waltz examined specimens and provided their taxonomic expertise. Two anonymous referees provided valuable suggestions for improvement of the manuscript. This research was supported by the Stroud Foundation, the Pennswood No. 2 Research Endowment, the Stroud Endowment for Environmental Research, and National Science Foundation Grant DEB-0424681.

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Received: 28 February 2005
Accepted: 11 November 2005