doi:10.1111/j.1420-9101.2009.01813.x

Evolutionary transitions among dioecy, androdioecy and hermaphroditism in limnadiid clam shrimp (Branchiopoda: Spinicaudata)

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Keywords:

Bayesian phylogenetics;
breeding system evolution;
Conchostraca;
maximum likelihood phylogenetics;
mixed mating system.

Abstract

Examinations of breeding system transitions have primarily concentrated on the transition from hermaphroditism to dioecy, likely because of the preponderance of this transition within flowering plants. Fewer studies have considered the reverse transition: dioecy to hermaphroditism. A fruitful approach to studying this latter transition can be sought by studying clades in which transitions between dioecy and hermaphroditism have occurred multiple times. Freshwater crustaceans in the family Limnadiidae comprise dioecious, hermaphroditic and androdioecious (males + hermaphrodites) species, and thus this family represents an excellent model system for the assessment of the evolutionary transitions between these related breeding systems. Herein we report a phylogenetic assessment of breeding system transitions within the family using a total evidence comparative approach. We find that dioecy is the ancestral breeding system for the Limnadiidae and that a minimum of two independent transitions from dioecy to hermaphroditism occurred within this family, leading to (1) a Holarctic, all-hermaphrodite species, *Limnadia lenticularis* and (2) mixtures of hermaphrodites and males in the genus *Eulimnadia*. Both hermaphroditic derivatives are essentially females with only a small amount of energy allocated to male function. Within *Eulimnadia*, we find several all-hermaphrodite populations/species that have been independently derived at least twice from androdioecious progenitors within this genus. We discuss two adaptive (based on the notion of 'reproductive assurance') and one nonadaptive explanations for the derivation of all-hermaphroditism from androdioecy. We propose that *L. lenticularis* likely represents an all-hermaphrodite species that was derived from an androdioecious ancestor, much like the all-hermaphrodite populations derived from androdioecy currently observed within the *Eulimnadia*. Finally, we note that the proposed hypotheses for the dioecy to hermaphroditism transition are unable to explain the derivation of a fully functional, outcrossing hermaphroditic species from a dioecious progenitor.

Introduction

Elucidating the forces that select for a separation of the sexes (i.e. into pure males and pure females, termed dioecy) relative to a combination of the sexes (i.e. cosexuals or hermaphrodites) is imperative for understanding breeding system evolution (Charnov *et al.*, 1976; Charlesworth & Charlesworth, 1978; Charlesworth, 1984; Schemske & Lande, 1985; Jarne & Charlesworth, 1993; Barrett, 2002; Wolf & Takebayashi, 2004). A useful approach to assessing these selective forces is to study clades in which transitions among breeding

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systems have occurred repeatedly (e.g. hermaphroditism to dioecy). Because numerous transitions from hermaphroditism to dioecy are evident in flowering plants (Weiblen *et al.*, 2000; Barrett, 2002), a good deal of theory has been developed to explain the likely evolutionary progression of this transition (reviewed in Charlesworth, 2006). Direct evolution of dioecy from hermaphroditism is not predicted to occur, but rather one of two temporary breeding systems is thought to be a likely intermediate stage in this transition (Lloyd, 1975; Charlesworth & Charlesworth, 1978; Charlesworth, 1984): gynodioecy (mixtures of females and hermaphrodites) or androdioecy (mixtures of males and hermaphrodites). A gynodioecious intermediate is predicted to be more common than an androdioecious intermediate (Lloyd, 1975; Charlesworth, 1984), and indeed gynodioecy is much more common in flowering plants than is androdioecy (Charlesworth, 1984; Pannell, 2002; Delph & Wolf, 2005).

Because of the relative frequency of the transition from hermaphroditism to dioecy in flowering plants, the evolutionary steps in this transition have been predicted in some detail. Charlesworth & Charlesworth (1978) proposed a plausible genetic model for the evolution of dioecy from hermaphroditism which suggested that the most likely transition would include a gynodioecious intermediate. They proposed that a recessive male sterility gene could spread in a partially selfing hermaphroditic population experiencing moderate to high inbreeding depression, thus producing females and hermaphrodites (i.e. gynodioecy). They suggested that a second mutation of a dominant modifier that reduced female function in the hermaphrodites could then spread in the gynodioecious population. This second mutation would eventually reduce female function to zero, and thus transform the hermaphrodites into males, resulting in dioecy. The spread of this second mutation would be greatly facilitated if it was tightly linked to the first, recessive male sterility gene (Charlesworth & Charlesworth, 1978).

Although the transition from hermaphroditism to dioecy has been thoroughly explored, the reverse transition, from dioecy to hermaphroditism, has not received nearly the level of detailed attention. Ghiselin (1974) provided several verbal models ('low-density', 'size-advantage' and 'gene-dispersal') outlining possible benefits for deriving hermaphroditism from dioecy. Charnov (1982) also outlined the conditions favouring hermaphroditism over dioecy using the concept of 'fitness sets'. However, neither author presented detailed outlines for how hermaphroditism could evolve from dioecy, and the notions of intermediate stages (e.g. androdioecy or gynodioecy) were never specifically considered.

The dearth of detailed discussions about a dioecy to hermaphroditism transition is not because such transitions are believed uncommon. Hermaphroditism is quite common in animals: when one excludes insects, up to one-third of animal species are hermaphroditic (Jarne & Charlesworth, 1993; Jarne & Auld, 2006). The distribution of hermaphroditism in animals is sporadic, with some higher taxa being primarily hermaphroditic (e.g. Platyhelminthes, pulmonate molluscs) and others having few hermaphroditic representatives (e.g. Echinoderms, Chordates; Ghiselin, 1974; Bell, 1982; Jarne & Charlesworth, 1993). Ghiselin (1974) has argued that the majority of these hermaphroditic animals are derived from dioecious ancestors (for an alternative perspective, see Eppley & Jesson, 2008; Lyer & Roughgarden, 2008), and thus these numerous species in disparate animal taxa suggest numerous dioecy to hermaphroditism evolutionary transitions. Therefore, understanding the details of the transition from dioecy to hermaphroditism should be quite important to those interested in the evolution of animal breeding systems.

One group of crustaceans, the Branchiopoda, displays a wide range of breeding systems (Sassaman, 1995; Dumont & Negrea, 2002): dioecy, androdioecy, hermaphroditism, parthenogenesis (i.e. asexual) and cyclic parthenogenesis (i.e. many rounds of parthenogenesis with a single episode of dioecy at the end of a growing season), and thus presents an opportunity to study many breeding system transitions within a single taxon. Because the basal clade in the Branchiopoda, the Anostraca (Negrea *et al.*, 1999), is almost entirely dioecious, it appears that androdioecy, hermaphroditism, parthenogenesis and cyclic parthenogenesis all have evolved from dioecy (although not necessarily directly) in this group. In fact, all of these breeding systems are found in what were historically termed the 'Conchostraca' or 'clam shrimp' (the Conchostraca have been determined to be a polyphyletic group and thus it has now been split into the orders Laevicaudata and Diplostraca; Fryer, 1987; Spears & Abele, 2000; Braband *et al.*, 2002). Sassaman (1995) outlined a scheme in which androdioecy, hermaphroditism and parthenogenesis could evolve (through a series of mutational steps) from a female-heterogametic, dioecious sex determining system (which Sassaman predicted to be the ancestral condition within the clam shrimp). Sassaman (1995) additionally predicted that cyclic parthenogenesis then evolved from parthenogenesis. Because of the breeding system diversity within clam shrimp, and because of our recent advances in understanding their biology and ecology, we believe this group presents an excellent opportunity to study the evolution of various breeding systems from a presumably dioecious ancestor.

Within the clam shrimp, one family, the Limnadiidae (Spinicaudata: Diplostraca), has three of the five above mentioned breeding systems: dioecy, hermaphroditism and androdioecy (Sassaman & Weeks, 1993; Sassaman, 1995; Weeks *et al.*, 2008). The Limnadiidae contains five extant genera: *Eulimnadia*, *Imnadia*, *Metalimnadia*, *Limnadia* and *Limnadopsis* (Baird, 1849; Straskraba, 1964). Of these, *Eulimnadia* is the most speciose (containing over 40 species that inhabit every continent except Antarctica;

Brtek, 1997) and is the best studied genus from a reproductive biology perspective (reviewed in Weeks *et al.*, 2006a). In the current study, we will outline the breeding system transitions inferred from a DNA sequence/morphology-based phylogeny of the Limnadiidae. Although the ancestral breeding system for the Limnadiidae has been assumed to be dioecy (Sassaman, 1995) and a preliminary phylogeny was erected for the family (Hoeh *et al.*, 2006), no ancestral character state reconstruction has been conducted to confirm or refute Sassaman's assertion. Our analyses indicate that dioecy is indeed the ancestral state for the Limnadiidae and that both androdioecy and hermaphroditism are derived states within this family. We combine these insights on breeding system transitions with previously published information about these crustaceans to consider hypotheses regarding the processes underlying transitions from dioecy to androdioecy and hermaphroditism in the Limnadiidae.

Methods

Specimen collection/rearing

We examined 173 individuals from 42 species/lineages, 10 genera and three families; these samples were collected from six continents (Table 1). Specimens were either adults preserved in 95% ethyl alcohol or were reared from eggs in the laboratory. Samples were either collected by us or sent to us by colleagues. For each of the populations that were reared from eggs, we collected soil from natural, dried field sites. We made soil collections by sampling at many spots across the dried pools and then homogenizing the soil in plastic bags. Approximately 500 mL of this field-collected soil was placed in the bottom of a 37-L aquarium and hydrated with deionized water. The aquarium was maintained under 'standard conditions' (Weeks *et al.*, 1997, 1999, 2001) of 25–28 °C, low aeration, constant light, and fed a mixture of baker's yeast and ground Tetramin™ flake fish food (Tetra Werke, Melle, Germany) (2.5 g of each suspended in 500 mL of water). Shrimp were reared to sexual maturity (based on the presence of eggs in the brood chamber for females/hermaphrodites and presence of claspers in males) and then preserved in 95% ethanol or frozen in a −80 °C freezer for morphological and molecular analyses, respectively.

Morphological analyses

The ethanol-fixed specimens were examined using a Wild M8 dissection stereomicroscope. To separate males from females/hermaphrodites, each specimen was examined for presence of eggs and elongated epipodites (females/hermaphrodites) or claspers (males). Because there are no recent keys for this family, species diagnostic characters were identified using descriptions from peer reviewed scientific literature, original descriptions, older keys and direct comparisons with previously identified material in public and private collections. Characters/character states were defined, scored and included in the phylogenetic analyses and their specifics are presented in Appendix S1.

Breeding system assignment

Breeding system determinations for 47 of the 54 limnadiid clam shrimp populations were identified in a recent study by Weeks *et al.* (2008). Breeding system determinations for four of the remaining seven populations were inferred using criteria outlined in that study, as follows. Weeks *et al.* (2008) concluded that within the Limnadiidae 'using simple sex ratios to infer breeding system can be valid if sex ratios are 1 : 1 or strongly female-biased'. Populations that contain 100% egg-bearing individuals are considered all-hermaphroditic while those that have male frequencies at 45% or above are considered dioecious (Weeks *et al.*, 2008). One of the seven populations noted above (i.e. that were not studied by Weeks *et al.* (2008)) had 0% males (represented by W149; *Eulimnadia cylindrova* from Desirade) and was thus considered hermaphroditic in the current study. Three of these seven populations were considered dioecious using the above noted 45% male criterion: (1) W161 from a population of *L. badia* collected from Western Australia – 46% males; (2) W198 from a population of *L. sordida* collected from Western Australia – 55% males; and (3) W299 from a population of *L. sordida* from collected Northern Territory, Australia – 56% males.

The remaining three populations (represented by W320, *E. africana* from Botswana; W225, *E. brasiliensis* from Brazil; and W246, *E. dahli* from Western Australia) all had natural sex ratios of 23–25% males and thus could not be classified using the above noted sex ratio criteria outlined by Weeks *et al.* (2008). All three populations had 3–8 hermaphrodites that produced male and hermaphroditic offspring in a 3:1 ratio. To date, all cases in which isolated hermaphrodites produced offspring with ~25% males have been found to be androdioecious (Sassaman, 1988; Sassaman & Weeks, 1993; Weeks *et al.*, 2006c, 2008). Therefore, we categorized these three remaining populations as androdioecious.

Breeding systems for most of the nonlimnadiid species included in our analyses were drawn from Sassaman (1995). The remainder was drawn from several other sources (Mattox 1950; Sassaman 1990; Tinti and Scanabissi 1996).

DNA sequencing

Total DNA was isolated from individual clam shrimp using the QIAGEN DNeasy Plant Kit (QIAGEN, Germantown, MD, USA). Portions of the nucleus-encoded 28S rDNA, the elongation factor 1-alpha (EF1α) and the

Table 1 Specimen information.

Family	Genus	Species	ID#	28S	EF1α	COI	BSys	Collection location
Cyzicidae	*Cyzicus*	*gynecia* (Mattox 1949)	NS30	AY851402			X	United States: PA
		gynecia (Mattox 1949)	**NS31**	AY851403	FJ499036		X	United States: PA
		gynecia (Mattox 1949)	NS36	AY851404	FJ499039		X	United States: PA
		gynecia (Mattox 1949)	NS37	AY851405	FJ499040		X	United States: PA
		lutraria (Brady 1886)	ZMUC CRU-9946	EF189639	EF189665	EF189592	D	N.S.W., Australia
		gifuensis (Ishikawa, 1895)	ZMUC CRU-9947	EF189640			D	Japan
		sp.	**W181**	FJ499303		FJ499176	D	Western Australia
		sp.	**W183**	FJ499304		FJ499177	D	Western Australia
		sp.	**W333**	FJ499305	FJ499120		D	Western Australia
		sp.	W340	FJ499306			D	N. Terr., Australia
		sp.	W345	FJ499307			D	N. Terr., Australia
		sp.	**W346**	FJ499308	FJ499121	FJ499221	D	South Australia
		sp.	W347	FJ499309	FJ499122	FJ499222	D	South Australia
	Eocyzicus	*digueti* (Richard 1895)	**NS52**	AY851406	FJ499042	FJ499133	D	Baja California
		digueti (Richard 1895)	**NS53**	AY851407	FJ499043	FJ499134	D	Baja California
		digueti (Richard 1895)	W219	FJ499300	FJ499090	FJ499188	D	United States: NM
		digueti (Richard 1895)	W220	FJ499301	FJ499091	FJ499189	D	United States: NM
		sp.	W298	FJ499302	FJ499112	FJ499213	D	South Australia
Leptestheridae	*Leptestheria*	*compleximanus* (Packard 1877)	**NS14**	AY851391	FJ499032	FJ499124	D	United States: NM
		compleximanus (Packard 1877)	**NS15**	AY851392	FJ499033	FJ499125	D	United States: NM
		compleximanus (Packard 1877)	**NS20**	AY851393		FJ499126	D	United States: NM
		compleximanus (Packard 1877)	NS32	AY851395	FJ499037	FJ499129	D	United States: NM
		compleximanus (Packard 1877)	NS33	AY851396	FJ499038	FJ499130	D	United States: NM
		compleximanus (Packard 1877)	**NS39**	AY851398	FJ499041	FJ499131	D	United States: NM
		compleximanus (Packard 1877)	W214	FJ499296	FJ499085	FJ499184	D	United States: NM
		compleximanus (Packard 1877)	W215	FJ499297	FJ499086	FJ499185	D	United States: NM
		dahalacensis (Rüppel, 1837)	**NS68**	AY851408	FJ499044	FJ499135	D	Austria
		dahalacensis (Rüppel, 1837)	**NS69**	AY851409	FJ499045	FJ499136	D	Austria
		dahalacensis (Rüppel, 1837)	ZMUC CRU-9945	EF189648	EF189670	AF526291	D	Austria
		kawachiensis Uéno, 1927	ZMUC CRU-9944	EF189649			D	Japan
		sp.	W217	FJ499298	FJ499088		D	United States: NM
		sp.	W218	FJ499299	FJ499089	FJ499187	D	United States: NM
Limnadiidae	*Eulimnadia*	*africana* (Brauer, 1877)	W261	DQ198215		FJ499195	A	Botswana
		africana (Brauer, 1877)	W285	FJ499232	FJ499104	FJ499202		South Africa
		africana (Brauer, 1877)	**W320**	FJ499233		FJ499220	A	Botswana
		agassizii Packard, 1874	W272	FJ499242		FJ499198	H	United States: MA
		agassizii Packard, 1874	W278	FJ499241		FJ499201	H	United States: MA
		brasiliensis Sars, 1902	W225	DQ198203			A	Brazil
		brasiliensis Sars, 1902	W228	FJ499245			A	Brazil
		brasiliensis Sars, 1902	W229	DQ198204	FJ499093		A	Brazil
		brasiliensis Sars, 1902	W230	FJ499246			A	Brazil
		braueriana Ishikawa, 1895	NS40	AY851425			A	Japan

Table 1 (*Continued*).

Family	Genus	Species	ID#	28S	EF1α	COI	BSys	Collection location
		braueriana Ishikawa, 1895	NS41	AY851426		FJ499132	A	Japan
		braueriana Ishikawa, 1895	ZMUC CRU-9949	EF189644	EF189667	EF189593		Japan
		colombiensis Roessler 1989	NS105	AY851414	FJ499048		H	Venezuela
		cylindrova Belk, 1989	NS11	AY851418			A	Baja California
		cylindrova Belk, 1989	NS16	AY851422			A	Baja California
		cylindrova Belk, 1989	NS17	AY851419			A	Baja California
		cylindrova Belk, 1989	NS65	AY851432			A	Galapagos
		cylindrova Belk, 1989	NS79	AY851440		FJ499138		Japan
		cylindrova Belk, 1989	NS80	AY851442		FJ499139		Japan
		cylindrova Belk, 1989	NS103	DQ198177				Venezuela
		cylindrova Belk, 1989	NS104	AY851413				Venezuela
		cylindrova Belk, 1989	**W147**	DQ198189		FJ499167		Martinique, FWI
		cylindrova Belk, 1989	**W149**	DQ198188		FJ499168	H	Desirade, FWI
		cylindrova Belk, 1989	W204	DQ198197			A	Japan
		cylindrova Belk, 1989	W205	DQ198198			A	Japan
		cylindrova Belk, 1989	W269	FJ499240	FJ499101		A	Galapagos
		dahli Sars, 1896	W101	DQ198175		FJ499142	H	Western Australia
		dahli Sars, 1896	W102	DQ198176		FJ499143	A	Western Australia
		dahli Sars, 1896	W103	DQ198177			A	Western Australia
		dahli Sars, 1896	W106	DQ198180		FJ499144	A	Western Australia
		dahli Sars, 1896	W107	DQ198181			H	Western Australia
		dahli Sars, 1896	W112	DQ198182			H	Western Australia
		dahli Sars, 1896	W113	DQ198183		FJ499148	H	Western Australia
		dahli Sars, 1896	W115	DQ198184		FJ499149	H	Western Australia
		dahli Sars, 1896	W231	DQ198205			A	Western Australia
		dahli Sars, 1896	W236	DQ198207			A	Western Australia
		dahli Sars, 1896	W238	DQ198208	FJ499094		A	Western Australia
		dahli Sars, 1896	W240	DQ198209	FJ499095		A	Western Australia
		dahli Sars, 1896	W242	DQ198210	FJ499096		A	Western Australia
		dahli Sars, 1896	W246	DQ198211			A	Western Australia
		dahli Sars, 1896	W296	FJ499228	FJ499111	FJ499211	H	Western Australia
		dahli Sars, 1896	W297	FJ499229		FJ499212	H	Western Australia
		diversa Mattox, 1937	NS8	AY851441			A	United States: AZ
		diversa Mattox, 1937	NS22	AY851420			A	United States: AZ
		diversa Mattox, 1937	NS23	AY851421			A	United States: AZ
		diversa Mattox, 1937	W132	AY851455	FJ499064		A	United States: IN
		diversa Mattox, 1937	W223	DQ198202			A	United States: IL
		diversa Mattox, 1937	W258	DQ198213			A	United States: NE
		diversa Mattox, 1937	W259	DQ198214			A	United States: NE
		diversa Mattox, 1937	W276	FJ499237		FJ499200		United States: FL
		diversa Mattox, 1937	W312	FJ499234	FJ499116	FJ499216	A	United States: IN
		diversa Mattox, 1937	W317	FJ499235	FJ499119	FJ499218	A	United States: MS
		diversa Mattox, 1937	W318	FJ499236		FJ499219	A	United States: MS

Table 1 (*Continued*).

Family	Genus	Species	ID#	28S	EF1α	COI	BSys	Collection location
		follisimilis Pereira & Garcia 2001	W321	FJ499238			A	United States: NM
		follisimilis Pereira & Garcia 2001	W322	FJ499239			A	United States: NM
		magdaliensis Roessler 1990	NS58	AY851430				United States: MA
		magdaliensis Roessler 1990	NS59	AY851431				United States: MA
		magdaliensis Roessler 1990	NS99	AY851445	FJ499047			Venezuela
		michaeli Nayar & Nair 1968	W348	FJ499243	FJ499123		H	Thailand
		michaeli Nayar & Nair 1968	W349	FJ499244			H	Thailand
		texana Packard 1871	W280	FJ499230	FJ499102		A	United States: NM
		texana Packard 1871	W281	FJ499231	FJ499103		A	United States: NM
		sp. 1	W170	DQ198190	FJ499073		A	United States: GA
		sp. 1	W209	DQ198200			A	United States: GA
		sp. 1	W252	DQ198212			A	United States: GA
		sp. 1	W253	FJ499226			A	United States: GA
		sp. 2	W293	FJ499223	FJ499109	FJ499208		N. Terr., Australia
		sp. 2	W294	FJ499224	FJ499110	FJ499209		N. Terr., Australia
		sp. 2	**W315**		FJ499117		A	N. Terr., Australia
		sp. 2	**W316**	FJ499225	FJ499118	FJ499217	A	N. Terr., Australia
		sp. 3	W274	FJ499227		FJ499199		Japan
	Imnadia	*yeyetta* Hertzog 1935	NS110	FJ499254			D	Austria
		yeyetta Hertzog 1935	**W125**	AY851449	FJ499059	FJ499156	D	Austria
		yeyetta Hertzog 1935	W72	FJ499255	FJ499050	FJ499141	D	Austria
		yeyetta Hertzog 1935	**W128**	AY851446	FJ499061	FJ499159	D	Austria
		yeyetta Hertzog 1935	**W129**	AY851450		FJ499160	D	Austria
		yeyetta Hertzog 1935	**W130**	AY851447	FJ499062	FJ499161	D	Austria
		yeyetta Hertzog 1935	**W131**	AY851448	FJ499063	FJ499162	D	Austria
		yeyetta Hertzog 1935		EF189646	EF189668	AF526289		Austria
	Limnadia	*badia* Wolf 1911	W124	FJ499256	FJ499058	FJ499155		Western Australia
		badia Wolf 1911		W135		FJ499065	FJ499163	Western Australia
		badia Wolf 1911	W136	FJ499257	FJ499066	FJ499164		Western Australia
		badia Wolf 1911	W144	FJ499258	FJ499068	FJ499166	D	Western Australia
		badia Wolf 1911	**W158**	FJ499259		FJ499170		Western Australia
		badia Wolf 1911	**W159**	FJ499267	FJ499070	FJ499171		Western Australia
		badia Wolf 1911	**W161**	FJ499260	FJ499071	FJ499172	D	Western Australia
		badia Wolf 1911	**W250**	FJ499261		FJ499191		Western Australia
		badia Wolf 1911	**W251**	FJ499262		FJ499192		Western Australia
		cygnorum (Dakin 1914)	**W193**	FJ499271	FJ499075			South Australia
		cygnorum (Dakin 1914)	**W194**	FJ499272	FJ499076			South Australia
		lenticularis Linnaeus 1761	NS24	AY851399	FJ499034	FJ499127	H	United States: FL
		lenticularis Linnaeus 1761	NS25	AY851400	FJ499035	FJ499128	H	United States: FL
		lenticularis Linnaeus 1761	W66	AY851401			H	United States: FL
		lenticularis Linnaeus 1761	**W154**	FJ499279	FJ499069	FJ499169		Italy
		lenticularis Linnaeus 1761	**W210**	FJ499282	FJ499081		H	Austria
		lenticularis Linnaeus 1761	**W211**	FJ499283	FJ499082		H	Austria

Table 1 (*Continued*).

Family	Genus	Species	ID#	28S	EF1α	COI	BSys	Collection location
		lenticularis Linnaeus 1761	**W212**	FJ499284	FJ499083	FJ499183	H	Austria
		lenticularis Linnaeus 1761	**W213**	FJ499285	FJ499084		H	Austria
		lenticularis Linnaeus 1761	**W216**	FJ499286	FJ499087	FJ499186	H	Japan
		lenticularis Linnaeus 1761	**W254**	FJ499280	FJ499097	FJ499193	H	Austria
		lenticularis Linnaeus 1761	**W255**	FJ499281	FJ499098	FJ499194	H	Austria
		lenticularis Linnaeus 1761	ZMUC CRU-9948	EF189651	EF189671			Austria
		sordida King 1855	**W110**	FJ499273	FJ499052	FJ499146	D	Western Australia
		sordida King 1855	**W111**	FJ499274	FJ499053	FJ499147	D	Western Australia
		sordida King 1855	W118	FJ499263	FJ499055	FJ499151	D	Western Australia
		sordida King 1855	W119	FJ499264	FJ499056	FJ499152	D	Western Australia
		sordida King 1855	W120	FJ499265		FJ499153	D	Western Australia
		sordida King 1855	W121	FJ499266	FJ499057	FJ499154	D	Western Australia
		sordida King 1855	W137		FJ499067	FJ499165	D	Western Australia
		sordida King 1855	**W197**	FJ499277		FJ499178	D	Western Australia
		sordida King 1855	**W198**	FJ499278	FJ499077	FJ499179	D	Western Australia
		sordida King 1855	**W299**	FJ499275	FJ499113		D	N. Terr., Australia
		sordida King 1855	W300	FJ499276	FJ499114		D	N. Terr., Australia
		stanleyana King 1855	**W179**	FJ499269		FJ499174	D	N.S.W., Australia
		stanleyana King 1855	**W180**	FJ499270	FJ499074	FJ499175	D	N.S.W., Australia
		urukhai Webb & Bell 1979	W169	FJ499268	FJ499072	FJ499173		N.S.W., Australia
		Limnadopsis	*birchii* (Baird 1860)		EF189652		AF526290	
		parvispinus Henry 1924	**W108**	AY851453	FJ499051	FJ499145	D	Western Australia
		parvispinus Henry 1924	**W109**	AY851451			D	Western Australia
		parvispinus Henry 1924	**W116**	AY851454	FJ499054	FJ499150		Western Australia
		parvispinus Henry 1924	**W126**	AY851452		FJ499157		Western Australia
		parvispinus Henry 1924	**W127**		FJ499060	FJ499158		Western Australia
		tatei Spencer & Hall 1896	**W201**	FJ499287	FJ499079	FJ499181	D	N. Terr., Australia
		tatei Spencer & Hall 1896	**W202**	FJ499288	FJ499080	FJ499182	D	N. Terr., Australia
		tatei Spencer & Hall 1896	**W290**	FJ499289	FJ499107	FJ499205		N. Terr., Australia
		sp. 1	**W305**	FJ499292	FJ499115	FJ499215		Western Australia
		sp. 2	**W222**	FJ499290	FJ499092	FJ499190	D	Western Australia
		sp. 3	**W303**	FJ499291		FJ499214		Western Australia
	Undescribed limnadopsoid species	sp.	W291	FJ499293	FJ499108	FJ499206		N. Terr., Australia
		sp.	W292	FJ499294		FJ499207		N. Terr., Australia
		sp.	W295	FJ499295		FJ499210		N. Terr., Australia
	Metalimnadia	sp.	NS109	AY851451	FJ499049	FJ499140	D	Brazil
		sp.	**W264**	FJ499247	FJ499099	FJ499196	D	Brazil
		sp.	**W265**	DQ198216	FJ499100	FJ499197	D	Brazil
	Undescribed eulimnadoid	sp. 1	NS74	AY851439	FJ499046	FJ499137		Mauritius
		sp. 2	**W199**	FJ499248	FJ499078	FJ499180		South Africa
		sp. 2	W284	FJ499249				South Africa
		sp. 2	W286	FJ499250				South Africa
		sp. 2	W287	FJ499251				South Africa

Table 1 (*Continued*).

Family	Genus	Species	ID#	28S	EF1α	COI	BSys	Collection location
		sp. 2	W288	FJ499252	FJ499105	FJ499203		South Africa
		sp. 2	W289	FJ499253	FJ499106	FJ499204		South Africa

GenBank accession numbers are shown for 28S, elongation factor 1-alpha (EF1α) and cytochrome *c* oxidase I (COI). ID#'s in bold were quantified for morphological characters. BSys, breeding system (A, androdioecy; D, dioecy; H, hermaphroditic; X, asexual).

mitochondrion-encoded cytochrome *c* oxidase I (COI) genes were polymerase chain reaction (PCR) amplified using the following primer pairs: 28S: D1F/D6R (Park & O'Foighil, 2000); EF1α: M44-1/3'EF1 (Braband *et al.*, 2002); COI: 5'Cox1CrustForward 5'-TCHACHAAYCAYA ARGAYATYGGNAC-3', MidCox1CrustForward 5'-TNCC NGTNYTDGCNGGNGCHATYAC-3', 3'Cox1LimnReverse 5'-TCDDYRTARCTRTGYTCWGCNGGRGG-3'. EF1α and 28S were chosen because of their phylogenetic utility in previous studies (EF1α: Braband *et al.*, 2002; 28S: Hoeh *et al.*, 2006), and COI because of its utility in many studies. Each PCR reaction consisted of 5 μL of 10× Qiagen PCR buffer, 1 μL of dNTPs (0.2 mM each), 2.5 μL of each primer (0.5 μM), between 1 and 5 μL of template DNA, 0.2 μL of Qiagen Taq polymerase (1 U), and enough H_2O to bring the total volume to 50 μL. PCR reactions were carried out in PTC-100 and PTC-200 thermal cyclers (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycler programs consisted of an initial incubation at 85 °C for 1 min, followed by 45 cycles of 94 °C for 0.5 min, annealing at 40 °C for 28S rDNA, 53 °C for EF1α and 46 °C for COI for 1 min, and extending at 72 °C for 1.25 min, followed by a final extension of 72 °C for 10 min. PCR products were purified using 1.5% NuSieve (GTG agarose; FMC Bioproducts, Rockland, ME, USA) low melting point gels. Sequencing-template purification was performed using the Wizard PCR preps DNA purification system (Promega, Madison, WI, USA). The mitochondrial and nuclear amplicons were characterized by cycle sequencing using the PCR amplification primers. The protocols for cycle sequencing of the amplicons are as presented in Folmer *et al.* (1994) and they include cycle-sequencing of both strands of each purified template using labelled primers. The separation of cycle-sequencing-reaction products was performed in 3.7% and 5.5% polyacrylamide gels on LI-COR (LI-COR Biosciences, Inc., Omaha, NE, USA) 4200L-2 and 4200S-2 automated DNA sequencers, respectively. The resulting sequences were aligned initially using AlignIR (v2.0; LI-COR Biosciences, Inc.) with subsequent refinement performed manually using MacClade v. 4.05 (Maddison & Maddison, 2002). All sequences generated for this project have been deposited in the GenBank database (see Table 1 for accession nos). The alignment of the COI and EF1α sequences utilized herein was straightforward since no indels have been detected at these loci in the clam shrimp sequences we have generated to date. However, the 28S rDNA sequences contained multiple indels and such areas of ambiguous alignment were deleted prior to phylogenetic analyses. The aligned 28S matrix is available from the authors.

Phylogenetic analyses

Phylogenetic analyses were conducted on a concatenated 3480-character data set that included the three afore-

mentioned genes (3453 characters: 28S = 962 bp, EF1α = 1039 bp, COI = 1452 bp) plus 27 morphological characters (Appendix S1) using Bayesian inference (BI) via MR. BAYES (v. 3.1.2; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The data set contained 167 terminals for which we generated sequences, plus an additional six terminals whose sequences were obtained from GenBank (Table 1). Two independent simultaneous analyses were performed using the GTR + G + I substitution model (Rodriguez *et al.*, 1990). Searches were conducted for 13.224 million generations with six search chains each, the molecular data were partitioned by gene region and by codon position (two gene regions × three codon positions for the COI and EF1α partitions and a single partition for 28S rDNA) yielding a total of eight partitions, and saving a total of 52 896 trees (one tree saved every 500 generations in each of the two analyses). To allow each partition to have its own set of parameter estimates, *revmat*, *tratio*, *statefreq*, *shape* and *pinvar* were all unlinked during the analysis. The analyses were terminated when the standard deviation of split frequencies fell below 0.02. The 10 448 postburnin trees (determined by examination of the log probability of observing the data × generation plot) were used to calculate the majority rule consensus tree. To obtain the most accurate branch length estimates possible, the option *prset ratepr* = *variable* was employed as per the recommendations of Marshall *et al.* (2006). A best maximum likelihood (ML) tree (using default settings except for the following: autoterminate run 1 000 000 generations postlast improved topology, lnL increase for significantly better topology = 0.0001 and score improvement threshold = 0.0005) and a 1000-replicate ML majority-rule bootstrap (Felsenstein, 1985) tree (using default settings except for the following: lnL increase for significantly better topology = 0.001 and score improvement threshold = 0.005), based on analyses of the concatenated three-gene matrix with no data partitioning, were generated using GARLI (Zwickl, 2006). All phylogenetic analyses included representatives of (1) each extant limnadiid genus, (2) the Leptestheriidae and (3) the Cyzicidae (all families are Branchiopoda: Spinicaudata) and designated representatives of the Cyzicidae as the outgroup (as per figures 7 and 8 in Richter *et al.*, 2007).

The estimation of ancestral breeding system character states (Table 1), based on the Bayesian topology with the highest overall posterior probability, was carried out using the ML algorithm in MESQUITE (v.2.5; Maddison & Maddison, 2008). The 173 terminal best BI tree was reduced to 79 terminals by first pruning out the terminals for which the breeding system character states were unknown and then by reducing duplicate non-*Eulimnadia* lineages to single representative individuals. The ML optimization utilized the Markov k-state one parameter model (Lewis, 2001) and incorporated branch length and parameter estimates from the Bayesian analyses. The use of a likelihood ratio test to calculate *P*-values for ancestral states is not possible because hypotheses regarding the likelihoods of each possible state at a given node are non-nested. Therefore, to make decisions regarding the significance of ancestral character states, Pagel (1999; following Edwards, 1972) recommended that ancestral character state estimates with a log likelihood two or more units lower than the best state estimate [decision threshold (*T*) set to *T* = 2] be rejected. Generally viewed as a conservative cutoff, this threshold has been used by numerous recent authors (e.g. Moczek *et al.*, 2006; Fernandez & Morris, 2007; Murphy *et al.*, 2007; Koepfli *et al.*, 2008). For the data presented herein, this protocol ensures that all of the character states judged to be significant have proportional likelihoods (PL) at least 10 times greater than that of any other state.

Results

The 173 terminal best BI tree (that with the highest posterior probability (PP) from our two independent analyses), with branch lengths, PPs (×100) and ML bootstrap information (1000 replicates) displayed, indicates strong support for limnadiid monophyly as well as for the monophyly of most traditional spinicaudate genera, such as *Eulimnadia*, *Metalimnadia*, *Imnadia*, *Limnadopsis*, *Leptestheria*, *Cyzicus* and *Eocyzicus* (Fig. 1). Additionally, two well supported, undescribed limnadiid clades, likely warranting generic rank, have been detected in South Africa (undescribed eulimnadioid lineage ZA, Fig. 1a) and Australia (undescribed limnadopsoid lineage AU, Fig. 1b). In contrast, representatives of the genus *Limnadia* occur in two distinct, well supported locations in the tree in Fig. 1: (1) in a clade (with terminals distributed in the Holarctic) sister to the genus *Imnadia* (Fig. 1b: node ❶) and (2) in a clade (with terminals distributed in Australia) sister to the genus *Limnadopsis* (Fig. 1b: node ❷). Taxonomic issues, such as the polyphyletic nature of the genus *Limnadia* and the undescribed limnadiid lineages, will be dealt with in separate manuscripts (D.C. Rogers *et al.*, unpublished data) and are not germane to the discussion of breeding system evolution in the Limnadiidae that follows below. Strongly supported intergeneric relationships displayed in Fig. 1 include the sister taxon relationships of *Eulimnadia* + *Metalimnadia* (Fig. 1a: node ❶) and 'Australian *Limnadia*' + *Limnadopsis* (Fig. 1b: node ❷). The above-described evolutionary relationships are also supported by the best ML tree (not shown).

Some species determinations within the Limnadiidae are likely problematic because of the lack of species monophyly sometimes displayed in Fig. 1 (e.g. *E. diversa*, *E. follisimilis*, *E. cylindrova* and *L. sordida*). Species and even generic determinations have been confusing in *Eulimnadia* and *Limnadia* for over a century, especially for Australian taxa (Sayce 1903; Henry 1924; Daday 1925; Straskraba 1964; Webb and Bell 1979; Belk 1989; Richter and Timms 2005). The specifics of these taxonomic issues

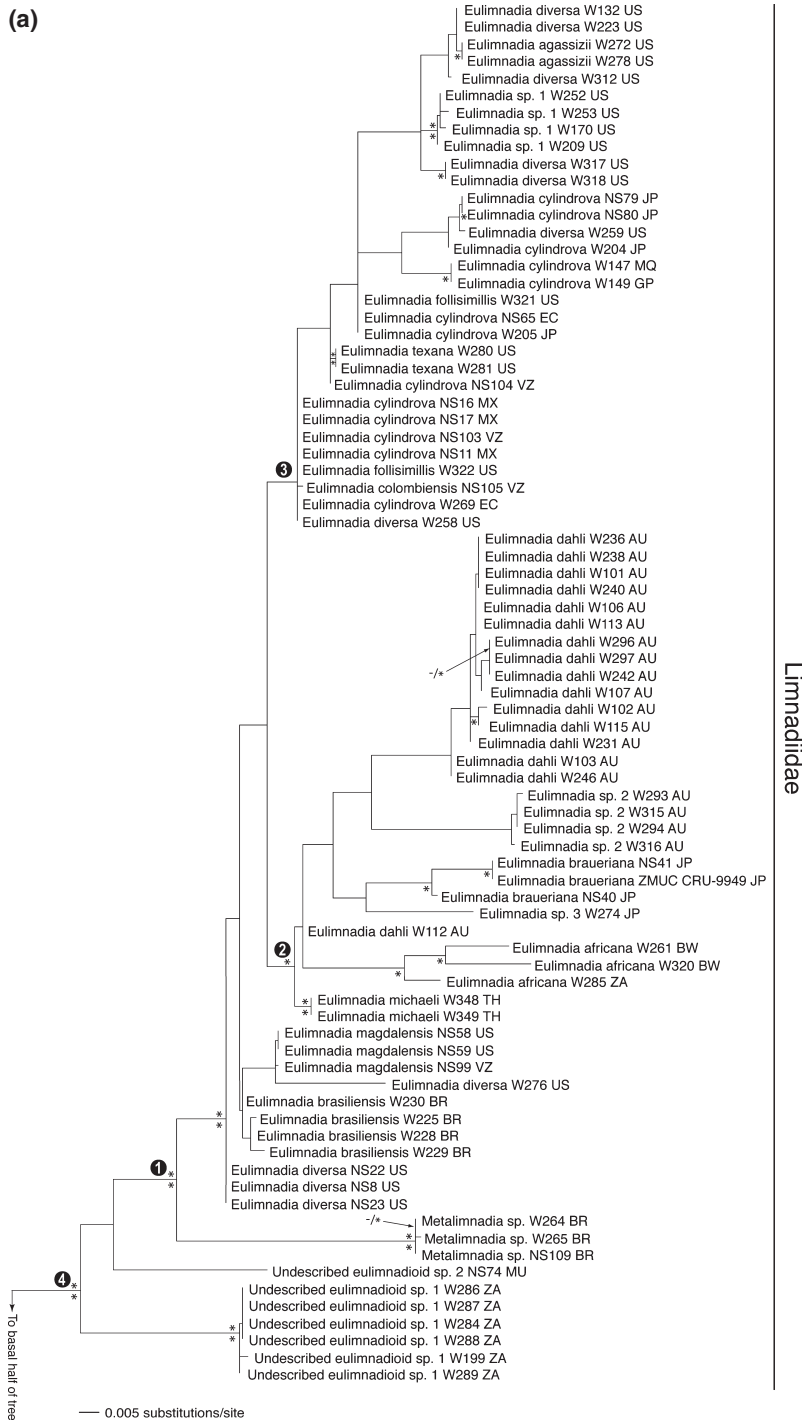

Fig. 1 Bayesian tree of highest posterior probability showing the apical (1a) and basal (1b) halves of the tree from a combined evidence analysis of 28S, elongation factor 1-alpha (Ef1α), cytochrome *c* oxidase I (COI) and morphology. Bayesian PP ≥ 95 and maximum likelihood (ML) bootstrap percentages ≥70 are denoted with asterisks above and below the branches, respectively. Codes after taxon names indicate individual specimen numbers (see Table 1) and two-letter country designations: Australia (AU); Austria (AT); Brazil (BR); Ecuador (EC); Guadeloupe (GP); Italy (IT); Martinique (MQ); Mauritius (MU); Mexico (MX); Japan (JP); South Africa (ZA); Thailand (TH); United States (US); Venezuela (VZ). Highlighted nodes are as follows: (1a: node ❶) – intergeneric relationship of *Eulimnadia* + *Metalimnadia*; (1a: nodes ❷ and ❸) – major lineages within *Eulimnadia* that contain one or more androdioecy-to-hermaphroditism transition; (1b: node ❶) – Holarctic *Limnadia*; (1b node ❷) – Australian *Limnadia*; and (1b: node ❸) – intergeneric relationship of Australian *Limnadia* + *Limnadopsis*.

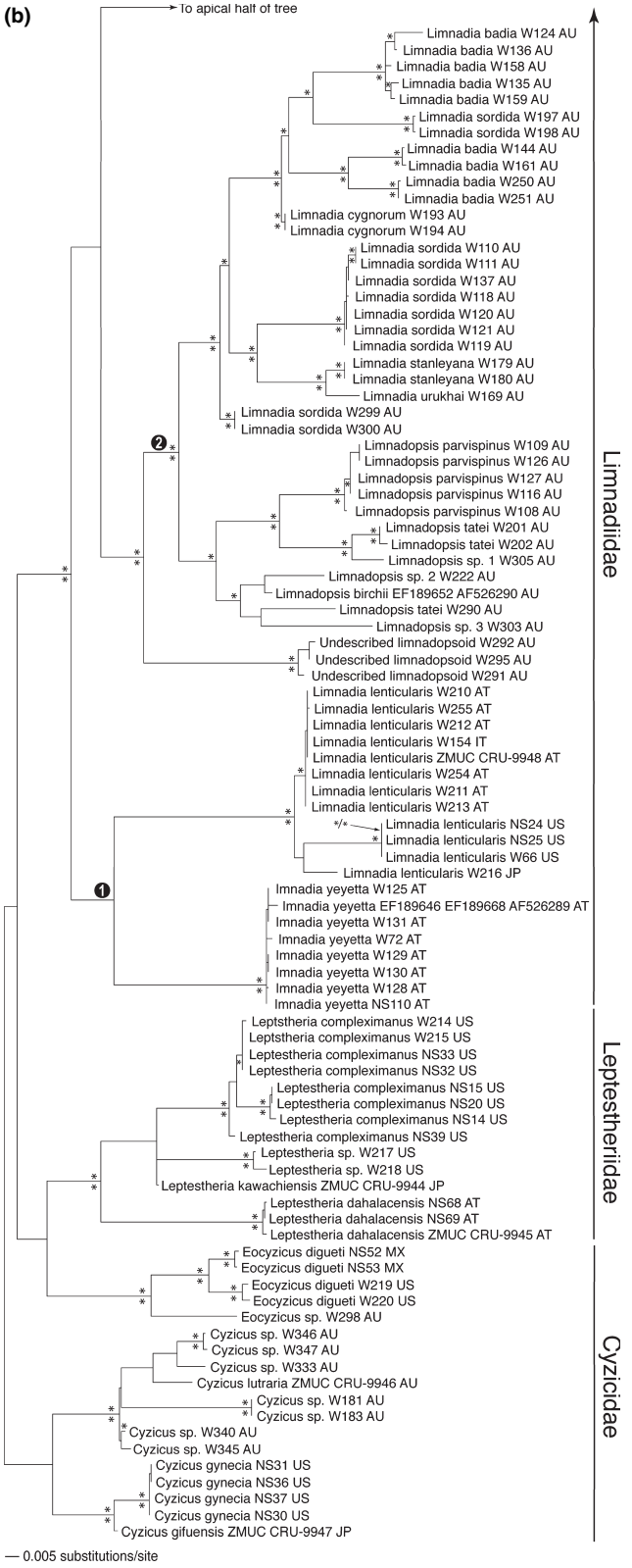

Fig. 1b (*Continued*).

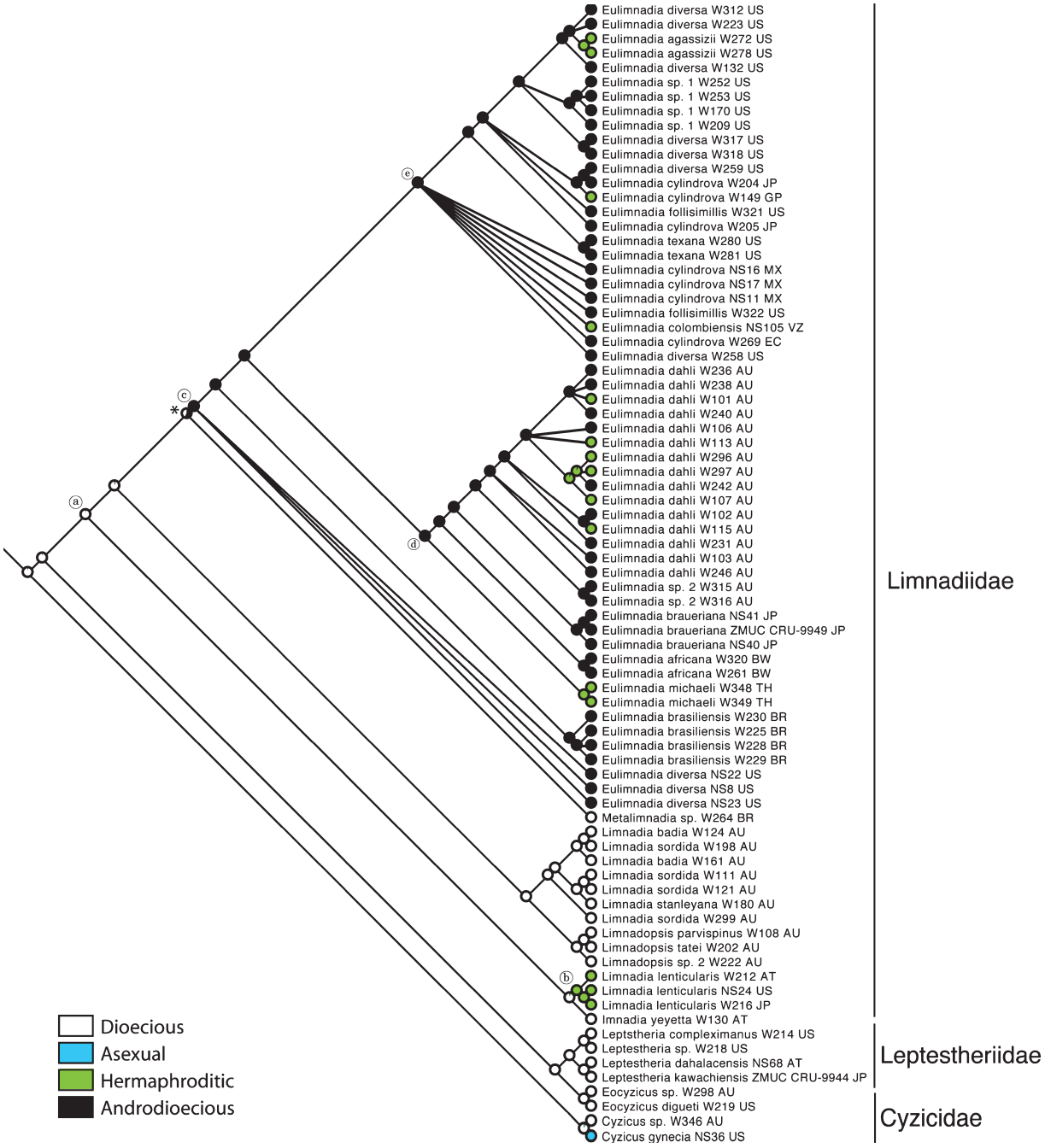

Fig. 2 Maximum likelihood optimization of breeding system on a pruned topology from Fig. 1 analysed with Mesquite using the Markov k-state one parameter model. Taxa pruned from Fig. 1 includes those from populations whose breeding system are undetermined, as well as duplicate non-*Eulimnadia* lineages. Significance of ancestral character state estimates determined by one character state having a log likelihood two or more units higher than all others. All nodes are significant for a single character state except a single node, denoted with an asterisk (*), which has two states (androdioecy and dioecy) significantly better than the others. Codes after taxon names indicate individual specimen numbers (see Table 1) and two-letter country designations: Australia (AU); Austria (AT); Brazil (BR); Ecuador (EC); Guadeloupe (GP); Italy (IT); Mexico (MX); Japan (JP); Thailand (TH); United States (US); Venezuela (VZ). Highlighted nodes are as follows: node ⓐ – dioecy is the inferred ancestral state for the Limnadiidae; node ⓑ – transition to all-hermaphroditism in the holartic *Limnadia*; node ⓒ – transition to hermaphrodites + males (androdioecy) in the *Eulimnadia*; nodes ⓓ and ⓔ major lineages within *Eulimnadia* that contain one or more androdioecy-to-hermaphroditism transitions.

will be the topic of a companion paper (Rogers *et al.* in preparation) and herein we will primarily concentrate on the inferred evolutionary transitions of the breeding systems within the Limnadiidae.

Figure 2 displays the ML estimation of breeding system ancestral states onto a 79 terminal topology that maintains the relative evolutionary relationships portrayed in the best 173 terminal BI tree (Fig. 1). Singular character state estimates for 57 of the 58 interior nodes in this topology were deemed significant by Mesquite. The internal nodes in Fig. 2 denote 'PL' for each of the four reproductive character states. Nodes that are primarily one colour usually signify a > 90% probability that the ancestral character was the type signified by the respective colours. There were only two nodes in which the PL of the most likely ancestral character state was < 90%: (1) the ancestral node for *Eulimnadia* + *Metalimnadia* sp. ($PL_{androdioecy} = 0.56$; $PL_{dioecy} = 0.40$; both of these states being significantly better than the other two possible states, but not significantly better than one another) and (2) the node defining the split between *Cyzicus* sp. and *C. gynecia* ($PL_{dioecy} = 0.87$). Even though the PL for the majority state at the latter was < 0.9, this state was judged by ML to be the single, significantly best state for this node, and the PL for this state was more than 13 times greater than the PL for any other state.

The breeding system ancestral states analysis indicates that dioecy was the breeding system of the limnadiid ancestral lineage (PL = 0.94; Fig. 2: node ⓐ). Furthermore, independent gains of hermaphroditic reproduction occurred in the ancestral lineage of Holarctic *Limnadia* (i.e. the *Limnadia* clade sister to *Imnadia*; Fig. 2: node ⓑ; Fig. 3: arrow A) and *Eulimnadia* (Fig. 2: node ⓒ; Fig. 3: arrow B). In the Holarctic *Limnadia*, the hermaphrodites replaced both males and females while in *Eulimnadia*, hermaphrodites replaced only females initially (yielding androdioecy) with later male loss in some populations (yielding all-hermaphroditism; Fig. 3: arrows B → C). Thus, within the typically androdioecious genus *Eulimnadia*, our ML optimization estimated that a shift from androdioecy to hermaphroditism has independently occurred seven times (Fig. 2; Fig. 3: arrow C). However, it should be noted that many of the nodes within *Eulimnadia* received low statistical support (BI PP < 0.95 and ML bootstrap percentage (BSP) < 70) as indicated by the relative paucity of asterisks on Fig. 1a. This topological instability can be accounted for when estimating the minimum number of breeding system shifts in *Eulimnadia*. Within *Eulimnadia*, there is a major subclade that received high Bayesian nodal support (Fig. 1a: node ❷, Fig. 2: node ⓓ) and contains four of the seven estimated independent transitions from androdioecy to hermaphroditism mentioned above. We could more conservatively estimate that this major subclade contains a single, independent transition by recognizing that the hermaphroditic lineages therein could actually form a clade. The same could be argued for the other three transitions occurring in the other major *Eulimnadia* subclade (Fig. 1a: node ❸, Fig. 2: node ⓔ). Thus, a conservative estimate of the minimum number of transitions from androdioecy to all-hermaphroditism within *Eulimnadia* would be two independent transitions. However, considering that there are some relatively long branch lengths separating some of the taxa within these subclades (e.g. the total branch length between *E. michaeli* and any one *E. dahli*), the actual number of androdioecy-to-hermaphroditism transitions within *Eulimnadia* likely lies between two and seven. The current ancestral states analysis suggests one single transition to asexuality from dioecy in the all-female *Cyzicus gynecia* (Fig. 2; Fig. 3: arrow D).

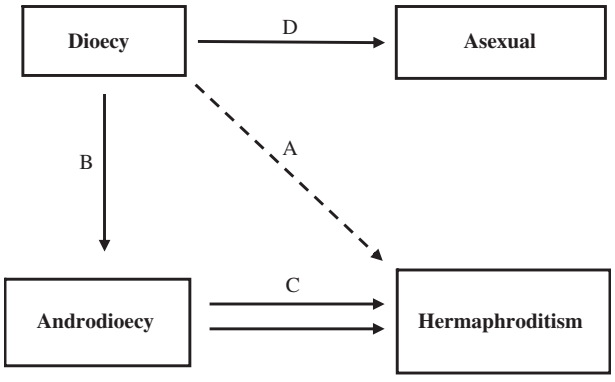

Fig. 3 Evolutionary transitions inferred from the analysis in Fig. 2. Arrow A: transition occurred in the ancestor to *Limnadia lenticularis*; arrow B: transition occurred in the ancestor to *Eulimnadia*; arrow C: transition occurred in the ancestor to some *Eulimnadia* species; arrow D: transition occurred in the ancestor to *Cyzicus gynecia*. The dashed arrow A denotes that although a possible direct pathway from dioecy to hermaphroditism may have occurred, an androdioecious intermediate is a more likely scenario (i.e. the B → C transition; see Discussion).

Discussion

To understand the evolution of hermaphroditism in animals, we need to discern the number and types of transitions from ancestral states, and determine the selective processes (and potential constraints) that shape these transitions. For the former, mapping breeding system onto a robust phylogeny to infer evolutionary transitions is most useful (Kiontke *et al.*, 2004; Sargent & Otto, 2004; Lopez-Vaamonde *et al.*, 2005; Surget-Groba *et al.*, 2006; Rossi *et al.*, 2007). Herein we have conducted such a phylogenetic comparison and below we will interpret these transitions by considering the selective regimes and the potential constraints that affect these transitions.

Breeding system transitions within the Limnadiidae

It has long been assumed that the ancestral breeding system for the Limnadiidae was dioecy (Sassaman, 1995).

Sassaman (1995) proposed a genetic model specifically for the clam shrimp by which parthenogenesis and androdioecy have directly evolved from dioecy and that selfing hermaphroditism and cyclic parthenogenesis were then derived from androdioecy and parthenogenesis, respectively. However, to date no one has conducted an ancestral character state reconstruction to confirm any of these assertions.

Using the character state optimization outlined in Fig. 2, we infer that the ancestral breeding system for the Limnadiidae is indeed dioecy (Fig. 2: ⓐ), as was suggested by Sassaman (1995). We further infer that there have been two separate derivations of hermaphroditism from dioecy: one in the progenitor to the all-hermaphroditic *L. lenticularis* (Fig. 2: ⓑ; Fig. 3: arrow A) and one in the progenitor to the hermaphroditic + male (i.e. androdioecious) *Eulimnadia* (Fig. 2: ⓒ; Fig. 3: arrow B). If a sister relationship existed between *Limnadia lenticularis* and *Eulimnadia*, the assertion that there were two independent derivations of hermaphroditism from dioecy would be questionable. However, there are two robustly supported nodes in Fig. 1a that reject this possibility: (1) (*Metalimnadia* +*Eulimnadia*) (Fig. 1a: node ❶) and (2) {undescribed eulimnadioid sp. 1 + [undescribed eulimnadioid sp. 2 + (*Metalimnadia* + *Eulimnadia*)]} (Fig. 1a: node ❹). Therefore, the inference of two independent derivations of hermaphroditism is robustly supported.

In the *Eulimnadia*, the hermaphroditic variants have outcompeted the females but have largely coexisted with males to form androdioecious populations (Fig. 2), which coincides with the assertions of Sassaman (Fig. 3: arrow C). In *Limnadia lenticularis*, our data suggest a direct derivation of all-hermaphroditism from dioecy (Fig. 3: dashed arrow A). There are no clear androdioecious close relatives to *L. lenticularis* (Fig. 2) and thus no evidence that this all-hermaphrodite species derived from an androdioecious progenitor. Nevertheless, there is good reason to suspect that such a progenitor may have initially evolved and has since gone extinct. We outline these arguments (largely drawn from Sassaman, 1995) below.

To understand the evolution of hermaphroditism in the Limnadiidae, Sassaman (1995) suggested that we use the genetic sex determining system first elucidated in *Eulimnadia texana* (Sassaman & Weeks, 1993) and assume it is shared among *Eulimnadia* more generally (Sassaman, 1995; Weeks *et al.*, 2008). In this genetic system, males are homogametic (ZZ) while hermaphrodites are of two genetic types: ZW (termed 'amphigenic') and WW ('monogenic'). Selfing ZW hermaphrodites produce one-quarter males while selfing WW hermaphrodites produce all hermaphrodites (Sassaman & Weeks, 1993). Sassaman suggested that the derivation of all-hermaphroditic limnadiid lineages is a simple product of selection for the WW hermaphrodites from within this mix of the three mating types (Fig. 3: arrow C).

We see evidence of Sassaman's supposition within the *Eulimnadia* (Fig. 2). Each of the major subclades within *Eulimnadia* (Fig. 2: nodes ⓓ and ⓔ) has experienced at least one derivation of all-hermaphroditism from androdioecy, and if our best estimate of phylogeny is correct (Fig. 1), as many as seven independent derivations of hermaphroditism have occurred among the *Eulimnadia* populations we sampled (Fig. 2). Additionally, two other *Eulimnadia* species have all-hermaphrodite populations from which data have not been analysed herein (*E. diversa* and *E. feriensis* Dakin 1914), and these all-hermaphrodite populations are much less common than their androdioecious conspecific counterparts (Sassaman, 1989; Weeks *et al.*, 2008). Thus, in the current and previous studies, it appears that all-hermaphrodite populations have been repeatedly derived from androdioecious populations, and we may expect this to have occurred in the development of all-hermaphroditism in *L. lenticularis* also (see 'Re-evaluation' section below).

Adaptive mechanisms promoting the evolution of hermaphroditism

Sassaman's (1995) model is primarily genetically based, and thus does not provide expected criteria under which one breeding system should be selected over another. However, there are two published mechanisms by which all-hermaphrodite populations may be expected to be derived from androdioecious progenitors. First, Chasnov (in press) suggested that outcrossing may be selected against in hermaphrodites which have < 50% inbreeding depression among selfed offspring. Chasnov & Chow (2002) additionally predicted that such hermaphrodites should be selected to reduce or eliminate outcrossing with males, leading to all-selfing, hermaphroditic populations. Such reduced outcrossing has apparently been selected in the androdioecious *Caenorhabditis elegans* (Chasnov & Chow, 2002; Chasnov *et al.*, 2007). If this phenomenon were occurring in *Eulimnadia*, we would then expect a lower propensity to mate and a general observation of lower inbreeding depression in the all-hermaphrodite compared with the androdioecious populations. At this point, we do not have the data needed to test this hypothesis, but this 'reduced outcrossing propensity' model could clearly explain the derivation of all-hermaphrodite populations from androdioecious progenitors in *Eulimnadia*.

A second hypothesis has been suggested by Pannell (1997, 2002): hermaphrodites are better early colonists and thus commonly are found in all-hermaphroditic, younger populations. Males are then later able to colonize these younger pools to re-establish androdioecy as the populations become larger and better established. There is strong evidence that this metapopulation hypothesis explains the mix of androdioecious and all-hermaphrodite populations of the plant *Mercurialis annua* (Obbard *et al.*, 2006; Dorken & Pannell, 2008; Pannell *et al.*, 2008). If this mechanism operates in *Eulimnadia*, we would then expect all-hermaphrodite populations to

be younger, have lower genetic diversity, and have higher among-population genetic differentiation (i.e. higher F_{ST}) than androdioecious populations (Pannell, 2002; Obbard *et al.*, 2006). Again, we do not yet have sufficient data to test these predictions, but clearly this hypothesis could well explain the observed patterns of sex ratio variation among populations in the genus *Eulimnadia*.

Both of the above models assume that hermaphroditism is selected within a dioecious species because of the advantages of 'reproductive assurance' (Baker, 1955) when population sizes are commonly low, such as in species that regularly colonize new habitats. Short-lived, ephemeral ponds are the typical habitat for these clam shrimp (Dumont & Negrea, 2002; Weeks & Bernhardt, 2004), and thus reproductive assurance is completely feasible as an important aspect of the life history of these branchiopod crustaceans.

If reproductive assurance is the primary force selecting hermaphroditism, as postulated, then the hermaphrodites should be primarily 'female-biased' because such low-density situations would disallow much fitness gain through male function (Pannell, 1997). In other words, the hermaphrodites should be primarily allocating reproductive investment to egg production and only produce enough sperm to ensure fertilization of their own eggs. This prediction is upheld in *Eulimnadia* as well as *L. lenticularis* hermaphrodites: hermaphrodites allocate only a small portion of their gonads to sperm production (Zaffagnini, 1969; Zucker *et al.*, 1997; Scanabissi & Mondini, 2002; Weeks *et al.*, 2005). Such female-biased allocation is also noted in androdioecious nematodes (Ward & Carrel, 1979) and fish (Harrington, 1963). Thus, the life history prediction of these two models that hermaphrodites will be female-biased is upheld in the well-studied androdioecious animal species noted to date.

Potential constraints on the evolution of hermaphroditism from dioecy

An alternate argument has been forwarded for the observation of female-biased hermaphroditism in these shrimp and the other androdioecious animals noted above: the development of a functional hermaphrodite from a sexually dimorphic ancestor may be constrained to be one that functions primarily as one sex, that sex being female (Weeks *et al.*, 2006a). If there are many physiological, morphological and/or behavioural traits that differ between males and females (i.e. the species is strongly sexually dimorphic), the odds of producing a hermaphrodite that fully captures all of the necessary phenotypes of both sexes to function equally well in both sexual roles might be prohibitively low. For example, clam shrimp males have male gonads, 'claspers' (used to attach to females during sperm transfer), elongate carapaces and male-specific behaviours (e.g. searching behaviour, faster swimming, etc.; Scanabissi Sabelli & Tommasini, 1994; Knoll, 1995; Olesen *et al.*, 1996; Medland *et al.*, 2000). Females have female gonads, ovoid carapaces, a 'brood chamber' to store eggs, extensions of their epipodites for egg attachment and female-specific behaviours (e.g. slow swimming, hole digging for egg laying, etc.; Scanabissi Sabelli & Tommasini, 1990; Dumont & Negrea, 2002; Zucker *et al.*, 2002). If each of these traits is encoded by one or more genes, the odds of mutations or re-arrangements of these genes to form a phenotype that successfully combines all traits from both sexes is miniscule. More commonly, a 'hermaphrodite' would likely be a dysfunctional combination of some subset of the sexual phenotypes of both sexes. For example, we have observed one case of an *E. texana* 'intersex' that had male claspers, male mating behaviour, and apparently functional ovotestes (Weeks *et al.*, 2006b). However, this intersex did not have a brood chamber nor epipodites for egg attachment; therefore all of its eggs were found in distorted clumps and all eggs proved to be inviable. Additionally, the individual had a normal *E. texana* hermaphrodite's ovotestes, which is highly skewed toward egg production (Zucker *et al.*, 1997), and thus could not produce enough sperm to effectively fertilize hermaphrodites. Thus, although this intersex was 'closer' to being fully competent in male and female roles than the common female-biased, self-compatible hermaphrodites (i.e. it had the claspers needed for pairing, had the appropriate mate searching behaviour, and produced fully yolked and shelled eggs), it still did not have all the needed character traits to be competent in either sexual role and therefore was sterile. Thus, a more parsimonious expectation for the formation of a functional hermaphrodite would be one that is primarily one sex but that had co-opted one or at most a few of traits of the opposite sex (e.g. through mutation or crossing over; Weeks *et al.*, 2006b). If this were true, the most likely arrangement to be selectively advantageous would be a female that could produce sperm but had no other male traits (Weeks *et al.*, 2006a). This would be more functional than a male that produced eggs, since egg production commonly needs extra traits to produce viable offspring, such as the brood chamber and hole-digging behaviour in the clam shrimp example noted above.

Thus, although the independent derivations of female-biased hermaphroditism within the Limnadiidae noted herein (i.e. in *Limnadia lenticularis* and *Eulimnadia*) is consistent with two models based on reproductive assurance (Pannell, 1997; Chasnov, in press), it can also be explained by a constraint argument based on the most parsimonious method to produce a hermaphrodite from a sexually dimorphic, dioecious progenitor (Weeks *et al.*, 2006a). Further data collection that can confirm/reject the additional predictions of the two selective models in nematodes, killifish and clam shrimp should resolve which of these explanations is most viable.

Re-evaluation of Sassaman's model of the evolution of hermaphroditism in the Limnadiidae

We can use the above discussion to construct an argument that is consistent with Sassaman's (1995) hypothesis for the development of hermaphroditism within the Limnadiidae. Let us assume that self-compatible hermaphroditism is selected from dioecy because of the benefits of 'reproductive assurance' in sperm-limited environments (Pannell, 1997; Wolf & Takebayashi, 2004; Chasnov, in press). A female-biased hermaphrodite is either specifically selected (Pannell, 1997; Chasnov, in press) or is the only viable mechanism to produce a functioning hermaphrodite in the Limnadiidae (Weeks *et al.*, 2006a). Such a female-biased, hermaphroditic variant arose twice within the Limnadiidae (Fig. 2). In *Eulimnadia*, this hermaphroditic variant then spread to displace females but was maintained with males, either because the correct balance of migration and colonization rates was achieved (Pannell, 1997, 2002) or because this migration/colonization process is combined with a constraint on the elimination of males because of the unique sex determining mechanism in this genus (Pannell, 2008). In *L. lenticularis*, the female-biased hermaphroditic variant spread to displace both females and males, either because very high levels of extinction and low migration rates caused most populations to be in a constant state of low abundance and recent establishment (Pannell, 1997) or because inbreeding depression among selfed offspring was below the threshold 50% level favouring selfing over outcrossing (Chasnov, in press). Chasnov argued that the latter scenario would be a two-step process, which would first manifest as hermaphrodites displacing females to form androdioecy and then later spreading to displace males once inbreeding depression is purged to the point where inbred offspring experience < 50% inbreeding depression. If this two-step process is valid, then the direct evolution of hermaphroditism from dioecy (Fig. 3: arrow A) did not occur but rather an androdioecious intermediate developed for some period of time and was later replaced by the all-hermaphrodite WW lineages, as predicted by Sassaman's (1995) model (Fig. 3: arrows B and C). Additionally, an argument can be made that some of the current populations/species of *Eulimnadia* may be undergoing Chasnov's second stage (i.e. elimination of males) that *L. lenticularis* underwent at some point in the more distant past.

Parthenogenesis derived from dioecy?

One last reproductive transition obvious in Fig. 2 is the derivation of parthenogenesis from dioecy in *Cyzicus gynecia* (Fig. 3: arrow D). Sassaman (1995) predicted that *C. gynecia* evolved directly from a dioecious ancestor, likely *C. mexicana*, by a mutation suppressing meiosis. Our data are certainly consistent with this prediction, although we cannot assess the underlying genetics of the reported asexuality in *C. gynecia*. Indeed, to date, no one has determined whether *C. gynecia* is truly parthenogenetic rather than being self-compatible hermaphrodites; determination of parthenogenesis has been only on the basis of an observed lack of males (Sassaman, 1995). Thus, it would be constructive to assess the genetics and anatomy of *C. gynecia* 'females' to check for levels of heterozygosity (parthenogenesis is commonly associated with high heterozygosity while selfing hermaphrodites are commonly completely homozygous; Bell, 1982) and the presence/absence of testicular tissue to determine the true mode of reproduction. Additionally, a population genetic comparison with *C. mexicana* (as suggested in Sassaman, 1995) and other *Cyzicus* species would allow a test of Sassaman's prediction that *C. gynecia* was recently derived from *C. mexicana*.

Conclusions

In conclusion, our data indicate that self-compatible hermaphroditism arose from dioecy independently twice within the Limnadiidae, likely because of the benefits of reproductive assurance in low-density environments. We suggest that the predictions of Sassaman (1995), that androdioecy and parthenogenesis are directly derived from dioecy (Fig. 3: arrows B and D, respectively) and that selfing hermaphroditism is secondarily derived from androdioecy (Fig. 3: arrow C), are true, although we cannot refute the possibility that the all-hermaphrodite *L. lenticularis* was directly derived from dioecy (Fig. 3: arrow A). In the limnadiid lineages examined to date, hermaphrodites are always 'female-biased' (i.e. produce few sperm and cannot outcross through male function). This type of hermaphrodite is consistent with other androdioecious systems in which males coexist with female-biased hermaphrodites (e.g. nematodes and killifish) and may be explained either using adaptive models which predict such female-biased hermaphroditism (Pannell, 1997, 2002; Chasnov, in press) or by a constraint argument based on the most parsimonious mechanism by which self-compatible hermaphroditism can be derived from a sexually dimorphic, dioecious ancestor (Weeks *et al.*, 2006a). Future studies should concentrate on testing the predictions of the two adaptive models combined with a comparative assessment of the validity of the constraint hypothesis. Additionally, although these models do predict a transitional pathway to produce hermaphrodites from dioecy, they are not sufficient to explain how fully functional hermaphrodites (i.e. that are competent in both male and female roles) can evolve from a dioecious ancestor. Because the majority of animal hermaphrodites appear to be derived from dioecious ancestors (Ghiselin, 1969, 1974; Jarne & Charlesworth, 1993; but see Eppley & Jesson, 2008; Lyer & Roughgarden, 2008 for an alternative interpretation), we need to expand our models to include an explanation of

the derivation of fully functional, outcrossing hermaphrodites from dioecious progenitors.

Acknowledgments

The authors thank: M. Hamer, L. Brendonck, N. Rabet, M. Grygier, B. Timms, A. Ohtaka, B. Lang, M. Hill, U. Balaraman, G. Pereira, S. Leslie, L. Sanoamuang, A. Ooyagi, J. Hoover, A. Ferreira, E. Eder, S. Richter, S. Wu, M. Cesari, F. Scanabissi, J. Garcia, D. Smith, A. Maeda-Martinez, Merlijn Jocqué and T. Spears for soil samples and/or preserved clam shrimp; C. Sassaman for help with species identifications; A. Crow, C. Komar, R. Posgai and B. Wallace for help with rearing clam shrimp in the wet lab; and R. Mitchell and N. Rabet for thoughtful comments on a previous version of this paper. Eric Chapman is supported by the Kentucky Agricultural Experiment Station State Project KY008043. This material is based upon work supported by the National Science Foundation under Grant No. DEB-0235301.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Characters and character states of the specimens coded for and present in the phylogenetic analyses.

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Received 20 May 2009; revised 30 June 2009; accepted 4 July 2009