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Genetic differentiation, behavioural reproductive isolation and mixis cues in three sibling species of monogonont rotifers

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Summary

1. Many aquatic species usually considered to be ‘cosmopolitan’ have been identified as cryptic species complexes, based on deep genetic differentiation. However, reproductive isolation among sibling cryptic species has rarely been studied, and interspecific hybridization is common in some taxa.
2. We investigated isolation mechanisms and possible introgression among three cyclical parthenogenetic rotifer species in the *Epiphanes senta* complex that are found in very different freshwater habitats: temperate floodplains, subtropical desert rock pools and a tropical alpine lake. Whereas *Epiphanes ukera* is reproductively isolated from *E. chihuahuaensis* and *E. hawaiiensis*, the latter hybridize under laboratory conditions.
3. While reproductive isolation is incomplete, RAPD profiles indicated unique genetic signatures and showed no evidence for introgression, indicating that these three species are diverging and have independent evolutionary trajectories.
4. Testing cues for sexual reproduction in these cyclic parthenogens demonstrated that mixis in *E. chihuahuaensis* and *E. ukera* is influenced by population density, whereas *E. hawaiiensis* females rarely produce mictic offspring regardless of density. Different mixis cues are likely to separate sexual periods and effectively cause reproductive isolation between the species. *Epiphanes ukera* and *E. chihuahuaensis* males display mate guarding behaviour, and *E. ukera* males distinguish between conspecific and heterospecific females in mate choice experiments. Geographic isolation, along with different cues for mixis induction and mate recognition, act as reproductive barriers among these sibling species.

Keywords

cryptic species; mixis induction; mating behavior; introgression; hybridization

Introduction

Cryptic speciation has been demonstrated in an increasing number of aquatic invertebrate taxa, including amphipods (Wellborn & Cothran, 2004, 2007), copepods (Thum & Derry, 2008; Thum & Harrison, 2009), bryozoans (Gómez *et al.*, 2007a, 2007c), cladocerans (reviewed in Fórro *et al.*, 2008) and rotifers (e.g., Gilbert & Walsh, 2005; Suatoni *et al.*, 2006; Mills, Lunt & Gómez, 2007; Fontaneto *et al.*, 2009; Walsh *et al.*, 2009). Many of

these studies have employed molecular markers (most commonly the mitochondrial *cox1* gene), sometimes combined with morphological analyses. In addition, combinations of molecular markers (nuclear DNA, mtDNA, microsatellites, AFLPs, RFLPs, RAPDs and allozymes) have been used successfully to explore and resolve genetic relationships among populations of morphologically cryptic species (e.g. Jones *et al.*, 2001; Hurwood *et al.*, 2003; Wilkerson *et al.*, 2005). Much less is known regarding the dynamics of genetic and reproductive processes involved in the production of cryptic species. In particular, very few studies have investigated reproductive isolation among cryptic species, especially in aquatic taxa (Knowlton, 2000). Evolutionary factors that ultimately lead to reproductive isolation and drive speciation in these species are linked to the ecological dynamics in communities and ecosystems (Pelletier, Garant & Hendry, 2009).

In many cryptic species complexes reproductive isolation is not complete and hybridization between sibling species does occur. In the presence of persistent gene flow, hybridization can have long-lasting consequences for the genetic architecture of populations (Schwenk, Brede & Streit, 2008). Interspecific hybridization is often restricted to relatively narrow hybrid zones between populations and is maintained by a balance between dispersal and selection against hybrids (Barton & Hewitt, 1985). In cladocerans, interspecific hybridization occurs frequently (Keller *et al.*, 2008; Brede *et al.*, 2009) but hybrid zones are unstable or absent (Petrusek *et al.*, 2008). Hybrids often have selective advantages over parental species in fluctuating environments and may replace them over time (Brede *et al.*, 2009). Much less is known about hybridization among sibling species in the Rotifera. There is little evidence that hybridization occurs frequently in this taxon under natural conditions, and to our knowledge only one study has indicated that introgression may have occurred among species within the genera *Polyarthra* and *Conochilus* (Pejler, 1956). Both cladocerans and monogonont rotifers are cyclic parthenogens that mostly reproduce parthenogenetically, whereas sexual reproduction (mixis) is temporally restricted. During periods of mixis in the population, some females undergo meiosis and produce haploid eggs which develop into males. Diapausing dispersal stages are then produced by fertilization. Genetic exchange among populations of coherent species, as well as hybridization between genetically distinct populations isolated in space, thus depends on dispersal as well as synchronized mixis of dispersers and established populations. Sexual reproduction is induced by environmental factors such as food quantity and quality, population density and photoperiod in both cladocerans (e.g., Kleiven, Larsson & Hobaek, 1992; Alekseev & Lampert, 2001) and rotifers (e.g. Gilbert, 1992, 2003; Stelzer & Snell, 2003). Thus both dispersers and established populations must respond to the same environmental cue(s) so that sexual reproduction and subsequent introgression can occur.

Studies of reproductive isolation in rotifers have focussed on the induction of mictic reproduction (e.g. Gilbert, 2003; Stelzer & Snell, 2006) and mating behaviour (Snell, 1989; Gómez & Serra, 1995; Gilbert & Walsh, 2005). In *Brachionus*, mictic reproduction is triggered by density-dependent chemical cue(s) (Gilbert, 1992; Stelzer & Snell, 2003). Gilbert (2003) found that genetically divergent lineages in the *B. calyciflorus* Pallas 1766 complex are unable to induce mixis across species boundaries (see Gilbert & Walsh, 2005). In contrast, Stelzer & Snell (2006) demonstrated that both closely related and genetically distant members of the *B. plicatilis* (Müller 1786) complex can induce mictic reproduction reciprocally, thus indicating that hybridization among sibling species is possible.

Male mating behaviour in some rotifers is initiated by a female glycoprotein (Snell & Stelzer, 2005). The specificity of the signal varies among species. For example, males in the *Brachionus plicatilis* complex may respond to females of sibling species, although to a much lesser extent than to conspecific females (Rico-Martínez & Snell, 1995). However, the

male response to the female in the *B. calyciflorus* complex is so specific that male mating behaviour is not initiated by congeneric females (Gilbert & Walsh, 2005).

Both mixis induction and mating behaviour can therefore act as reproductive barriers or allow hybridization among sibling species, depending on the degree of differentiation that has evolved in a species complex. Other isolating mechanisms such as post-zygotic barriers have rarely been investigated in the Rotifera (Suatoni *et al.*, 2006) and the extent of hybridization in natural populations has rarely been studied (Gomez, Temprano & Serra, 1995, Ortells, Gómez & Serra, 2003).

Schröder & Walsh (2007) recently identified *Epiphanes senta* (Müller 1773) as a species complex and described three species based on morphological differences (trophi and diapausing eggs), *cox1* sequence divergence and partial reproductive isolation. *Epiphanes ukera* Schröder & Walsh 2007 occurs in temporary and permanent habitats in eastern Germany. *Epiphanes chihuahuaensis* Schröder & Walsh 2007 primarily inhabits shallow rock pools and other temporary habitats in the northern US Chihuahuan Desert. *Epiphanes hawaiiensis* Schröder & Walsh 2007 is known from a single population in a permanent alpine lake on the main island of Hawaii. Mixis induction is influenced by crowding in *E. ukera* (Schröder & Gilbert, 2004), but cues initiating sexual reproduction in the other two species have not been investigated. In two of these species (*E. ukera* and *E. chihuahuaensis*), males display a mating behaviour that is unique among rotifers (Schröder, 2003) and is similar to mate guarding found in other taxa. Males of these species will actively attend embryos about to hatch from eggs and, as soon as the female emerges, the male attempts to mate. In previously conducted cross-mating experiments with conspecific and heterospecific matings (Schröder & Walsh, 2007), we found that the German species is reproductively isolated from the two other species. Mictic Hawaiian and Texan females crossed with German males produced only male offspring and no diapausing eggs, indicating that females were not fertilized. Mating trials with Texan males and German females led to production of male offspring and, in some cases, to the production of unviable diapausing eggs or even death of the female. However, in crosses of Hawaiian males and Texan females, females produced viable diapausing eggs that could be induced to hatch (F₁ offspring). In conspecific mating trials all species produced viable diapausing eggs. Here we present further evidence of the divergence of these species by investigating aspects of reproductive isolation (induction of sexual reproduction, mating behaviour, mate choice) and the genetic composition of populations. We tested the hypothesis that differences in mating behaviour and selective mate choice lead to behavioural reproductive isolation among species. Further, we explored the possibility that different cues induce mixis in *E. hawaiiensis* and *E. chihuahuaensis*, thus effectively preventing hybridization between the two species. RAPD (Randomly Amplified Polymorphic DNA) markers were used to determine genetic structure within and among populations and to investigate potential dispersal or introgression among these species.

Methods

Sampling and culturing

Populations of three species in the *Epiphanes senta* complex were sampled in Germany (*E. ukera*), in the northern Chihuahuan Desert (*E. chihuahuaensis*; Texas, United States) and on the main island of Hawaii (*E. hawaiiensis*; United States). Three German populations were sampled: individuals from a population in the inundated Oder River floodplain were collected at two sites near Schwedt, Brandenburg (53°02.071'N, 14°15.626'E and 53°00.627'N, 14°15.992'E) and two other populations were sampled in small ponds near the floodplain (52°58.507'N, 14°09.663'E and 52°56.331'N, 14°01.324'E). Individuals from Texan populations were taken from three temporary rock pools (huecos) at Hueco Tanks

State Park and Historic Site (El Paso County, Texas, USA; 31°55.485'N, 106°02.538'W). *Epiphanes hawaiiensis* individuals were collected in Lake Waiau (19°48.675'N, 155°28.642'W), an alpine lake 237 m below the summit of Mauna Kea. Additional information on sampling is given in Schröder & Walsh (2007).

Clonal lineages were established from individual females of the three species that were collected in the field (*E. ukera*: six lineages from the floodplain, three and four lineages from two ponds, respectively; *E. chihuahuensis*: 15, 17 and 25 lineages from three rock pools, respectively; and *E. hawaiiensis*: 34 lineages). Mass cultures of clones were fed *Cryptomonas erosa* var. *reflexa* Marson *ad libitum* and maintained in modified MBL (Marine Biological Laboratory) medium (Stemberger, 1981) at 12°C with a 12h L:12h D photoperiod. Under these conditions, all clonal lineages reached high densities ($> 1000 \text{ L}^{-1}$).

Induction of sexual reproduction

As crowding is known to induce sexual reproduction in *E. ukera* (Schröder & Gilbert, 2004; Schröder & Walsh, 2007), this potential stimulus was also tested for representative clones of *E. hawaiiensis* and *E. chihuahuensis*. For these experiments we followed the design of Gilbert (2004) and Schröder & Gilbert (2004). All experiments were started with neonate females ($< 24 \text{ h}$ old) which were collected from cultures initiated 3 days earlier. Animals were fed $3 \times 10^4 \text{ cells mL}^{-1}$ and were maintained at 15 °C with a photoperiod of 12h L:12h D.

Low density treatments for *E. hawaiiensis* consisted of 15 mL medium per female, while females in high density treatments were cultured in 1 mL medium. For *E. chihuahuensis*, the low density treatment was conducted in a much larger volume (50 mL MBL) due to their high frequency of mixis (unpublished observations). The high density treatment consisted of 2 mL of medium per female per well.

Data from both experiments were analysed by t-tests on arcsine transformed percentages of the mictic offspring produced by each female. In addition, differences in the allocation to mictic versus amictic offspring over the reproductive lifespan were analysed by a logistic regression model (nominal logistic fit) with density and days of reproduction as main and interaction effects. All statistical tests were done using the statistical software package JMP (S.A.S Institute, 2001).

Mate choice experiments with *E. ukera* and *E. chihuahuensis*

To test the hypothesis that males respond to a specific cue that is produced only by conspecific developing embryos, mate choice experiments were conducted with males from both *E. ukera* and *E. chihuahuensis*. Reciprocal tests with *E. hawaiiensis* were not possible due to extremely infrequent periods of mixis. A female embryo with visible movement of the coronal cilia was selected from each species. These two embryos were placed in close proximity into a well, filled with 50µl of MBL medium, of a tissue culture plate. Then a male (age $\leq 2 \text{ d}$) of either the German or the Texan species was added. The male was observed until it encountered an egg and displayed typical egg recognition behaviour (Schröder, 2003). Starting from that point, the time that the male spent displaying this behaviour toward each of the two eggs was recorded over a 10 min period. Then the male was removed and a male of the other species was introduced into the same assay and its behaviour was recorded. Since the criterion of moving cilia provides a rough estimate of an embryo's age, and age can be critical in eliciting the egg recognition behaviour of the male (Schröder, 2003), the same pair of eggs was tested with males from both species. Twenty males of a clone from each species were tested in this manner. In order to avoid a bias created by the time that elapsed during a replicate, a male of *E. ukera* was tested first in 10

replicates and a male of *E. chihuahuaensis* was tested first in the remainder. Differences in the total amount of time directed toward each of the two eggs were analysed by a Student's t-test (JMP software, SAS Institute, 2001).

Mating behaviour in no-choice experiments

Mixis occurred frequently in mass cultures of German and Texan clones but only sporadically in the Hawaiian clones. Mating behaviour was observed and recorded during mictic intervals. Hawaiian males collected during sporadic mictic periods were used in the cross-mating experiments and too few were available to conduct mate-choice experiments as described above for *E. ukera* and *E. chihuahuaensis*. To test whether males displayed mating behaviour towards eggs or newly hatched females from different species, their behaviour was observed in the cross-mating experiments (see Schröder & Walsh, 2007) and is summarized below. Here we analysed whether mate guarding behaviour occurred towards eggs from other species. Male behaviour was observed for 5 min and the occurrence of the behaviour was recorded as present or absent. Differences in behaviour among species were analysed using a Kruskal-Wallis test (JMP software, SAS Institute, 2001).

Genetic differentiation among populations and species

RAPD-PCR was used to determine genetic variation within and among species (Williams *et al.*, 1990). Established clonal lineages derived from individual females isolated from field samples were used to obtain DNA. DNA template was extracted from 10–20 females of each lineage using a proteinase K lysis buffer (5% proteinase K solution [20mg/mL] in 1X PCR buffer: 3µl per female) incubated at 65°C for 1.5 h with subsequent enzyme deactivation at 95°C for 15 min. Samples were then frozen at –70°C for at least one hour, thawed and homogenized with a sterile glass pestle.

PCR reactions were run in duplicate for each sample to verify reproducibility of RAPD bands. All reactions were carried out in a 25 µl reaction volume containing 2.5 µl 10X PCR buffer, 0.75 µl MgCl₂ (50 mmol L⁻¹), 2.5 µl dNTP mix (1.25 mmol L⁻¹ each of dATP, dCTP, dGTP, dTTP), 1.5µl primer (5 µmol L⁻¹), 0.75 µl Taq polymerase (5 units µl⁻¹), and 3 µl template. Reactions were run on a thermocycler (Techne TC-412) with an initial denaturation at 80°C for 15 min, 45 cycles of 1 min at 94°C, 1 min at 35°C, 1 min at 72°C, and a final extension of 1 min at 72°C. PCR products were separated by electrophoresis at 100 V for 10 min and 60 V for 4 h in 1X TAE buffer on a 1.8 % 3:1 agarose gel stained with ethidium bromide (37.5 µg 100 mL⁻¹). In an initial screening process, 14 10-mer primers (Operon Technologies) were tested for polymorphisms and reproducibility in banding pattern. Three primers were selected for the final analysis: OP-H7 (5'-CTGCATCGTG-3'), OP-H16 (5'-TCTCAGCTGG-3') and OP-H18 (5'-GAATCGGCCA-3').

Digital images of RAPD bands were analysed with GeneImagIR 3.56 (Scanalytics, Inc.) using a 1KB plus DNA ladder (Invitrogen) to determine fragment size. Bands of different sizes were considered different loci and their presence or absence was scored across all genotypes. Mean error rates per locus were calculated according to Pompanon *et al.* (2005). Clonal lineages originating from a total of 104 individuals were analysed, 57 from *E. chihuahuaensis*, 34 from *E. hawaiiensis* and 13 from *E. ukera*.

A UPGMA cluster analysis, using a model of evolutionary change for restriction sites (Nei & Li, 1979), was conducted using FreeTree (Hapl, Pavlíček & Flegr, 2001).

Genetic variation within and between populations was analysed using AMOVA (Analysis of Molecular Variance) implemented in Arlequin 3.11 (Excoffier, Laval & Schneider, 2005). Three hypotheses of population grouping were tested: the first assumed that all three species are separate entities. The second assumed the German and the Texan species were one group

(based on the shared trait of guarding behaviour) and the Hawaiian species as a separate group. The third hypothesis also assumed two groups, one consisting of the Hawaiian and the Texan species (based on the assumption that introgression occurs) and a separate group consisting of the German species. Gene diversity (Nei, 1987) was also computed with the Arlequin software (Excoffier, Laval & Schneider, 2005).

Bayesian analysis of population structure was conducted using Structure 2.3 (Pritchard, Stephens & Donnelly, 2000). The analysis was based on a model of K populations with admixture and α starting at 1.0. The analysis was run for $K = 1$ to $K = 8$ genetic clusters to test a range for K larger than the actual number of sampled populations ($n = 7$). The LOCPRIOR model was used to accommodate for the relatively small dataset (Hubisz *et al.*, 2009). Initially the model was run eight times to test for stability: four runs assuming allele frequencies among populations are independent; four runs assuming allele frequencies are correlated among populations. Based on these results, an additional 11 runs were conducted under the assumption of independence of allele frequencies. In all runs, the burn-in period was 130,000 iterations and data were collected over an additional 100,000 iterations.

Because the likelihood distribution often reaches an asymptote once the most likely K value has been reached in the Structure analysis (Evanno, Regnaut & Goudet, 2005), the statistic ΔK was calculated to further validate the estimated K .

Results

Induction of sexual reproduction

Production of mictic offspring in the Hawaiian species was very low. Density did not affect sexual reproduction (t-test; $t = 0.159$, $df = 18$, $P = 0.695$). Under high density conditions, 1.5% of offspring were mictic while 2.1% were mictic under low density conditions (Fig. 1a). Further, no pattern of differential investment in mictic and amictic offspring was discernable. Only one (high density) or two females (low density) produced few mictic offspring during the experiment, thus no statistical analysis could be done.

In contrast, mictic offspring production was much higher and density-dependent in the Texan species. Females produced a significantly higher proportion of mictic offspring at a density equivalent to 500 individuals L^{-1} than at density equivalent to 20 individuals L^{-1} (t-test; $t = 8.307$, $df = 22$, $P = 0.0087$; Fig. 1b). Overall offspring production was higher under high density conditions. However investment in amictic offspring dropped to almost 0 after the third day of reproduction in these treatments, whereas under low density conditions investment was nearly constant from the third to the sixth day of reproduction (Fig. 2). There was also a significant interaction of density effects and reproductive period (Table 1).

Mate choice experiments with *E. ukera* and *E. chihuahuaensis*

Males of the Texan species displayed mate guarding behaviour, as was previously described for the German species (Schröder, 2003). When offered a choice between female embryos of their own and the German species, Texan males did not differentiate between the two eggs. They attended eggs from both populations for equal amounts of time (t-test; $t = 0.15$, $df = 38$, $P = 0.706$) over a 10 min period (Fig. 3a). They attended eggs from their own species for 223.8 ± 50.0 s (mean $\pm$ 1 s.e.) and eggs from the German species for 197.9 ± 46.0 s (mean $\pm$ 1 s.e.). In contrast, German males displayed mate guarding behaviour and attended eggs only from their own population (Fig. 3b). When they made contact with an egg from the Texan species, they only briefly examined the egg with their corona and then swam until they encountered and subsequently guarded a conspecific egg. They spent 497.7 ± 41.5 s (mean $\pm$ 1 s.e.) attending their own eggs, and 0.5 ± 0.4 s (mean $\pm$ 1 s.e.) examining eggs

from the Texan population. This difference is highly significant (t-test; $t = 143.9$, $df = 38$, $P < 0.0001$).

Mating behaviour in cross-mating experiments

Eggs are deposited individually by *E. chihuahuensis* females and are not generally aggregated. In cross-mating experiments, German males never attended eggs from the Texan species, nor did they copulate with hatching Texan females ($n = 24$). Texan males, however, always attended eggs from the German species and mated with the hatching females ($n = 24$).

Unlike males from the German and the Texan species, Hawaiian males were never observed displaying mate guarding behaviour. Instead, males copulated with free-swimming females that had hatched from their eggs. In this species, male eggs are deposited in clusters with both mictic and amictic eggs from other females. Males were frequently observed mating with females hatching from the same cluster.

In cross-mating experiments, Hawaiian males did not guard female eggs of either the Texan ($n = 8$) or the German species ($n = 18$). However, males were observed copulating with newly hatched females of both species when they encountered them in the assay. Differences in the occurrence of mate guarding behaviour towards eggs from the other species were highly significant (Kruskal-Wallis-Test: $df = 3$, $\chi^2 = 73$, $P < 0.0001$) since only Texan males displayed mate guarding behaviour towards heterospecific developing females.

Genetic differentiation among populations and species

RAPD analysis resulted in 42 loci, 41 of which were polymorphic. All 42 loci had reproducible banding patterns in duplicate samples. Four loci were specific for the Hawaiian species while the German species had two unique loci, and none were found for the Texan species. The average error rate for all 42 loci was 1.9%.

Within species, the percentage of polymorphic loci was lowest in the Hawaii population (7 %), ranged from 43 % – 52 % in the Texan populations and from 33 % – 36 % in the German populations. Gene diversity was 0.68 in Lake Waiau and ranged from 0.72 – 0.88 in the Texan populations and from 0.86 – 1.0 in the German populations.

UPGMA cluster analysis revealed three distinct clades corresponding to the three geographic regions, with genetic distances of 0.38 to 0.44, and bootstrap values of >86 % (Fig. 4). Genetic differentiation within the population of the Hawaiian species was low, bootstrap values of groups within this clade were <51 %. Genetic structure within Texan and German species was more defined. The Texan species was divided into two clusters with a genetic distance of 0.31 with a bootstrap support of 78 and 91 %, respectively. These clusters were also identified by Bayesian analysis (Fig. 5). The German species consisted of two groups separated by a genetic distance of 0.18 (bootstrap support 78 and 83 %). AMOVA showed that most of the genetic diversity was due to variation within populations (Table 2). Genetic variation among populations and within regions was significant ($P < 0.0001$) and accounted for 13.6 – 21.1 % of the total variation. However, only the model where all three regions were treated as separate entities explained genetic variation among regions ($P = 0.0477$).

Bayesian analysis of population structure produced stable results in independent runs under the assumption that allele frequencies are independent among populations while assuming correlated frequencies caused dramatic shifts in posterior probabilities for K during data collection. Using the model with independent allele frequencies, three to four genetic clusters among the 104 individuals were identified. The likelihood distribution for K reached

a maximum at $K = 4$, with $\ln Pr(X|K)$ being only slightly smaller for larger K . The posterior probability was highest for $K = 4$, ΔK had highest values for $K = 3$, followed by $K = 4$ (see the electronic Appendix S1 for details). When $K = 4$ (Fig. 5), all Hawaiian individuals have a very high probability (average probability $P = 0.999$) of belonging to a single genetic cluster as do individuals collected from German populations ($P = 0.993$). Probabilities for assignment of the Texan individuals to these genetic clusters were <0.0003 on average. The Texan species was split into two clusters (inferred ancestry from one of these clusters with P ranging between 0.743 and 0.998), while German or Hawaiian individuals were assigned to these clusters with a probability of <0.003 on average. Although Texan individuals clustered in two genetic groups unique to *E. chihuahuaensis*, these clusters did not correspond to the sampled populations. Both clusters were present in all sampled rock pools in varying frequencies. At $K > 4$, individuals were still assigned to four clusters with high probabilities, assignments to clusters five to eight occurred with probabilities ≤ 0.009 (see electronic Appendix S2).

Only one individual had a probability higher than 0.01 of being assigned to a cluster other than their actual origin. Ancestry of this individual from the German species was inferred to originate from the Hawaiian cluster with a probability of 0.017 and from one of the two Texan clusters with probabilities of 0.010 and 0.002, respectively.

Discussion

Cryptic speciation has mostly been inferred based on deep divergences of genetic lineages, often without further investigation of reproductive isolation. Genetic distances among *E. ukera*, *E. chihuahuaensis* and *E. hawaiiensis*, as reflected in partial *cox1* sequences (Schröder & Walsh, 2007), are comparable in magnitude to those found in other cryptic complexes of monogonont rotifers (Gómez *et al.*, 2002; Gilbert & Walsh, 2005; Suatoni *et al.*, 2006). However, cross-breeding experiments have shown that hybridization between *E. chihuahuaensis* and *E. hawaiiensis* is possible (Schröder & Walsh, 2007). Laboratory mating experiments resulted in viable diapausing eggs and several hybrid clones were maintained for several generations under laboratory conditions (unpublished observations).

Although reproductive isolation is not complete among these three *Epiphanes* species, genetic exchange between allopatric lineages is not evident based on the RAPD analysis. Analysis of Molecular Variance does not indicate any coherent structure among the three species and supports the supposition that each is a separate genetic entity. Furthermore, Bayesian analysis indicates that introgression has not occurred between *E. hawaiiensis* and *E. chihuahuaensis*. Not a single clone was assigned to a species other than its own with a probability higher than 0.017, indicating that hybridization and gene flow among the two species is highly unlikely.

This is quite different from cladocerans, where introgression among sibling species is a frequent phenomenon (e.g. Petrusek *et al.*, 2008; Taylor, Sprengler & Ishida, 2005). Within species complexes of the Rotifera, introgression and interspecific hybridization have not yet been documented. For example, *B. ibericus* and the 'Almenara'-clade in the *B. plicatilis* complex are distinct lineages with a genetic distance of ~23% in *cox1* sequences, but they form viable F_1 hybrids in cross-breeding experiments (Suatoni *et al.*, 2006). However, in natural coexisting populations of these two lineages, no evidence for hybridization has been found (Ortells *et al.*, 2000; Ortells, Gómez & Serra, 2003). Species of the *E. senta* complex may provide another example of the lack of introgression, although it is uncertain whether members of the complex are sympatric or strictly allopatric.

Bayesian analysis further demonstrates genetic differentiation within *E. chihuahuensis*: in this species, individuals are assigned to two genetic clusters with high probability, but they are allied in clusters with individuals of populations in neighbouring pools (Fig. 5). If gene flow occurs among populations of the Texan species it would also be expected that individuals with mixed ancestry would exist. These were rarely found in our samples, although some individuals with mixed ancestry were identified in both clusters (Fig. 5). Genetic differentiation within *E. chihuahuensis* may indicate the existence of additional reproductively isolated lineages. Using only reproducible RAPD loci kept the average error rate for all loci low and in a range comparable to AFLP analyses (Bonin *et al.*, 2004), but also reduced the number of loci which limited the power of the analysis. Therefore, further studies are necessary to determine whether *E. chihuahuensis* comprises a species complex.

Importantly, our results show that several factors may contribute to the lack of hybridization and gene flow among three allopatric *Epiphanes* species. Differences in environmental cues involved in mixis induction, mate recognition and mating behaviour separate the three species and, along with geographic separation, may lead to reproductive isolation and its reinforcement.

Induction of sexual reproduction

Mixis induction, as a form of intraspecific communication, is likely under stabilizing selection (García-Roger *et al.*, 2009). García-Roger *et al.* (2009) demonstrate that different species of the *B. plicatilis* complex continue to respond to the same chemical mixis cue even though they are reproductively isolated species. In contrast, in two sibling species of the *Brachionus calyciflorus* complex (Gilbert, 2003) mixis induction has been demonstrated to require different cues. Still, these species require a cue that is directly related to high density. While in *Brachionus* the density-dependent cue seems to be conserved, in the *E. senta* complex this trait may have been lost in the Hawaiian species.

Our experiments showed that sexual reproduction in *E. chihuahuensis* is significantly influenced by population density, similar to *E. ukera* (Schröder & Gilbert, 2004). *Epiphanes chihuahuensis* inhabits temporary desert rock pools that fill and dry within short time periods. Thus, rapid production of new diapausing eggs through sexual reproduction is of selective advantage in these habitats. Mictic reproduction in natural populations often occurs soon after rock pools have filled with water during seasonal rains and mixis rates are usually very high (unpublished observations). Similarly, the proportion of mictic offspring in our experiment was high even under low density conditions. In contrast, mictic offspring production in *E. hawaiiensis* was very low under laboratory conditions and population density did not have a significant effect in inducing mixis.

Although induction of sexual reproduction could be tested with only a few representatives for each species, we have cultured numerous clones of all three species for years. In cultures of *E. chihuahuensis* and *E. ukera*, mixis is frequently observed at high densities. This suggests that population density as a mixis cue is prevalent in both species and is probably present in cryptic lineages within these species. Patterns of sexual reproduction in natural populations of *E. hawaiiensis* are unknown, but in laboratory cultures under very high density conditions ($> 8,000 \text{ L}^{-1}$), females reproduce primarily asexually and sexual reproduction occurs only sporadically for short periods. Therefore, cues inducing mictic reproduction in *E. hawaiiensis* do not occur frequently under laboratory conditions. Assuming natural populations of *E. hawaiiensis* and *E. chihuahuensis* respond to different cues, they would be effectively reproductively isolated when occurring in sympatry – unless mixis occurred in both populations simultaneously in response to different environmental conditions.

Observations of mating behaviour and mate choice experiments

In addition to previous cross-breeding experiments (Schröder & Walsh, 2007), mate choice experiments conducted in this study demonstrate behavioural reproductive isolation of *E. ukera* from the other two species. The cue(s) that elicits mate guarding behaviour are probably chemical in nature (Schröder, 2003). Cues released by female embryos of *E. ukera* must be different from those of female *E. chihuahuaensis* embryos, since the response of *E. ukera* males is asymmetrical. However these differences are probably small since *E. chihuahuaensis* males do not differentiate between conspecific and heterospecific female eggs. Even though mate guarding behaviour has been lost or did not evolve in the Hawaiian species, Hawaiian males copulate with *E. ukera* or *E. chihuahuaensis* females they encounter. The mate recognition factor causing Hawaiian males to copulate with a female may be the same cue that elicits mate guarding behaviour in *E. chihuahuaensis* and *E. ukera*, but evokes a different form of mating behaviour. This behaviour, however, does not lead to reproductive isolation between *E. hawaiiensis* and *E. chihuahuaensis*, since males of both species respond indiscriminately to heterospecific females and production of viable hybrid diapausing eggs is possible. In contrast, pre- or post-zygotic reproductive isolation mechanisms prevent hybridization between *E. chihuahuaensis* or *E. hawaiiensis* males and *E. ukera* females (Schröder & Walsh, 2007).

Behavioural reproductive isolation has also been demonstrated among species of other cryptic complexes (Gómez & Serra, 1995; Gilbert & Walsh, 2005; Glatzel & Königshoff, 2005). In most of these cases, the behavioural pattern is the same across species in the complex, but males differentiate between conspecific and congeneric females. This is not the case for these *Epiphanes* species, where two species exhibit mate guarding behaviour (*E. ukera* and *E. chihuahuaensis*) while it is lacking in *E. hawaiiensis*. Mictic and amictic females of *E. hawaiiensis* deposit their eggs in clusters together with eggs deposited by other females. Males hatching from eggs readily encounter females hatching from the same cluster. In contrast, females of *E. chihuahuaensis* and *E. ukera* deposit their eggs individually. Males spend considerable amounts of time searching for females and precopulatory mate guarding may increase male fitness (Schröder, 2003). Thus differences in mating behaviour may have arisen as adaptations to ecological conditions.

Geographic separation

Geographic distance among studied populations of both *E. ukera* and *E. chihuahuaensis* is small (ranging from only ~30 m – 20 km) compared to distances among the localities of the three species (5,000 – 12,000 km). Unlike members of other cryptic species complexes, where individual species often have wide regional distributions and sometimes occur in sympatry with sibling species (e.g. Ortells, Gómez & Serra, 2003; Gómez *et al.*, 2007b), species of the *Epiphanes senta* complex have disjunct distribution patterns in space and time (see Schröder & Walsh, 2007). Although Chihuahuan desert aquatic habitats have been widely sampled (Walsh *et al.*, 2007; Wallace *et al.*, 2008), *E. chihuahuaensis* has been found in few locations. Populations of *E. ukera* are known only from several locations in eastern Germany, but other regions of Europe have not been sampled since the description of the species. Similarly, the only Pacific islands sampled were those of the Hawaiian archipelago where *E. hawaiiensis* was found (Jersabek, 2003). Therefore, current distribution ranges for these species probably reflect sampling effort rather than actual biogeographic patterns. Nonetheless, members of the *E. senta* species complex are less commonly found than those of the *B. plicatilis* or *B. calyciflorus* complexes. These disjunct distribution patterns may further limit dispersal and gene flow among populations and prevent introgression between species.

In conclusion, our results confirm that *E. chihuahuaensis*, *E. hawaiiensis* and *E. ukera* are distinct genetic lineages. UPGMA analysis of RAPD data indicates that genetic distance between *E. ukera* and *E. chihuahuaensis* is less than between the *E. hawaiiensis* and the other two species. This is consistent with the facts that males from both the German and Texan species display the same mating behaviour, and that sexual reproduction is induced by cues related to density in both species. In contrast, mate guarding behaviour is not apparent in the Hawaiian species, and sexual reproduction is induced by cues other than population density. However, genetic distances among the three species for the mitochondrial *cox1* gene are different: in this case *E. hawaiiensis* is more similar to *E. ukera* (uncorrected *P* values are 0.109 between *E. hawaiiensis* and *E. ukera* and 0.125 between *E. ukera* and *E. chihuahuaensis*; Schröder & Walsh, 2007). Inconsistencies in the two types of genetic data may be resolved by more detailed analyses using additional polymorphic markers. Nonetheless both RAPD and mtDNA sequences indicate that all three *Epiphanes* lineages have evolved independently for long periods of time. Lack of hybridization may be due in part to the large distances that separate the three species and effectively limit the number of possible colonization events. In addition, habitat characteristics of the three species differ greatly so that local adaptation may prevent successful colonization events by sibling species, consistent with the hypothesis that persistent founder effects determine community composition (De Meester *et al.*, 2002). Because these three taxa occur in such different habitats (temporary rock pools, seasonal floodplains, a permanent alpine lake), local selective pressures associated with these habitats may have led to genetic and reproductive differentiation and, ultimately, to the formation of new species.

Understanding complex genetic and ecological interactions among species is necessary to understand fully the dynamics of freshwater aquatic systems. Cryptic species that are adapted to very different environments, as are those of the *E. senta* complex, will affect these systems differently, and thus contribute uniquely to their complexity and diversity. For instance, sex is induced differently in *E. chihuahuaensis* and *E. hawaiiensis* and this will probably influence population dynamics differentially. Prevailing parthenogenetic reproduction in *E. hawaiiensis* will lead to larger population sizes and increased clonal selection through intraspecific competition, thus decreasing genetic diversity of active populations, and subsequently that of egg banks. In contrast, sexual reproduction at low densities in *E. chihuahuaensis* will limit population size and intraspecific competition, thereby increasing genetic diversity in the egg banks that will establish future populations. Similarly, predator-prey interactions and other food web dynamics are likely to be affected by demographic differences among cryptic species. Thus, evolutionary changes leading to cryptic speciation may change ecological interactions within aquatic communities and selective processes affecting other species (Fussmann, Loreau & Abrams, 2007; Pelletier, Garant & Hendry, 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Alekseev V, Lampert W. Maternal control of resting-egg production in *Daphnia*. *Nature*. 2001; 414:899–901. [PubMed: 11780060]
- Barton NH, Hewitt GM. Analysis of hybrid zones. *Annual Review of Ecology and Systematics*. 1985; 16:113–148.
- Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P. How to track and assess genotyping errors in population genetics studies. *Molecular Ecology*. 2004; 13:3261–3273. [PubMed: 15487987]
- Brede N, Sandrock C, Straile D, Spaak P, Jankowski T, Streit B, Schwenk K. The impact of human-made ecological changes on the genetic architecture of *Daphnia* species. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:4758–4763. [PubMed: 19273852]
- De Meester L, Gómez A, Okamura B, Schwenk K. The Monopolization Hypothesis and the dispersal-gene flow paradox in aquatic organisms. *Acta Oecologica - International Journal of Ecology*. 2002; 23:121–135.
- Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*. 2005; 14:2611–2620. [PubMed: 15969739]
- Excoffier L, Laval G, Schneider S. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*. 2005; 1:47–50. [PubMed: 19325852]
- Fontaneto D, Kaya M, Herniou EA, Barraclough TG. Extreme levels of hidden diversity in microscopic animals (Rotifera) revealed by DNA taxonomy. *Molecular Phylogenetics and Evolution*. 2009; 53:182–189. [PubMed: 19398026]
- Forró L, Korovchinski NM, Kotov AA, Petrusek A. Global diversity of cladocerans (Cladocera; Crustacea) in freshwater. *Hydrobiologia*. 2008; 595:177–184.
- Fussmann GF, Loreau M, Abrams PA. Eco-evolutionary dynamics of communities and ecosystems. *Functional Ecology*. 2007; 21:465–477.
- Garcia-Roger EM, Dias N, Carmona MJ, Serra M. Crossed induction of sex in sympatric congeneric rotifer populations. *Limnology and Oceanography*. 2009; 54:1845–1854.
- Gilbert JJ. Rotifera. In: Adiyodi, KG.; Adiyodi, RG., editors. *Reproductive Biology of Invertebrates*. Oxford & IBH Publishing Co; New Delhi: 1992. p. 115–136.
- Gilbert JJ. Specificity of crowding response that induces sexuality in the rotifer *Brachionus*. *Limnology and Oceanography*. 2003; 48:1297–1303.
- Gilbert JJ. Population density, sexual reproduction and diapause in monogonont rotifers: new data for *Brachionus* and a review. *Journal of Limnology*. 2004; 63 (Suppl I):32–36.
- Gilbert JJ, Walsh EJ. *Brachionus calyciflorus* is a species complex: Mating behavior and genetic differentiation among four geographically isolated strains. *Hydrobiologia*. 2005; 546:257–265.
- Glatzel T, Königshoff D. Cross-breeding experiments among different populations of the ‘cosmopolitan’ species *Phyllognathopus viguieri* (Copepoda: Harpacticoida). *Hydrobiologia*. 2005; 534:141–149.
- Gómez A, Hughes RN, Wright PJ, Carvalho GR, Lunt DH. Mitochondrial DNA phylogeography and mating compatibility reveal marked genetic structuring and speciation in the NE Atlantic bryozoan *Celleporella hyalina*. *Molecular Ecology*. 2007a; 16:2173–2188.
- Gómez A, Montero-Pau J, Lunt DH, Serra M, Campillo S. Persistent genetic signatures of colonization in *Brachionus manjavacas* rotifers in the Iberian Peninsula. *Molecular Ecology*. 2007b; 16:3228–3240.
- Gómez A, Serra M. Behavioral reproductive isolation among sympatric strains of *Brachionus plicatilis* Müller 1786: insights into the status of this taxonomic species. *Hydrobiologia*. 1995; 313/314:111–119.
- Gómez A, Serra M, Carvalho GR, Lunt DH. Speciation in ancient cryptic species complexes: evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). *Evolution*. 2002; 56:1431–1444. [PubMed: 12206243]
- Gomez A, Temprano M, Serra M. Ecological genetics of a cyclical parthenogen in temporary habitats. *Journal of Evolutionary Biology*. 1995; 8:601–622.

- Gómez A, Wright PJ, Lunt DH, Cancino JM, Carvalho GR, Hughes RN. Mating trials validate the use of DNA barcoding to reveal cryptic speciation of a marine bryozoan taxon. *Proceedings of the Royal Society of London, Series B*. 2007c; 274:199–207.
- Hapl V, Pavlíček A, Flegr J. Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. *International Journal of Systematic and Evolutionary Microbiology*. 2001; 51:731–735. [PubMed: 11411692]
- Hubisz MJ, Falush D, Stephens M, Pritchard JK. Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*. 2009; 9:1322–1332. [PubMed: 21564903]
- Hurwood DA, Hughes JM, Bunn SE, Cleary C. Population structure in the freshwater shrimp (*Paratya australiensis*) inferred from allozymes and mitochondrial DNA. *Heredity*. 2003; 90:64–70. [PubMed: 12522427]
- Jersabek, C. Freshwater Rotifera (Monogononta) from Hawai'i - a preliminary checklist. In: Evenhuis, NL.; Eldredge, LG., editors. *Records of the Hawaii Biological Survey*. Bishop Museum Press; Honolulu: 2003. p. 46-72. Bishop Museum Occasional Papers
- Jones CS, Rollinson D, Mimpfoundi R, Ouma J, Kariuki HC, Noble LR. Molecular evolution of freshwater snail intermediate hosts within the *Bulinus forskalii* group. *Parasitology Supplements*. 2001; 123:S277–292.
- Keller B, Wolinska J, Manca M, Spaak P. Spatial, environmental and anthropogenic effects on the taxon composition of hybridizing *Daphnia*. *Philosophical Transactions of the Royal Society of London, Series B - Biological Sciences*. 2008; 363:2943–2952.
- Kleiven OT, Larsson P, Hobaek A. Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos*. 1992; 65:197–206.
- Knowlton N. Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*. 2000; 420:73–90.
- Mills S, Lunt DH, Gómez A. Global isolation by distance despite strong regional phylogeography in a small metazoan. *BMC Evolutionary Biology*. 2007; 7:225. [PubMed: 17999774]
- Nei, M. *Molecular evolutionary genetics*. Columbia University Press; New York: 1987.
- Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*. 1979; 76:5269–5273. [PubMed: 291943]
- Ortells R, Gómez A, Serra M. Coexistence of cryptic rotifer species: ecological and genetic characterisation of *Brachionus plicatilis*. *Freshwater Biology*. 2003; 48:2194–2202.
- Ortells R, Snell TW, Gómez A, Serra M. Patterns of genetic differentiation in resting egg banks of a rotifer species complex in Spain. *Archiv für Hydrobiologie*. 2000; 149:529–551.
- Pejler B. Introgression in planktonic Rotatoria with some points of view on its causes and conceivable results. *Evolution*. 1956; 10:246–261.
- Pelletier F, Garant D, Hendry AP. Eco-evolutionary dynamics. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2009; 364:1483–1489.
- Petrusek A, Seda J, Macháček J, Ruthová S, Smilauer P. *Daphnia* hybridization along ecological gradients in pelagic environments: the potential for the presence of hybrid zones in plankton. *Philosophical Transactions of the Royal Society of London, Series B - Biological Sciences*. 2008; 363:2931–2941.
- Pompanon F, Bonin A, Bellemain E, Taberlet P. Genotyping errors: causes, consequences and solutions. *Nature Reviews Genetics*. 2005; 6:847–859.
- Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics*. 2000; 155:945–959. [PubMed: 10835412]
- Rico-Martínez R, Snell TW. Male discrimination of female *Brachionus plicatilis* Müller and *Brachionus rotundiformis* Tschugunoff (Rotifera). *Journal of Experimental Marine Biology and Ecology*. 1995; 190:39–49.
- S.A.S Institute. JMP statistical software package, version 4.0.4. SAS Institute, Inc; Cary, NC: 2001.

- Schröder T. Precopulatory mate guarding and mating behaviour in the rotifer *Epiphanes senta* (Monogononta, Rotifera). *Proceedings of the Royal Society of London, Series B*. 2003; 270:1965–1970. [PubMed: 14561311]
- Schröder T, Gilbert JJ. Transgenerational plasticity for sexual reproduction and diapause in the life cycle of monogonont rotifers: Intraclonal, intraspecific and interspecific variation in the response to crowding. *Functional Ecology*. 2004; 18:458–466.
- Schröder T, Walsh EJ. Cryptic speciation in the cosmopolitan *Epiphanes senta* complex (Monogononta, Rotifera) with the description of new species. *Hydrobiologia*. 2007; 593:129–140.
- Schwenk K, Brede N, Streit B. Introduction. Extent, processes and evolutionary impact of interspecific hybridization in animals. *Philosophical Transactions of the Royal Society of London, Series B - Biological Sciences*. 2008; 363:2805–2811.
- Snell TW. Systematics, reproductive isolation and species boundaries in monogonont rotifers. *Hydrobiologia*. 1989; 186:299–310.
- Snell TW, Stelzer CP. Removal of surface glycoproteins and transfer among *Brachionus* species. *Hydrobiologia*. 2005; 546:267–274.
- Stelzer CP, Snell TW. Induction of sexual reproduction in *Brachionus plicatilis* (Monogononta, Rotifera) by a density-dependent chemical cue. *Limnology and Oceanography*. 2003; 48:939–943.
- Stelzer CP, Snell TW. Specificity of the crowding response in the *Brachionus plicatilis* species complex. *Limnology and Oceanography*. 2006; 51:125–130.
- Stemberger RS. A general approach to the culture of planktonic rotifers. *Canadian Journal of Fisheries and Aquatic Sciences*. 1981; 38:721–724.
- Suatoni E, Vicario S, Rice S, Snell T, Caccone A. An analysis of species boundaries and biogeographic patterns in a cryptic species complex: The rotifer *Brachionus plicatilis*. *Molecular Phylogenetics and Evolution*. 2006; 41:86–98. [PubMed: 16815046]
- Taylor DJ, Sprengler HL, Ishida S. Geographic and phylogenetic evidence for dispersed nuclear introgression in a daphniid with sexual propagules. *Molecular Ecology*. 2005; 14:525–537. [PubMed: 15660943]
- Thum RA, Derry AM. Taxonomic implications for diaptomid copepods based on contrasting patterns of mitochondrial DNA sequence divergences in four morphospecies. *Hydrobiologia*. 2008; 614:197–207.
- Thum RA, Harrison RG. Deep genetic divergences among morphologically similar and parapatric *Skistodiaptomus* (Copepoda: Calanoida: Diaptomidae) challenge the hypothesis of Pleistocene speciation. *Biological Journal of the Linnean Society*. 2009; 96:150–165.
- Wallace RL, Walsh EJ, Schröder T, Rico-Martínez R, Ríos-Arana JV. Species composition and distribution of rotifers in Chihuahuan Desert waters of México: is everything everywhere? *Verhandlungen der Internationalen Vereinigung für theoretische und angewandte Limnologie*. 2008; 30:73–76.
- Walsh EJ, Schröder T, Arroyo ML, Wallace RL. How well do single samples reflect rotifer species diversity? A test based on interannual variation of rotifer communities in Big Bend National Park (Texas, USA). *Hydrobiologia*. 2007; 593:39–47.
- Walsh EJ, Schröder T, Wallace RL, Rico-Martínez R. Cryptic speciation in *Lecane bulla* (Monogononta: Rotifera) in Chihuahuan Desert waters. *Verhandlungen Internationale Vereinigung für theoretische und angewandte Limnologie*. 2009; 30:1046–1050.
- Wellborn GA, Cothran RD. Phenotypic similarity and differentiation among sympatric cryptic species in a freshwater amphipod species complex. *Freshwater Biology*. 2004; 49:1–13.
- Wellborn GA, Cothran RD. Niche diversity in crustacean cryptic species: complementarity in spatial distribution and predation risk. *Oecologia*. 2007; 154:175–183. [PubMed: 17684770]
- Wilkerson RC, Foster PG, Li C, Sallum MAM. Molecular phylogeny of neotropical *Anopheles* (*Nyssorhynchus*) *albitarsis* species complex (Diptera: Culicidae). *Annals of the Entomological Society of America*. 2005; 98:918–925. [PubMed: 18079976]
- Williams JGK, Kubelik RR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*. 1990; 18:6531–6535. [PubMed: 1979162]

Fig. 1. Proportions of mictic offspring production under low and high density conditions in (a) *E. hawaiiensis* and (b) *E. chihuahuensis*. Bars represent means $\pm$ 1 S.E. Proportions of mictic offspring production in *E. chihuahuensis* under low density and high density conditions are significantly different (**: $P = 0.0087$).

Fig. 2.

Amictic and mictic offspring allocation during the reproductive lifetime of *E. chihuahuensis* females under low density and high density conditions. [Symbols represent means of the number of deposited eggs per day (± 1 S.E.)]. Effects of time of reproduction and interaction effects between density and time of reproduction on amictic and mictic offspring allocation are significant (see Table 1).

Fig. 3. Mate guarding behaviour displayed by (a) Texan and (b) German males towards conspecific and heterospecific female embryos. Bars represent the mean time (± 1 S.E.) that males spent on eggs during the trial period. Differences in male behaviour of *E. ukera* are significant (****; $P < 0.0001$).

Fig. 4. Cluster analysis (UPGMA) based on the Nei & Li (1979) model for genetic variation in restriction sites of endonucleases. Bootstrap values (> 50%) are given above nodes. Colour codes of clades represent clusters identified in the Bayesian analysis (see Fig. 5).

Fig. 5.

Bayesian analysis of population structure for $K = 4$. Each individual is represented by a bar that is partitioned into four fractions representing the individual's probability of belonging to one of the four clusters. The individuals are grouped by region and sampling sites.

Table 1

Nominal logistic fit for the propensity of mictic and amictic offspring production in *Epiphanes chihuahuaensis* with density and time of reproduction as effects.

Whole model test				
	-log likelihood	df	χ^2	P
Full model	533.22	15	290.51	< 0.0001
Reduced model	678.48			
Difference	145.26			
Wald tests of effects				
		df	Wald χ^2	P
Density		1	0.744	0.3885
Time of reproduction		7	93.337	< 0.0001
Density * time of reproduction		7	72.417	< 0.0001

Hierarchical analysis of genetic variation among six populations of three *Epiphanes* species under three models of geographic clustering based on mating behaviour, reproductive isolation and *cox1* sequence similarity.

Table 2

source of variation	three separate regions			Hawaii – (Chihuahuan Desert – Oder River)			(Hawaii – Chihuahuan Desert) – Oder River		
	% variation	P	fixation indices	% variation	P	fixation indices	% variation	P	fixation indices
among regions	8.75	0.0477	0.088 (Φ_{CT})	10.09	0.1603	0.101 (Φ_{CT})	1.27	0.8616	0.013 (Φ_{CT})
among populations within regions	13.56	<0.0001	0.149 (Φ_{SC})	13.91	<0.0001	0.155 (Φ_{SC})	21.05	<0.0001	0.208 (Φ_{SC})
within populations	77.69	<0.0001	0.223 (Φ_{ST})	76.0	<0.0001	0.240 (Φ_{ST})	80.22	<0.0001	0.198 (Φ_{ST})