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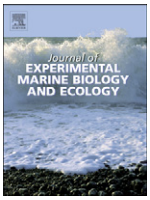

Multigenerational life history traits of the copepod *Tigriopus* from the Northwest Pacific rim in crossbreeding experiments

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ABSTRACT

We screened life-history variables (survival, first batch hatchability, metamorphosis rate, sex ratio) during several generations following crossbreedings of *Tigriopus japonicus* (3 localities in Korea, Japan, and Hong Kong) and *Tigriopus thailandensis* (1 population from 1 locality in Thailand). Crossbreeding results showed incomplete reproductive separation of populations of *T. japonicus* and *T. thailandensis*. Life history variables obtained during the present study indicated both higher performance and lower survival and fecundity—depending on populations—as well as faster and slower metamorphosis rates of pure lines compared to hybrid lines. These contrasting effects were particularly evident in the crossings of geographically more distant populations, indicating that their genetic compatibility was changed by showing both hybrid vigor and hybrid breakdown effects. In the present study, the fitness of hybrids was equal to or greater than that of the control lines in the F_1 generation and decreased from the F_2 generation with the exception of that of the TJJ×TT hybrids. The hybrid fitness changes did not correlate with genetic divergence of the copepods. Although TJJ×TT crosses were successfully mated and brought forth offspring in the present study, their first hybrid generations did not grow to adults and died. Survival percentage dropped drastically for both mating combinations, TJH-M×TT-F ($P<0.01$) and TJH-F×TT-M ($P<0.05$) and no 3rd generation (F_3) was produced for this mating pair combination. Metamorphosis rate was similar to the above survival percentage. Sex ratios of TJH-M×TT-F in F_2 generation were significantly lower than that of the control lines ($P<0.05$). No successful breeding of hybrid lines in the F_3 generation was indicated since the hybrids were not grown to copepodid stages. Fecundity, survival, metamorphosis rate, sex ratio and number of successful breeding lines of the hybrid and the control lines for *T. japonicus* Japan strain (TJJ) and *T. thailandensis* showed little changes in successive generations. In the crossbreeding of TJJ and TT, the fecundities of both females (F_0) which were mated with different species were significantly higher than that of the control line ($P<0.05$). Although F_1 hybrids (TJJ-F×TT-M) showed similar survivorship and metamorphosis rate to control lines, none of the surviving copepods reached the adult stage during a 16-day period contrary to the control lines. Similarity scores and genetic distance between three *T. japonicus* populations and *T. thailandensis* using partial mitochondrial COI and complete ITS1, -2 rDNA sequences revealed large genetic differences. *Tigriopus* from Hong Kong showed the highest DNA similarity (in terms of COI and ITS) and lowest genetic distance to *T. thailandensis*. *Tigriopus* from Japan and from Korea both showed a lower DNA similarity and higher genetic distance compared to *T. thailandensis*. Comparison of CO1 amino acid (aa) sequence from different isolates shared the highest similarity between *T. japonicus* from Japan with *T. japonicus* from Hong Kong with 97.0 and the lowest score was obtained from the geographically most distant populations, *T. japonicus* from Busan and *T. thailandensis* from Bangkok with 87.0. Comparison of CO1 and ITS protein and DNA similarity (%) and calculated genetic distance showed that *T. thailandensis* had a protein similarity of 100% with *T. japonicus* from Hong Kong, and also a highest DNA similarity of COI and ITS (of 94.6% and 98.4%, respectively). This correlated with the lowest genetic distance of 0.05% and 0.006% for COI and ITS, respectively, of this geographically closest *Tigriopus* populations. The overall analysis showed that *T. japonicus* populations from Japan and Korea were comparatively similar but genetically distant to *T. thailandensis*. The present crossbreeding experiment demonstrated that 2 populations of *Tigriopus* that were separated as the 2 species, *T. japonicus* and *T. thailandensis* solely on morphological grounds belonged in fact to one biological species.

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1. Introduction

Knowlton (2000) suggested that in several marine groups a morphological approach has led to excessive lumping of cryptic (=sibling) biological or molecular-criterion species (Knowlton, 2000). Cryptic species are a common phenomenon in copepods. Examples of cryptic marine copepod species were shown by Frost (1989) for seven biochemically and genetically distinct species of *Pseudocalanus* in northern oceans. McKinnon et al. (1992) demonstrated biochemically different sibling species of the calanoid *Acartia* in Australia. Ganz and Burton (1995) showed outbreeding depression in populations of *Tigriopus californicus*. Lee (2000) examined the global distribution of the calanoid copepod *Eurytemora affinis* and demonstrated by mitochondrial DNA and interpopulation mating experiments that this presumably uniformly distributed species actually comprised of several reproductively isolated cryptic species. Lee and Frost (2002) found reproductively isolated populations when crossbreeding individuals of *E. affinis* were collected several thousand of kilometers apart. The same holds for the harpacticoid copepod *Cletocamptus deitersi* (see Rocha-Olivares et al., 2001) where genetic lineages indicated by morphological distinctions were also proven to be different species (Gómez et al., 2004). Whether the reverse could be true and populations are separated on morphological grounds that in fact are little genetically isolated and no reproductive isolation occurs remains to be tested in most of these cases.

Crossbreeding experiments are operational means for proving or disproving infertility or reproductive isolation (Edmands, 2002). In this way, crossbreeding experiments can prove a species to be a biological species (following the biospecies concept *sensu* Mayr, 1942). Crossbreeding experiments are commonly restricted to taxa that are breedable under laboratory conditions or in captivity. In the case of successful interbreeding of individuals that belong to disjunct populations and successful reproduction of the F_1 generation, the representatives are defined as belonging to the same biological species. The interpretation of failing experiments or the production of sterile offspring is more problematic. Such “negative results” do not necessarily indicate distinctly separate evolutionary species since the reproductive performance of individuals could be disturbed by artificial experimental conditions. At the same time, testing individuals show forced reproduction at captivity conditions—that would not cross at natural field conditions. The prospective explanatory strength of the outcome of crossbreeding experiments has, therefore, to be seen in proportion.

Crossbreeding between populations can lead to an increase in survival and reproduction (Darwin, 1876)—an effect also called hybrid vigor (Birchler et al., 2003). Such enhanced fitness is generally attributed to a heterozygous condition where dominant alleles can effectively mask deleterious recessives (Burton, 1997; Mingroni, 2004). Hybridization between populations can also result in a reduction of fitness, referred to as outbreeding depression (Dobzhansky, 1947; Edmands, 2001). In this case, a reduction of fitness is attributed to a break up of co-adapted gene combinations, resulting in some individuals which are homozygous for one parent's genes at one locus and the other parent's gene at another locus (Noor and Feder, 2006). A reduction of fitness is explained by the loss or breakup of locally co-adapted gene complexes (Burton, 1990; Ellison and Burton, 2008). Outbreeding depression can also be viewed as genetic costs of dispersing (Brown, 1991) since individuals choosing mates from different populations may have lower fitness than individuals choosing mates from the same population. There is considerable concern about the negative effects of crossbreeding. Crossbreeding attempts may result from accidental introductions, translocations due to species transfer (Wyngaard, 1998) or to compensate for inbreeding (Burton, 1990). Since crossbreeding may cause serious problems, such as in applied fields like conservation management of endangered and threatened populations, husbandry, and aquaculture, a more detailed understanding of outbreeding depression is critical for the understanding of gene flow between populations.

A serious effect of mating among close relatives is inbreeding depression, commonly causing a fitness reduction within the inbred lines (Futuyma, 1998). Since homozygosity—particularly of recessive mutations—increase with inbreeding, this leads to the expression of mostly deleterious mutations with effects like reduced survivorship, growth rates and fertility (Edmands, 1999; Willett and Burton, 2004). We used the harpacticoid copepod *Tigriopus* in our efforts to test whether there was indeed genetic isolation between the populations that were belonging to *T. japonicus* and *T. thailandensis* which has been described as a new species in good standing (Chullasorn et al., 2012). *Tigriopus* has favorable biological attributes that allow its use as a marine model organism (e.g. small size, high fecundity, short life-cycle, sexual reproduction, distinct life stages—see Raissudin et al., 2007). Representatives of *Tigriopus* breed year-round, particularly under controlled laboratory conditions, and show both out- and inbreeding depression (Brown, 1991). *Tigriopus* combines a wide geographic distribution (Handschumacher et al., 2010) with restricted dispersal (Burton and Lee, 1994; Johnson, 2001) and high ecophysiological amplitude (Damgaard and Davenport, 1994; Davenport et al., 1997). Its wide distribution, high density in nature and available knowledge about its cultivation facilitate the use of this organism for evolutionary (Burton, 1998; Edmands, 1999), molecular (Lee et al., 2007, 2008), or toxicological research (Forget et al., 1998, 2003; Kwok and Leung, 2005) in laboratories worldwide.

The aim of the present study was to test for reproductive isolation and genetic compatibility across several generations among *T. japonicus* and *T. thailandensis* using crossbreeding experiments. *T. thailandensis* is described as a species in good standing on morphologically grounds (Chullasorn et al., 2012). We were combining mates from disjunct geographic populations of *T. japonicus* and *T. thailandensis* and compared life table results of different crossings to show whether reproductive isolation was established between both species that are defined as yet on morphological grounds only.

2. Materials and methods

2.1. Copepod collection and maintenance

Copepods used in this study were collected from four locations in Korea and Thailand as summarized in Table 1. Single gravid females were isolated and the isofemale lines were cultured in 1 L beakers with the chlorophyte alga *Tetraselmis suecica* Kylin (Butch) in an incubator kept at 20 °C at a 16 h light:8 h dark cycle. The culture medium was changed with fresh UV treated and filtered (1 μm) seawater of 32 PSU salinity every 10th day. These lines were maintained in the laboratory for more than 1 year before experimental crosses were begun (average generation time was 14.1 days at 20 °C, see Lee et al., 2008). *Tetraselmis suecica* were cultivated in 2 L transparent glass bottles with Walne's medium (Walne, 1970) using filtered seawater (1 μm mesh of glass fiber filter) that was then autoclave sterilized. The phytoplankton cultures were incubated at 20 °C, with a 24-h light photoperiod at 4000 lux.

Table 1
Characteristics of *Tigriopus* spp. used in the present study and COI DNA sequence GenBank accession numbers.

Species	Isolation locality	Abbreviation	GenBank accession no.
T. japonicus	Busan, South Korea	TJK	DQ225119
T. japonicus	Nagasaki, Japan	TJJ	DQ225126
T. japonicus	Clear Water Bay,Hong Kong	TJH	DQ225127
T. thailandensis	Near Bangkok, Thailand	TT	EU520442