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Original Research Paper

Growth Characteristics of *Platymonas subcordiformis* and *Oxyrrhis marina* in Their Co-culture Systems

Xinlong An†, Xuemei Li, Zhixia Li and Yanling Zhang
Ocean College, Agricultural University of Hebei, Qinhuangdao 066003, Hebei, China
†Corresponding author: Xinlong An

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ABSTRACT

This study was aimed to investigate the growth characteristics of living diet *Platymonas subcordiformis* for aquaculture animals and its harmful organism *Oxyrrhis marina* in co-culture systems to provide experimental evidences for discussing their succession processes. The colour changes of culture media of *P. subcordiformis* and growth characteristics of *O. marina* and *P. subcordiformis* in co-culture were analysed by the combined methods of macro-observation, microscopic examination and microscopic counting. The results showed that with the decrease of the initial density of *O. marina* cell (0.65×10^4 cells mL^{-1}, 0.37×10^4 cells mL^{-1} and 0.11×10^4 cells mL^{-1} in *P. subcordiformis* culture media), the time that the population of *O. marina* reached the stationary phase required longer, were 4d, 5d and 7d after inoculated by *O. marina*, respectively, and the death time of all cells of *P. subcordiformis* became longer, were 9d, 10d and 12d after inoculated by *O. marina*, respectively. After inoculation 9d, pale pink appeared in upper layer of culture media of *P. subcordiformis* in flasks and the density of *O. marina* was 2.10×10^5 cells mL^{-1}. The results also indicated that the variation tendency of cell densities of *O. marina* and *P. subcordiformis* cultured in aquariums were consistent with that in flasks basically, pink flocs appeared in upper layer of culture media of *P. subcordiformis* after inoculation 12d and the density of *O. marina* was 2.10×10^6 cells mL^{-1}. The experimental results showed that the feeding of *O. marina* on *P. subcordiformis* was affected by the initial concentration of *O. marina*, and *P. subcordiformis* populations were evolved to *O. marina* populations during the 15 days culture period, the colour of culture media appeared pale pink to pink depends on density of *O. marina* was confirmed, too. Also, the colour changes of *P. subcordiformis* culture media are indications of pollution by *O. marina*, which will be important for culture process of *P. subcordiformis*.

INTRODUCTION

Oxyrrhis marina Dujardin, a common heterotrophic dinoflagellate in many intertidal and coastal habitats, is typically regarded as cosmopolitan or globally distributed (Watts et al. 2011). On the basis of published data, the *O. marina* morphospecies is best described as broadly distributed, inhabiting areas of the Atlantic and Pacific coasts of the USA, the Gulf of Mexico, the Atlantic coasts of Europe, the Mediterranean and Baltic Seas, Persian Gulf, the Indian Ocean and the western Pacific (Watts et al. 2011). In China, *O. marina* is widely distributed in coastal environments of Qingdao, Qinhuangdao, Shenzhen and Shanghai (An et al. 2011b, Ke et al. 2011). Owing to easy cultivation, *O. marina* has been used as a model organism to examine the feeding responses of heterotrophic protists to many marine microalgae, such as *Chattonella marina* (An et al. 2015), *Isochrysis galbana* (Martel 2006), *Dunaliella tertiolecta* (Hartz et al. 2008, Hartz et al. 2011), *Rhodomonas salina* (Florian et al. 2010), *Chlorella pyrenoidosa* (An et al. 2012a), *Platymonas subcordiformis* (An et al. 2011a), *Karenia mikimotoi* (An et al. 2012c), bacteria (An et al. 2015), fungi (Droop 1959, Jeong et al. 2010) and so on.

Platymonas subcordiformis is the earliest living diet for aquaculture animals cultured in China. *O. marina* is one of important harmful organisms on *P. subcordiformis* for feeding it directly (An et al. 2012b), and changing rules of culture media colour of *P. subcordiformis* could indicate the feeding process of *O. marina* on it. But the growth characteristics of *O. marina* and *P. subcordiformis* quantificationally in their co-culture systems have not been reported, so it lacks theoretical basis for guiding the productive practices in hatcheries.

The goal of this study is to investigate the growth characteristics of *P. subcordiformis* and *O. marina* in co-culture to provide experimental evidences quantitatively for discussing serious consequences of *P. subcordiformis* culture media contaminated by *O. marina*.

MATERIALS AND METHODS

The Source of samples: Wild populations of *O. marina* and *P. subcordiformis* were collected from the coastal waters near Qinhuangdao in the Bohai Sea in 2010 (39°48' N;

119°42' E) and in 2007 (39°50' N; 119°39' E), China, respectively.

Isolating, identification and culture of *O. marina* and *P. subcordiformis*: *O. marina* and *P. subcordiformis* were identified based on external morphology by light microscopy, respectively. Then the population of *O. marina* was cultured in seawater (the salinity of seawater was 32 ‰) on a diet of a natural bacterial assemblage grown in starch-enriched seawater in a 1000 mL conical flask (Lowe et al. 2005), with culture temperature 20°C and light intensity 60 μE m^{-2} s^{-1}. The population of *P. subcordiformis* was cultured in f/2 medium, culture conditions as above.

Co-culture of *O. marina* and *P. subcordiformis* in conical flasks: For the experiment, initial concentrations of *O. marina* and *P. subcordiformis* were established using an autopipette to deliver predetermined volumes of known cell concentrations to the flasks. Triplicate 250 mL conical flasks (mixtures of *O. marina* and *P. subcordiformis*), and triplicate control flasks (*P. subcordiformis* only) were set up for each predator-prey combination. The flasks were filled to capacity with filtered seawater, placed on rotating wheels at 0.9 rpm for 3 min in the morning, in the afternoon and in the evening respectively, and incubated at 20°C under illumination of 60 μE $m^{-2}s^{-1}$ in a 12:12h light:dark cycle. To determine actual predator and prey densities at the beginning of the experiment, a 10 mL aliquot was removed from each flasks, fixed with 5% acid Lugol's solution and examined with a compound microscope to determine predator and prey abundance by enumerating cells in three 1 mL Sedgwick-Rafter counting chambers (SRCs). The flasks were filled again to capacity with freshly filtered seawater, capped, and placed on rotating wheels with the environmental conditions described above. After counting, the initial concentrations of *O. marina* were 0.65×10^4 cells mL^{-1} (in No.1 flasks), 0.37×10^4 cells mL^{-1} (in No.2 flasks) and 0.11×10^4 cells mL^{-1} (in No.3 flasks), respectively, and initial concentration of *P. subcordiformis* was 5.60×10^5 cells mL^{-1}.

Co-culture of *O. marina* and *P. subcordiformis* in aquariums: For the experiment, aquariums were washed clearly and added seawater, and then sterilized by NaClO for active chlorine 25g m^{-3}. Residual chlorine was neutralized by $Na_2S_2O_3$ after aeration for 8~10 h, then residual chlorine was tested after aeration for 4~6 h again. There was an airstone, a thermometer and a heating rod in each aquarium, and 2 lights along both sides of each aquarium respectively. After sterilization, *P. subcordiformis* in exponential phase was inoculated according to ratio of 1:5 volumes of *P. subcordiformis* and seawater. Triplicate 15L aquariums (mixtures of *O. marina* and *P. subcordiformis*), and triplicate 15L control aquariums (*P. subcordiformis* only) were set up for each predator-prey combination, mixed evenly for 3 min in the morning, in the afternoon and in the evening respectively, and incubated at 20°C under illumination of 60 μE $m^{-2}s^{-1}$ in a 12:12h light:dark cycle. To determine actual predator and prey densities at the beginning of the experiment, a 10 mL aliquot was removed from each flask, fixed with 5% acid Lugol's solution and examined with a compound microscope to determine predator and prey abundance by enumerating cells in three 1 mL SRCs. Then the aquariums were filled again to capacity with freshly filtered seawater and incubated with the environmental conditions described above. After counting, the initial concentration of *O. marina* was 0.06×10^4 cells mL^{-1}, and initial concentration of *P. subcordiformis* was 3.09×10^5 cells mL^{-1}.

Taking pictures and microscopic counting: All experimental flasks and aquariums were incubated for 15days, photos were taken before rotation/mixing and counted microscopically after rotation/mixing every day.

RESULTS AND DISCUSSION

Colour changes of *Platymonas subcordiformis* culture media after inoculated by *Oxyrrhis marina* in conical flasks: Photos of colour changes of *P. subcordiformis* culture media inoculated by *O. marina* were taken before rotation in the morning every day; the initial concentration of *O. marina* was 0.65×10^4 cells mL^{-1}, and initial concentration of *P. subcordiformis* was 5.60×10^5 cells mL^{-1} (Fig. 1). Colours of *P. subcordiformis* culture medium inoculated by other two initial concentrations of *O. marina* changed similar to above. The culture medium was deep grass green before inoculation by *O. marina* (Fig. 1A). After 1 day, transparencies of culture media of all experimental flasks increased and colours became lighter (Fig. 1B). On the 2nd day, transparencies increased and colours became lighter obviously, and a little precipitates on the bottoms of all experimental flasks appeared (Fig. 1C). After 7 days, colours of culture media of all experimental flasks became lighter, transparencies increased and precipitates increased obviously (Fig. 1D). On the 9th day, transparencies increased more obviously, a little precipitate existed still and pale pink appeared in the upper layer of culture media of *P. subcordiformis* (Fig. 1E). There were *O. marina* and a very few *P. subcordiformis* in the pale pink culture media, and the density of *O. marina* was 2.10×10^5 cells mL^{-1} obtained by microscopic counting. After rotation for 3 mins, the colour of pale pink receded and became lower than that before rotation; the density of *O. marina* was 6.46×10^4 cells mL^{-1} then.

Colour changes of culture media of *O. marina* were re-

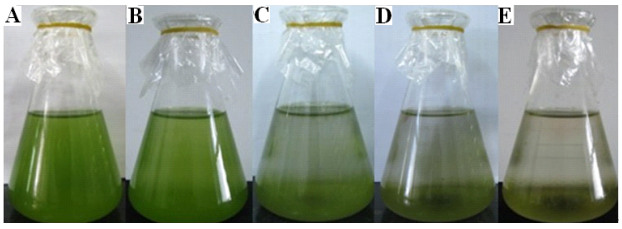

Fig. 1: Color changes of culture media of *P. subcordiformis* inoculated by *O. marina* in conical flasks. Notes: A: culture medium of *P. subcordiformis*; B-E: culture media of *P. subcordiformis* inoculated by *O. marina* for 1, 2, 7 and 9 days, respectively.

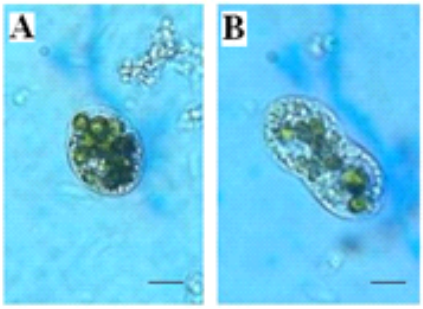

Fig. 2: Feeding of *O. marina* on *P. subcordiformis.*
Notes: A: a vegetative cell of *O.marina*, B: a dividing cell of *O. marina*. bar = 15 μm.

searched in the world. The changes of transparencies and colours of *P. subcordiformis* culture media inoculated by *O. marina* were consistent with that of *Karenia mikimotoi* (An et al. 2012c). Lowe et al. (2011) revealed that *O. marina* appears colourless but with pink pigmentation that was apparent in concentrated cultures. As revealed in our study, on the 9th day, pale pink appeared in the upper layer of culture media of *P. subcordiformis* inoculated by *O. marina* and the density of *O. marina* was 2.10×10^5 cells mL^{-1}, while after rotation for 3 mins, pale pink in the upper layer receded, the density of *O. marina* was 6.46×10^4 cells mL^{-1}. Zhang et al. (2014) revealed that in the culture conditions of regular shaking in every morning, afternoon and evening once, culture media of *O. marina* in flask did not appear pink when their density ranged from 4.8×10^4 to 7.8×10^4 cells mL^{-1}. According to the colour changes of *P. subcordiformis* culture media in conical flasks, the colour changes were indication of contamination with *O. marina*.

We also found that *P. subcordiformis* could be seen clearly whether in a vegetative (Fig. 2A) or a dividing (Fig. 2B) *O. marina* cell.

An et al. (2012b) revealed that the feeding method of *O. marina* on *P. subcordiformis* is filter feeding, that is to say, *O. marina* could engulf the whole cells of *P. subcordiformis* directly. In feeding process of *O. marina* on *P. subcordiformis*, feeding currents moved from above the predator toward the singular depression of the predator along the flow lines, and then *O. marina* intercepted and ingested a single *P. subcordiformis* cell in the feeding current. Li et al. (2013) revealed that under suitable environmental conditions, binary fission is the most common reproductive mode of *O. marina*, the number of cells increased rapidly through this mode of reproduction. In this experiment, phenomenon of dividing *O. marina* cells containing *P. subcordiformis* was consistent with that dividing *Noctiluca scintillans* cells containing *P. subcordiformis* (Qi et al. 1994).

Density changes of *Platymonas subcordiformis* and *Oxyrrhis marina* in conical flasks: Density changes of *O. marina* and *P. subcordiformis* cultured in conical flasks for 15 days are shown in Fig. 3. With increasing elapsed incubation time, the concentrations of *P. subcordiformis* in the experimental flasks decreased continuously, the concentrations of *O. marina* in the experimental flasks increased and became stable at 4d in No. 1 flasks (Fig. 3A), 5d in No. 2 flasks (Fig. 3B) and 7d in No. 3 flasks (Fig. 3C), respectively. With increasing elapsed incubation time, the concentrations of *O. marina* in No. 1 flasks continuously increased and reached to the maximum 6.46×10^4 cells mL^{-1}at 9d, but then continuously decreased and down to 4.42×10^4 cells mL^{-1} at 15d; while the concentrations of *P. subcordiformis* decreased obviously after inoculated by *O. marina* at the 2nd day and down to 0.90×10^2 cells mL^{-1} at 15d (Fig. 3A). With increasing elapsed incubation time, the concentrations of *O. marina* in No. 2 flasks continuously increased and reached to the maximum 5.90×10^4 cells mL^{-1} at 9d, but then continuously decreased and down to 4.36×10^4 cells mL^{-1} at 15d; while the concentrations of *P. subcordiformis* decreased obviously after inoculation by *O. marina* at the 3rd day and down to 0.10×10^3 cells mL^{-1} at 15d (Fig. 4B). With increasing elapsed incubation time, the concentrations of *O. marina* in No. 3 flasks continuously increased and reached to the maximum 5.35×10^4 cells mL^{-1} at 9d, but then continuously decreased and down to 4.63×10^4 cells mL^{-1} at 15d; while the concentrations of *P. subcordiformis* decreased slowly after inoculation by *O. marina* at the 3rd and down to 0.11×10^3 cells mL^{-1} at 15d (Fig. 3C).

The results showed that the feeding of *O. marina* on *P. subcordiformis* was affected by the initial concentration of *O. marina*. With the decrease of the initial density of *O. marina*, the time that the populations of *O. marina* reached the stationary phases required longer after inoculation by *O. marina* respectively, and the death time of all cells of *P.*

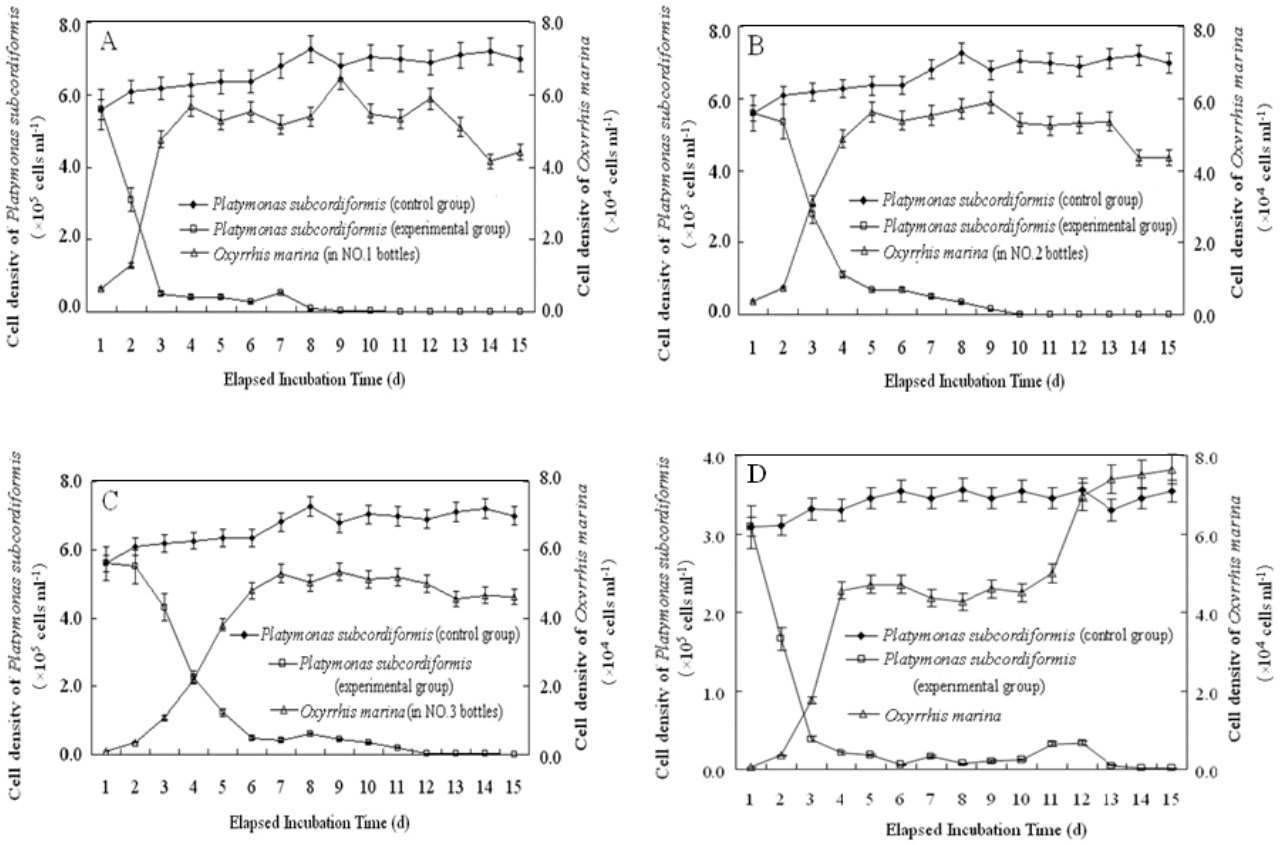

Fig. 3: Growth Characteristics of *Oxyrrhis marina* and *Platymonas subcordiformis* in co-cultures in flasks and aquariums.

subcordiformis became longer after inoculation by *O. marina* respectively. During the 15 days culture period, all *P. subcordiformis* populations were evolved to *O. marina* populations within the scope of initial densities designed in the paper.

Colour changes of *Platymonas subcordiformis* culture media after inoculation by *Oxyrrhis marina* in aquariums: Photos of colour changes of *P. subcordiformis* culture media inoculated by *O. marina* were taken before mixing in the morning every day, the initial concentration of *O. marina* was 0.06×10^4 cells mL^{-1}, and initial concentration of *P. subcordiformis* was 3.09×10^5 cells mL^{-1} (Fig. 4). The culture media were deep grass green before inoculation by *O. marina* (Fig. 4A). After 2~5 days, transparencies of culture media increased and colours were chartreuse, *P. subcordiformis* attached to the walls of aquariums and precipitates on the bottom of aquariums appeared (Fig. 4B~4D). On the 10th day, transparencies increased and pale pink appeared in the upper layer of culture media of *P. subcordiformis* (Fig. 4E). During the 12th~15th days, deep pink of culture media maintained (Fig. 4F). On the 12th day, cells attaching to the walls of aquariums and precipitates on the bottoms of aquariums decreased obviously, lots of banded pink flocs appeared in the upper layer of culture media after stopping aeration for 5 mins (Fig. 5). The density of *O. marina* in the pale pink culture media was 2.10×10^6 cells mL^{-1} obtained by microscopic counting, higher than that after mixing, 7.40×10^4 cells mL^{-1}.

Density changes of *Platymonas subcordiformis* and *Oxyrrhis marina* in aquariums: Density changes of *O. marina* and *P. subcordiformis* cultured in aquariums for 15 days are shown in Fig. 3. With increasing elapsed incubation time, the concentrations of *P. subcordiformis* in the aquariums decreased continuously until disappear, the concentrations of *O. marina* in the aquariums increased gradually. After inoculated by *O. marina* 1~3 days, the concentration of *P. subcordiformis* decreased from 3.09×10^5 cells mL^{-1} to 0.38×10^5 cells mL^{-1}; the concentration of *O. marina* increased exponentially from 0.06×10^4 cells mL^{-1} to 4.56×10^4 cells mL^{-1}, and was stable during 4~10 days and no significant difference ($P>0.05$). On the 11th and 12th day, concentrations of *P. subcordiformis* increased from 0.12×10^5

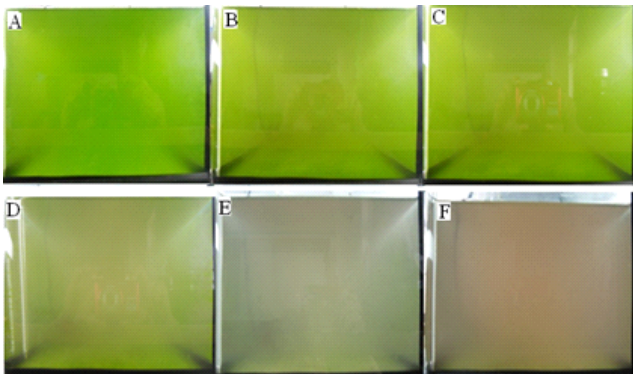

Fig. 4: Color changes of culture media of *P. subcordiformis* inoculated by *O. marina* in aquariums. Notes: A: culture medium of *P. subcordiformis*; B-F: culture media of *P. subcordiformis* inoculated by *O. marina* for 2, 3, 5, 10 and 12 days, respectively.

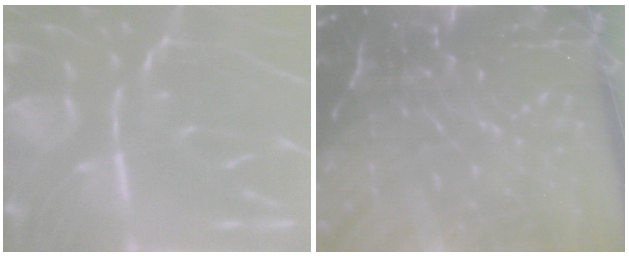

Fig. 5: Pink flocs of *O. marina* in aquariums.

cells mL^{-1} to 0.33×10^5 cells mL^{-1} and suddenly for cells attaching to the walls fell off, accordingly, concentrations of *O. marina* increased significantly from 4.51×10^4 cells mL^{-1} to 6.96×10^4 cells mL-1 (Fig. 3D). Similar to culture media of *P. subcordiformis* inoculated by *O. marina* in conical flasks, all *P. subcordiformis* populations were evolved to *O. marina* populations within the scope of initial densities designed in aquariums during the 15 days culture period.

In the aquariums, the density of *O. marina* in the pale pink culture media was 2.10×10^6 cells mL^{-1}, higher than that after mixing, 7.40×10^4 cells mL^{-1}. Begun et al. (2004) revealed that the highest density of *O. marina* in a pink bloom in the water of Amursky Bay (Sea of Japan) caused by *O. marina* was 4.43×10^5 cells mL^{-1}. So the density of *O. marina* was one of the important factors of determining whether pink appears in the culture media or not, and pink may appear when the density of *O. marina* increases to 10^5 cells mL^{-1}. In this paper, *w*ith increasing elapsed incubation time, the concentrations of *P. subcordiformis* in the aquariums decreased continuously until disappear, the concentration of *O. marina* in the aquariums increased gradually, colours of *P. subcordiformis* culture media changed from deep grass green to pale pink until pink. It is revealed that during the 15 days culture period, all *P. subcordiformis* populations were evolved to *O. marina* populations within the concentrations designed in co-culture in this experiment.

CONCLUSIONS

1. Density of *O. marina* is one of the important factors of determining whether pink appears in the culture media or not, pink appears when the density of *O. marina* increases to 10^5 cells mL^{-1}.
2. Within the concentrations designed in this experiment, the feeding of *O. marina* on *P. subcordiformis* was affected by the initial concentration of *O. marina*. During the 15 days culture period, all *P. subcordiformis* populations were evolved to *O. marina* populations.
3. The feeding of *O. marina* on *P. subcordiformis* is the most important reason for succession of *P. subcordiformis* population to *O. marina* population.

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