

Summary

Comparative morphological and molecular phylogenetic studies on divergence and differentiation of two closely-related intertidal hermit crabs, *Pagurus lanuginosus* and *Pagurus maculosus* (Crustacea: Anomura: Paguridae)

「酷似する 2 種の岩礁潮間帯性ヤドカリ *Pagurus lanuginosus* および *Pagurus maculosus* (甲殻類: 異尾類: ホンヤドカリ科) における分岐と分化に関する形態的・分子系統学的研究」

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INTRODUCTION

Pagurus lanuginosus De Haan, 1849 and *Pagurus maculosus* Komai and Imafuku, 1996 are intertidal hermit crabs. They have very similar morphological characters except the color of chromatophore scattered on the pereopods and head, in which chromatophores are white in one form but black in another (Imafuku and Ikeda 1995). Therefore, they have been long-time considered as color morphs under the name of *Pagurus lanuginosus*. In 1996, Komai and Imafuku reported some subtle differences in morphological characteristics and pre-mating behavior between these two color morphs. Thus, they described the white morph as a new species, *P. maculosus*, and referred the black-spot type as *P. lanuginosus* (*sensu stricto*).

Though the color of chromatophore is the most traceable feature to distinguish these two species, previous studies did not describe its pattern in details. Therefore, in the present study, I extensively investigated the distribution pattern of chromatophore on the surface of body and appendages in adults. In addition, a comparative study of mouthparts morphology of adult was conducted with the assumption that there may have differences between the two species. Since, it is reported by Imafuku and Ikeda (1995) that the geographical distribution of these two species is different as, the *P. maculosus* prefers open oceanic wave-interacting areas whereas the *P. lanuginosus* prefers rather wave-protected areas like gulf. Further, the developmental pattern of chromatophore during larval stages was never been investigated. Therefore, a comparative study of larval morphological characters was conducted to identify differences between the two species. Furthermore, the taxonomic relationship of these two species has never been evaluated from a molecular phylogenetic viewpoint. To infer the relationship between the two species, molecular phylogenetic analyses have been conducted based on mitochondrial

DNA sequences. Moreover, their conspicuous chromatophore color pattern becomes of particular interest to examine the molecular basis of different color formation in hermit crab lineage. Therefore, to identify the candidate genes for different color in chromatophores, a comparative transcriptome analysis was conducted.

MATERIALS & METHODS

Specimens of *Pagurus lanuginosus* and *P. maculosus* with other congeneric species of *Pagurus* hermit crabs were collected widely from Japanese intertidal areas during spring to autumn (mainly in the breeding season) in 2013 to 2014. Species identification was done according to the Book, “*Japanese Crustacean Decapods and Stomatopods in Color Vol. I. Macrura, Anomura and Stomatopoda* (Miyake 1991). Specimens were preserved either into ethanol or by frozen for phylogenetic study. However, for transcriptomic study, specimens were kept alive. To compare larval morphology, the ovigerous female were reared in the laboratory until hatch.

Images of living adults were taken under a zoom stereomicroscope (Nikon SMZ1500). For SEM observations, specimens were rinsed in distilled water and dehydrated through a graded ethanol series. Specimens were further dehydrated by freeze dryer (ES 1030, Hitachi). After being dried, specimens were attached to metal stubs and sputter-coated with gold by ion sputter (E 1030, Hitachi). Observation were made in a SEM (S 4300, Hitachi). For comparative study of larval morphology, larvae were reared up to crab-1 stage in the laboratory with artificial environmental condition. Specimens of each larval stage were dissected under a zoom stereomicroscope (Nikon SMZ1500) and temporary slides were prepared in pure glycerin. The illustration of the appendages were done from the pictures taken with a digital camera (Evolt, E-300, Olympus) attached to the microscope. The phylogenetic relationship of the two species was investigated based on mitochondrial genes, sequenced by next-generation technologies. RNA-seq was done for conducting comparative transcriptome analyses of the two species to identify the candidate genes responsible for the different color of chromatophores.

RESULTS & DISCUSSION

P. lanuginosus has black chromatophores on olive green or brownish grey background whereas and *P. maculosus* has white chromatophores on dark brown and bluish grey background. There were no considerable differences in chromatophore distribution. However, differences in setal types were observed in mandible and maxillule of *P. maculosus*. Such changes also observed in larval morphology. For example, at each larval stage, the number

of setae was higher in *P. maculosus* than that in *P. lanuginosus*. These results may indicate that the mouthpart morphology of *P. maculosus* has been specialized for their habitat. The results of molecular phylogeny showed that *P. lanuginosus* and *P. maculosus* are two monophyletic and sister taxa. The nucleotide diversity between these two species was 4% at mitochondrial DNA and it was estimated that they have been diverged about 4 to 5 million years ago. To investigate the candidate genes for different color in chromatophore, *Drosophila melanogaster* pigment-genes as used as reference. Because the causal genes of pigmentation of other crab species have not been well studied. The results showed that the overall gene expression patterns between the two species were almost similar, however, there were differences in terms of pigment related genes expression. For example, melanin interfering gene like 'Bm-ebony' was expressed in *P. maculosus* but was absent in *P. lanuginosus*. Some other melanogenesis genes were significantly more expressed in *P. lanuginosus* than in *P. maculosus*. These genes could be the candidates for the black color pattern.

要旨

十脚甲殻類の分類において、種の同定および記載には伝統的に形態形質が用いられてきた。しかしこの分類群は大変に種の多様性が高く、形態形質が酷似するが行動や生態が明らかに異なり生殖的に隔離されている種、すなわち隠蔽種が知られている。このような隠蔽種についての、正確な同定や生態調査は、生物の種分化や表現型可塑性の進化や発達の過程の解明における良い研究材料となる。

本研究は、日本およびその近海に分布する岩礁潮間帯にすむ外部形態の類似する 2 種のヤドカリ、*Pagurus lanuginosus* (ケアシホンヤドカリ) および *Pagurus maculosus* (ホシゾラホンヤドカリ) を対象に、各種の成長段階における形態比較、並びに系統分類学的研究を行うことで、この 2 種の系統関係について明らかにし、種分化や表現型可塑性について考察を行った。

研究対象とした種の成体について、2 種を区別する形態的特徴は、歩脚並びに頭部に散在する斑点の色が異なることが知られているが、その詳細な分布パターンについての報告はない。このため、まず 2 種の成体の、より詳細な形態的差異を明らかにするために、斑点の分布やその他の形質について生時の写真に基づく比較を行った。その結果、鉗脚上面、第 2 脚、第 3 脚の両側面の色彩に大きな違いがあった。さらに、この 2 種は生息場所に違いが見られるが、生息環境の選好性についてその違いが餌の選好性に反映するという点に着目し、摂餌器官である口器について、SEM による詳細な観察を行った。その結果、一部の毛の構造に若干の違いが認められるものの、全体としては顕著な違いは認められなかった (1 章)。

十脚甲殻類の分類において、幼生の形態は成体と比較した場合、機能的、構造的差異があるため、重要な分類形質となりうることが示唆されているが、この点に着目した研究例は少ない。このため、対象 2 種のヤドカリの幼生について、それぞれ詳細な形態観察を行い、その特徴を記載した上で、それぞれの比較を行った。特に、*P. maculosus* の幼生形態については初の報告となる。その結果、この 2 種の幼生には形態、色彩においても違いがあることが明らかになった (2 章)。

形態的特徴に基づく分類では *P. lanuginosus* と *P. maculosus* は非常に近縁であると考えられていたが、分子系統学的な視点に基づく系統関係、ヌクレオチド多様性は明らかにされていなかった。そこでミトコンドリア遺伝子の複数座位を用いて分子系統解析を行い、2 種の系統関係を明らかにした。また、この 2 種に加え国内で見られる *Pagurus* 属の数種を解析に加えることで、国内の本属の系統生物地理を明らかにした。その結果日本の *Pagurus* 属は 2 つのクレードに分けられ、*P. lanuginosus* と *P. maculosus* は姉妹群を成し、非常に近縁な関係にあることが示された。またこの 2 種は、約 4 百万～5 百万年前に分化したことが示唆された (3 章)。

また、*P. lanuginosus* と *P. maculosus* を区別する際に最も有効な形質は、体に散在する斑点の色彩である。したがって、この 2 種において、どのような遺伝子が斑点の色彩をはじめとする多様な表現型に影響しているのかを確かめた。まず初めに、2 種の色彩に関与していると予想される遺伝子を特定するため、トランスクリプトーム解析による比較を行った。その結果、黒斑を持つ個体は白斑のものに比べて、メラニン形成関連遺伝子が優位に発現していた他、白斑個体では ‘*Bm-ebony*’ と呼ばれるメラニン阻害遺伝子が発現していた(4 章)。