Comprehensive comparison of four species of Onchidiidae provides insights on morphological and molecular adaptations of invertebrates from shallow seas to wetlands

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Background. The Onchidiidae family provides ideal species of marine invertebrates for the study of the evolution from seas to wetlands. However, different species of Onchidiidae have rarely been considered in comparative studies.

Methods.A total of 40 samples were collected from four species (10 specimens per onchidiid). In addition, we systematically investigated the histological and molecular differences to elucidate the morphological foundations underlying these adaptations.

Results. Histological analysis enabled the structural comparison of respiratory organs (gill, lung-sac, dorsal skin) among onchidiids. Transcriptome sequencing of four representative onchidiids was performed to further expound the molecular mechanisms with their respective habitats. Twenty-six Single nucleotide polymorphism (SNP) markers of *Onchidium struma* presented the DNA polymorphism determining some visible genetic traits. Non-muscle myosin heavy chain II (NMHC II) and myosin heavy chain (MyHC) played an essential role in amphibian developmental processes and are expressed differentially invarious onchidiids and tissues. The species with higher terrestrial ability and higher integrated expression of *Os-MHC* (NMHC II gene) and MyHC gene illustrated the expression level associated with the evolutionary degree.

Conclusions. The present study indicates that different adaptions occurred in four species in various environments.We hope to provide a valuable reference point and a source of inspiration for amphibian investigatorsstudying the morphological characteristics and molecular mechanisms underlying the transition of invertebrates from shallow seas to wetlands.

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Abstract

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Keywords: Onchidiidae; morphological characteristics; transcriptome sequencing;single

nucleotide polymorphism loci;gene expression.

Introduction

Environmental adaption that is considered to be the result of natural selection has been illustrated by physiological and molecular mechanisms. In addition, studies of these adaptive traits that evolved along processes are of importance in understanding the evolution of respiration, movement and other unique characters. Some vertebrates, such as mudskippers (You *et al*. 2014) and lungfish (Zardoya and Meyer. 2014), developed terrestrial adaptations that enable them to spend a considerable amount of time on land. However, few systematic studies have tried to pinpoint the mechanism of adaptive evolution in invertebrates, and the molecular and morphological bases of adaptive evolution remain largely unknown.

The family Onchidiidae (Gastropoda: Eupulmonata: Onchidioidea) provides ideal invertebrate models for studying amphibian adaptations, as there are few groups that possess both aquatic-living organisms and primarily terrestrial-living pulmonate organisms. The family Onchidiidae, of the higher clades of eupulmonates, is mainly composed of intertidal marine, shell-less, air-breathing slugs. Other than the family Ellobiidae, Onchidiidae is the only family that has a free-life veliger stage in Eupulmonata (Bouchet and Rocroi, 2005). Onchidiidae species are widely distributed in the intertidal zone of the South China Sea, the East China Sea and South Yellow Sea, and estuarine mangrove areas (Shen *et al*., 2004). Six species in five genera are known from China (Sun *et al*. 2014), and four main species ofOnchidiidae are widely distributed in China, namely, *Peronia verruculata*, *Paraoncidium reevesii*, *Onchidium struma* and *Platevindex mortoni*.Their habitats range from shallow sea waters to intertidal zones up to supratidal zones, which shows a gradual distribution from sea to wetland.*O. struma*, which mainly lives in wetlands, cannot stay in the water for a long time; *P. reevesii*, which is mainly aquatic, can submerge itself under the seawater for a long time and feed on algae on the coral reef surface; and *P. mortoni*can live both in shallow sea water and in wetlands and has the ability to burrow in mud and climb on rocks. As the only species that has dendritic gills as a respiratory organ when submerged, *P. verruculata* is predominantly an aquatic organism (Fig. 1).

Members of Onchidiidae have three respiratory organs, which include dendritic gills, lung-sac and skin. Mainly aquatic veligers use gills to breathe, and these gills eventually degrade to "lung sac" breathing after metamorphosisas an adaptation to wetland habitat. A few inferior species still use dendritic gills, and their respiratory methods are closer to the subclass Opisthobranchia (Winston *et al*.,2008). The different habitats lead to the evolution of different

breathing patterns to adapt to different environments (Xu *et al*., 2004; Pinchuck and Hodgson, 2010). Therefore, Onchidiidae is a useful group that can be used to gain insights on the morphological and molecular differences underlying the terrestrial adaptations of amphibious invertebrates.

Using multiple species creates a representation of a continuum of adaptions that reflect one species being more terrestrial than others. However, very little is known about the genetic basis and histological basis of these different adaptations. Here, we compare the tissue morphology of four representative species, referred to as *Peronia verruculata*, *Paraoncidium reevesii*, *Onchidium struma* and *Platevindex mortoni*. We also report transcriptome sequencing and de novo analysis of the four species. Moreover, to further improve our understanding of the population structure of *O. struma* and the differences in epidermis morphology, muscle formation, blood vessel development and cuticularization from other species, single nucleotide polymorphism loci were developed and characterized.In the current study, genes related to environmental adaption were identified based on transcriptome data. Interestingly, onchidiids express their trait-associated genes in various tissues that are suited to their specific living habitats. These comparative analyses are carried out to demonstrate the seawater-to-land transition that occurred in Onchidiidae.

Materials and methods

Sample collection

Adult individuals used in this study were collected between May and November. *Onchidium struma* were collected from Shanghai (31°33′N, 121°48′E);*Paraoncidium reevesii* and *Platevindex mortoni* were collected from Xiamen(24°27′N, 118°04′E), Fujian Province; and *Peronia verruculata* were collected from Zhanjiang (21°11′N,110°24′E), Guangdong Province. In this study, all specimens from the four species (10 specimens per onchidiid) were fed with cornflour and reared at room temperature. Samples were maintained until use.

Stereomicroscope, light microscope and scanning electron microscopy

Three fresh adult specimens of each species used in this experiment were anaesthetized by ether, and details of their external morphology were observed using an Olympus SZX16 stereomicroscope. Dorsal and ventral skin from four species of Onchidiidae were dissected into small pieces and were fixed in Bouin's fluid and embedded in paraffin wax (Wang and Tang, 2007). Sections (5~6μm) were cut on a Leica RM2035 microtome, stained with haematoxylin-eosin and observed under a Nikon Eclipse Ni light microscope.

Ten sections were selected randomly from each specimen to collect measurements on the

thickness of the skin, epidermis, dermis, stratum compactum and stratum spongiosum at six sites. In addition, the number of granular glands and mucous glands were counted per cm of skin, and the dimensions (length and width) of the granular glands and mucous glands were measured using a Cellsens Entry Version 1.12 mounted on an Olympus BX53 microscope. Finally, the date was analyzed using the date analysis software, JMP Version 10.0 (Lu, 2006).

For scanning electron microscopy (SEM) of Onchidiidae, the tissues were fixed in amixture of methanol and glutaraldehyde for one week and then preserved in 75% alcohol. After this procedure, the materials were washed 3 times in phosphate buffer (pH=7.0) for 15 min each, cleaned in an ultrasonic water bath for 2~3 min and then dehydrated in a series of increasingly concentrated ethanol solutions (30%, 50%, 70%, 80%, 90%, 100% ethanol), with 15 min per solution; finally, samples were prepared for SEM using critical-point drying. Specimens were sputter coated with gold using DMX-220 ion-plating equipment and then examined by SEM.

Transcriptome sequencing and sequence analysis

Total RNA was extracted by standard molecular biology techniques, and the cDNA library was sequenced in Genergy Biotechnology Company (Shanghai, China) using Illumina Hiseq™ 2000 (Illumina, Inc. USA).Raw data were removed and then assembled by using the short reads assembling program-Trinity(Grabherr *et al.*,2011; Knowles and McLysaght, 2009).

Functional annotation of the transcriptome was done using the Blast2GO software (Conesa *et al*., 2005; Conesa and Götz. S, 2008; Götz. S *et al*., 2008).For annotation, BLASTX alignment (e value<1e-5) between unigenes and protein databases, such as UniProt (www.uniprot.org) and NCBI NR (NCBI non-redundant nucleotide database, (http://www.ncbi.nlm.nih.gov/), was performed, and the best aligning results were used to annotate the protein function. Unigenes annotation provided functional annotation of unigenes, including protein sequence similarity, GO (Gene ontology, http://www.geneontology.org/GO.slims.shtml) (Ashburner *et al.*, 2000) functional classification, and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) pathway analysis (Kanehisa*et al.*, 2010).

SNP markers development in*Onchidium struma*

Potential SNP loci of *Onchidium struma* that differed from those in *Peronia verruculata, Paraoncidium reevesii* and *Platevindex mortoni* invascularization, muscle development, cuticularization and epidermis formation were selected. Primer pairs were designed by Primer Premier 5.0 (http://www.premierbiosoft.com) and synthetized by Map Biotech(Shanghai China). Then, primer pairs were tested in 10 individuals as preparatory screening. The primers that produced clearly defined bands were further tested for polymorphism in 60 individuals.

Primary data analysis of ABI3730XL sequencing was performed with GeneMapper 4.0 (Applied Biosystems Co., Ltd., USA). Calculations of the number of alleles (Na), the observed heterozygosity (H_O), the expected heterozygosity (H_E) and the deviations from Hardy-Weinberg equilibrium (HWE) for each locus were performed by Popgene32 (Version 1.32). A Bonferroni correction was used to correct the results. The polymorphism information content was calculated by Cervus 3.0 (http://www.fieldgenetics.com/pages/home.jsp).

Cloning and quantitative analysis of *Os-NMHC* gene and *MyHC* gene

The dorsal skin, ventral skin, foot skin, lung-sac, ganglion and ventricle were sampled from the four species, and samples were immediately flash frozen in liquid N_2 and kept at -80ºC until use. Total RNA was extracted from those tissues with Trizol (TakaRa, Japan), according to the manufacturer's protocol. The specific primers for cloning the full-length cDNA of *Os-NMHC* and *MyHC* are provided in Table 6.The cDNA was synthesized from the dorsal skin mRNA by using an RT REASER kit with a gDNA Eraser (TakaRa, Japan), and the 3' end and 5' end of the cDNA were obtained by the RACE technique (TakaRa, Japan). The PCR product was ligated into pGEM-T Easy vector (Promega, USA) and transformed into the competent *Escherichia coli* DH5-α cell. Using blue-white selection and PCR identification, positive clones were picked up and were sequenced. At the same time, cDNAs of other tissues were synthesized for RT-PCR analysis of *Os-NMHC* gene expression. In addition, the constitutive expression gene, 18S, was used as an internal control to verify the fluorescent real-time RT-PCR reaction.

The expressions of *Os-NMHC* and *MyHC* transcript in different tissues were studied by means of fluorescent real-time RT-PCR. Quantitative RT-PCR was carried out using the Light Cycler® 480 II (Roche, Swiss) with a QuantiFast® SYBR® Green PCR kit (Qiagen, Germany). All primers used in this process are shown in Table 6.

Results

Comparison of morphological characteristics of four species

The stereomicroscope revealed that the nodular papillae in the dorsal of *Onchidium struma* were the most obvious of the four species of Onchidiidae. Furthermore, the most striking difference between *Peronia verruculata* and the other three species was the dendritic gills located at the back end of the body. *P. verruculata* have dendritic gills at the back end of their bodies (Fig. 1) so they can breathe well when submerged. Moreover, the skin on its gill is thin, so the gills have better permeability than the other parts of the back skin, although they also have thicker cuticular membranes than the other three species (Fig. 2).The surface of Onchidiidae species is covered with a layer of cuticular membrane, which turns purple after staining. The epidermis of

Onchidiidae always has 2~3 layers of cells, and the epidermis of *P.verruculata* is highly keratinized. Cells of each layer are abundant and are closely arranged in *O. struma* and *Platevindex mortoni,* but they are arranged sparsely in *P. verruculata* and *Paraoncidium reevesii*. We also measured the dorsal skin thickness of the four species (Table 1) and found that *P. verruculata* had the thickest dorsal skin, and *P. reevesii* had the thinnest dorsal skin. There is a certain number of granular glands and mucous glands in the skin of Onchidiidae (Fig. 2), and both are multicellular glands.

Onchidium struma had the most developed lung sacs, closely followed by *Platevindex mortoni* and *Peronia verruculata*; *Paraoncidium reevesii* had the least developed lung sacs (Fig. 3). The structural differences among lung sacs of the four species in the Onchidiidae family are shown in Table 2. Specifically, *Onchidium struma*has developed reticular septa, secondary septa and third septa, while *Paraoncidium reevesii*only possesses undeveloped reticular septa (Table 2). In conclusion, the developed degree of lung sacs in the four species of Onchidiidae is, in order, *Onchidium struma*, *Peronia verruculata*, *Platevindex mortoni* and *Paraoncidium reevesii*.

Transcriptome analysis

A series of sequencing libraries were constructed from the RNA of dorsal skin from four species of Onchidiidae. To guarantee the quality of data used for analyses, adaptor sequences, low-quality bases and short reads were removed. After this filtering, we generated 60,219,324; 89,062,542; 62,624,204; and 61,663,900 reads for *Platevindex mortoni*, *Paraoncidium reevesii*, *Onchidium struma*and *Peronia verruculata*, respectively(Table 3). 131,325(*Platevindex mortoni*), 233,625(*Paraoncidium reevesii*), 416,848(*Onchidium struma*) and 263,097(*Peronia verruculata*) unigenes were annotated successfully by GO annotation. These annotated unigenes were classified into three categories: BP(biological process), CC(cellular compartment) and MF(molecular function)(Table 4).

In addition to GO analysis, KEGG pathway mapping based on the enzyme commission (EC) numbers for assignments, which is an alternative approach to categorize gene functions with an emphasis on biochemical pathways, was also carried out for the assembled sequences. After analysis, we determined that unigenes participated in 129, 136, 138 and 134 pathways in *Platevindex mortoni*, *Paraoncidium reevesii*, *Onchidium struma* and *Peronia verruculata*, respectively. To determine the phylogenetic relationships between Onchidiidae and its orthologs in other mollusks and amphibians, we constructed a phylogenetic tree by using the Mrbayes version3.2 (Fig. 4).

Developing SNP markers of *Onchidium struma*.

Single nucleotide polymorphism (SNP) is an important molecular marker. The developed SNPs of Onchidiidae were critical for understanding their respiratory manners and amphibious features. The proposed sites in the transcriptome sequences of *Onchidium struma* were searched using Samtools, and 152,212 SNP were detected after analysis. Forty-two pairs of primers were successfully amplified among 57, of which 26 pairs were identified (Table 5). In total, the observed and expected heterozygosities ranged from 0.2553 to 1.0000 and from 0.0000 to 0.7447, respectively (Table 5). No genetic linkage was observed among these loci. Fifteen loci with '*' significantly departed from HWE after the Bonferroni correction ($P<0.05$).Among the 26 SNP loci, 3 loci (S_Unigene508_c0_seq1_142, S_Unigene685_c0_seq1_3534, and S_Unigene508_c0_seq1_283) were related to epidermis formation, 1 locus (S_Unigene3026_c0_seq1_3726) was related to epidermis formation and muscle formation, 3 loci (S_Unigene512_c0_seq1_971, S_Unigene512_c0_seq1_5524, and S_Unigene512_c0_seq1_5912) were related to vascularization and muscle formation simultaneously, 1 locus (S_Unigene11849_c0_seq1_804) was related to formation of blood vessels and skin, and the others loci were related only to vascularization.

Non-muscle myosin heavy chain II

Non-muscle myosin heavy chain II (NMHC II) is from the non-muscle myosin II (NM II), which is composed of a pair of heavy chains and two pairs of light chains (Bresnick A R, 1999). NM II has three critical functions: cell adhesion, cell motility and cytokinesis (Matsha, *et al.*, 2012). In mammals, NMHC II have three isoforms, referred to NMHC IIA, NMHC IIB and NMHC IIC (Conti, *et al.*, 2008; Vicetemanzanares, *et al*., 2009). However, *Xenopus* has two isoforms, II-A and II-B, and does not appear to have II-C (Lynne M.2008). In vertebrates, *Drosophila* has only a single isoform of NH II (Peralta, *et al*., 2007). According to the evolutionary level of Onchidiidae, we speculated that Onchidium contained at least one isoform.

The specific expression of a gene in tissues is normally related to its function in those tissues, and different tissues reveal the different adaptions of the four species. To investigate the tissue-specific expression, the mRNA levels of expression were quantified by qRT-PCR in the dorsal skin, ventral skin, lung-sac, ganglion and ventricle samples from the four species. The SNPs reflect the genetic differences in the four species of Onchidiidae. However, the expression differences of genes related to phenotype remain unknown. According to a histological study from Onchidiidae and transcriptome data from us, we determined *Os-NMHC* further reveals adaption from seas to wetlands in the four species of Onchidiidae. *Onchidium struma* has a higher evolution level, as is evident by their ability to live in more complex environments (Wei LL, 2013). We obtained full-length cDNA, submitted it to the GenBank database and obtained an

accession number (KU663401).

The expression of *Os-NMHC* was determined in various tissues from four species of adult Onchidium. Compared with the expression of *Os-NMHC* in all tested tissues, we found it displayed significantly strong expression in the lung-sac, which reflected the tissue-specific expression. *Os-NMHC* has the highest expression level in ganglion from *Platevindex mortoni* but is not found in almost any tissues of *Peronia verruculata.* The result of expression showed differences in the same tissues from different species ($P<0.05$).

Myosin heavy chain

Myosin heavy chain is the primary protein in muscle and is a tissue-specific protein (Talmadge, *et al*., 1993). In addition, myosin heavy chain protein is related to the contraction of muscle, which is critical for analyzing the muscle adaptation. We cloned *MyHC* gene (GenBank accession number: KU550708)in four species and compared the expression levels in four types of tissues among the four species. The results showed that the expression level of *MyHC* gene in *Onchidium struma* was the highest, while its expression level was the lowest in *Paraoncidium reevesii*; additionally, the expression levels between them were significantly different ($P<0.05$). *Platevindex mortoni* showed the highest expression level in both the ventral skin and foot. In the comparison of expression level from lung-sacs, *P. mortoni*had the highest expression level of *MyHC*, was closely followed by *O. struma*, and *P. reevesii* had the least expression.

To further analyze the relative expression of the *MyHC* gene in 3 different tissues from four Onchidiidae species, we found that the expression levels were associated with their living habitats. *O. struma* and *P. mortoni* are mainly terrestrial and need to burrow in mud and climb rocks to avoid tidewater. However, *P. reevesii* and *P. verruculata* are mainly aquatic, and their movement requirements are lower. *O. struma* had a high expression level of *MyHC* in the dorsal skin, foot and lung-sac, which was suited with their terrestrial adaption. *P. mortoni* expressed a high level in the foot, and they frequently climb trees. The epidermis of *P. verruculata* had the highest level of keratinization; thus, they had a high expression of the *MyHC* gene in their dorsal skin. We speculated the expression level of this gene was related to their respiration ability, moisture retention and defense capacity.

Discussion

Skin is an important respiratory organ for onchidiids. If skin has lower keratinization, it will have better permeability, which is beneficial for breathing in Onchidiidae species. If skin has higher keratinization, this feature will benefit individuals by retaining moisture and protecting against predators.

Onchidium struma, which is mainly terrestrial, has relatively weak respiratory function inits skin. Its epidermis is thick and has the function of retaining moisture and protecting against

predators, which matches its terrestrial characteristics(Quay, 1972; Arey and Barrick, 1942). For the mainly aquatic *Paraoncidium reevesii*, the dorsal skin is thin and is easily permeable, so its respiratory function is strong. However, another aquatic species, *Peronia verruculata*, has a higher level of keratinization of the epidermis, but their gill skin is thin and is suitable for breathing when submerged(Table 1). In *Platevindex mortoni*, the thickness of their skin and the number of blood sinus are all at intermediate levels. This species lives mostly in the supratidal zone and mudbank, can stay in the sea for a long time, and can even climb trees.

The secretions from the mucous glands are slimy and smooth, which can reduce the friction between skin and water and is also beneficial for gas exchange and ion transportation (Wu, 2011). Meanwhile, the dense distribution of blood sinus is one of the hallmarks of aquatic species (Tang *et al*, 1999; Cao *et al*, 2011). In addition, there is only a small amount of blood sinus in the stratum spongiosum of *Onchidium struma*, though blood sinus in other species are abundant.

The sequence of the diameters of the sac room and the small room are also in the same order. Dayrat called the respiratory organ of *Onchidium vaigiense* as lung sac, which is similar to the breathing bag of limacine (Dayrat, 2010). The results of lung sacs in amphibians showed that developed lung sacs are more suitable for terrestrial life (Hu *et al.*, 1998). The efficiency of the lung sac respiration depends on its superficial area. Thus, species with larger superficial areas have stronger respiratory capacities. Because of the well-developed reticular diaphragm, thin connective tissue, rich blood capillary and the largest superficial area of lung sacs, *O. struma* has the strongest respiratory capacity for wetland living among the four species. In conclusion, the developed degree of lung sacs in the four species of Onchidiidae was, in order, *Onchidium struma*, *Peronia verruculata*, *Platevindex mortoni* and *Paraoncidium reevesii*.

The evolution of Onchidiidae and their amphibious features are reflected by their morphological characteristics. As a typical amphibious mollusk, transcriptome sequencing data provide the base to further study the genes related to their morphological differences and amphibious features. The next generation of sequencing technology makes it feasible and convenient to analyze and investigate transcriptomes of non-model organisms, and it provides the large-scale sequence data, which are valuable for further studies to understand biological processes, such as metabolic process, signal transduction, and so on (Huang *et al*., 2013). In this study, our data provide the best transcriptomic resource currently available for these four species. The transcriptome data were provided by the Illumina HiSeq™ 2000 sequencing, and the sequences were assembled and functionally annotated. Based on these annotated unigenes, the analyses of GO and KEGG assignments were performed. This study established an excellent

resource for future genetic or genomic studies on the analyses of this family's variation and offered a significant platform for functional genomics and comparative genomic studies for mollusks.

In this study, SNP loci for *O. struma* were developed based on the transcriptome sequencing comparison with the other species. These genomic regions were related to the vascularization and the formation of muscle and cuticle. These loci were easy to mutate and may reflect strong directional selection, which is important for *O. struma* evolution. Those loci can be reference genes and verified in other species. Those SNPs may have experienced geographical selection and could reflect some directional selections for Onchidium adaptive evolution (Yoshiura K, *et al*., 2006).

We all know that the dorsal skin plays a significant role in defending against predators and protecting against moisture loss. Meanwhile, developed ventral skin and foot skin are important for locomotion, and NMHC II could influence axon growth (Hur EM, *et al*., 2011).

In some species, such as *Drosophila* (Crish, *et al*., 2013), NMII is related to dorsal skin and is implicated in epidermal barrier functions (Sumigray, *et al.*, 2012). Therefore, for studies on the adaption of epidermis, NMHC II has an important role in skin development. Our study revealed that species that are mainly terrestrial (e.g., *Onchidium struma* and *Platevindex mortoni*) have developed epidermis that can retain water and defend against predators. In addition to its pronounced expression in skin (dorsal skin, ventral skin and foot skin) from species that are mainly terrestrial, we also found *P. mortoni* had high expressions in the ventricle and ganglion. Previous studies found that the mutation of NMHC II affects the development of the heart (Tullio, *et al.*, 1997; Lu, *et al*., 2008). This evidence explains how both *O. struma* and *P. mortoni* had the ability to adapt to terrestrial environments due to the heart and ganglion being closely associated with feeding habits (Grega and Prior, 1985; Welsford and Prior, 1991). The higher-level expression of *Os-MHC* in the dorsal skin, ventral skin, foot skin and lung-sac indicates that species that are mainly terrestrial (e.g., *O. struma* and *P. mortoni*) can easily adapt to complicated land environments.

Myosin heavy chain protein is associated with muscle, and analyzing the expression levels provides information on a species' habitat. *O. struma* and *P. mortoni* mainly live in wetlands, and they must burrow in mud and climb rocks to avoid tidewater. *P. reevesii* and *P. verruculata*, which are mainly aquatic, do not require strong locomotive abilities. Moreover, the environment in which *P. reevesii* lives is more complex than the environments of the other species, and *P. reevesii* can even climb trees. Therefore, *P. reevesii* has the strongest requirement of a developed

foot, and its expression quantity of *MyHC* is much higher than those in *P. reevesii* and *P. verruculata.*

Conclusion

This study provides a comprehensive insight to elucidate the adaptation of invertebrates, and Onchidiids are a typical group of invertebrates that are widely found. Onchidiids can breathe with skin, lung sacs and gills. They can breathe through gills and skin when under seawater, can breathe through lung sacs and skin when in wetlands with amphibious habitats, and can provide insights into better understanding the histological structural adaptation underlying the seawater-to-land transition of marine invertebrates.

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Figure 1

Habitats of the four species in the family Onchidiidae.

The drawing of the habitats of Onchidiidae was created using Photoshop. *Onchidium struma* spend most of their time in wetlands, *Platevindex mortoni* can live well in both water and wetlands, and*Paraoncidium reevesi*i and *Peronia verruculata* predominantly dwell in water. Note: The picture with the red square highlights the dendritic gills in the dorsal skin of *Peronia verruculata.*

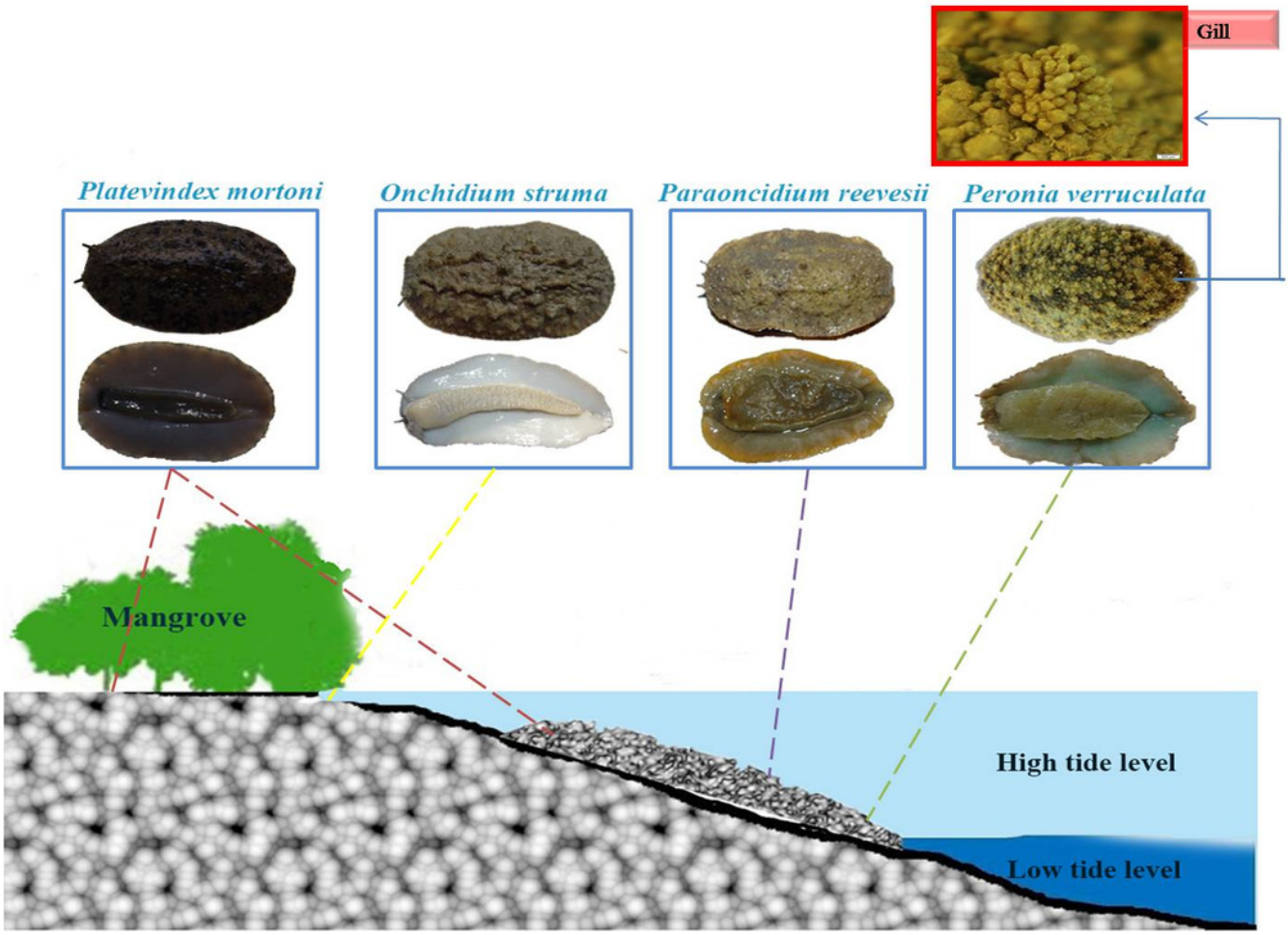

Figure 2

Light microscopy of the dorsal skin of four species in the Onchidiidae.

(**A-D**)An overview of dorsal skin in (**A**)*Onchidium struma*(×40), (**B**)*Paraoncidium reevesii*(×40), (**C**)*Platevindex mortoni*(×40) and (**D**)*Peronia verruculata*(×40). (**E-H**)Dermis layer of (**E**)*O. struma*(×40), (**F**) *P. reevesii*(×40), (**G**)*P. mortoni*(×40)and (**H**)*P. verruculata*(×40). (**I-L**)Histological observation of glands in four species of Onchidiidae. (**I**)*O. struma*(×40), (**J**). *P. reevesii*(×40), (**K**)*P. mortoni*(×40), and (**L**)*P. verruculata*(×40).

E. Epidermis; D. Dermis; SS. Stratum spongiosum; SC. Stratum compactum; CM. Cuticular membrane; SCO. Stratum comeum; SGR. Stratum granulosum; SGE. Stratum germinativum; MG. Mucous gland; GG. Granular gland; PC. Pigment cell; MF. Muscle fiber; BS. Blood sinus; CP. Calcium particle.

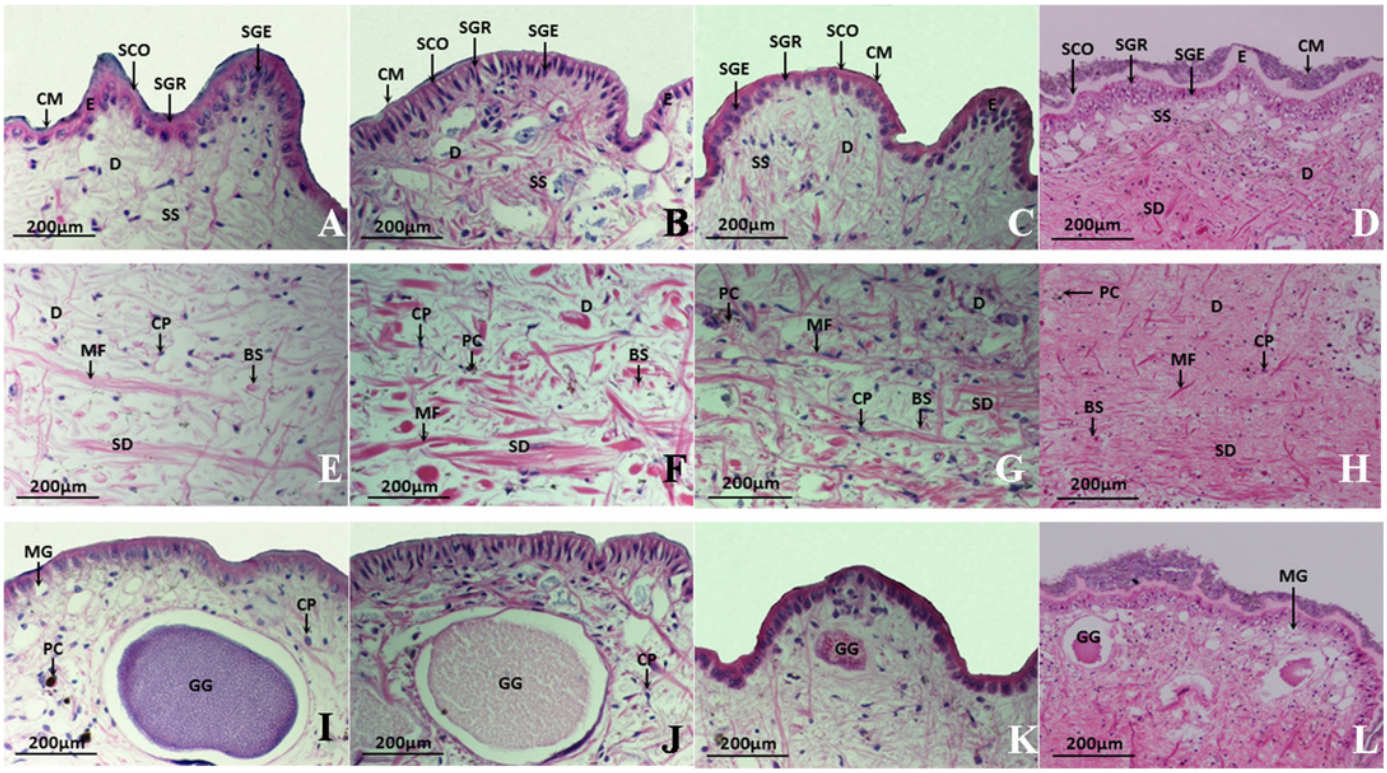

Figure 3

SEM observation of lung sac of four species in the family Onchidiidae.

(A-B). *Paraoncidium reevesii*; (C-D). *Platevindex mortoni*; (E-F). *Peronia verruculata*; (G-H). *Onchidium struma*

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

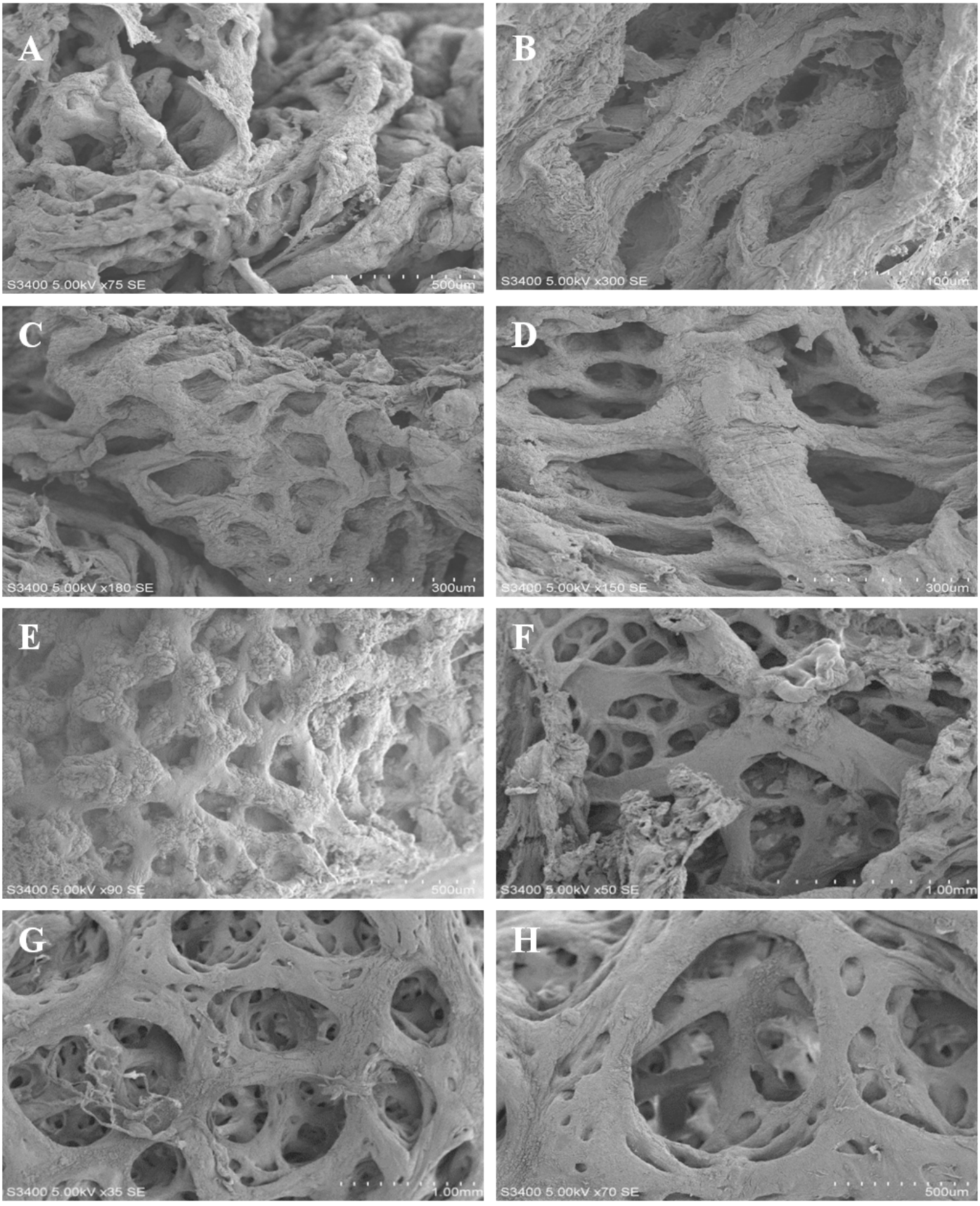
A
S3400 5.00kV x75 SE
500um
B
S3400 5.00kV x300 SE
100um
C
S3400 5.00kV x180 SE
300um
D
S3400 5.00kV x150 SE
300um
E
S3400 5.00kV x90 SE
500um
F
S3400 5.00kV x50 SE
1.00mm
G
S3400 5.00kV x35 SE
1.00mm
H
S3400 5.00kV x70 SE
500um

Figure 4

Phylogenetic analysis of 13 species.

The colored names highlight the main objects of this research.The phylogenetic tree was inferred by using Bayesian method and conducted in MrBayes version 3.2.4. This tree was generated using 18 S sequences.

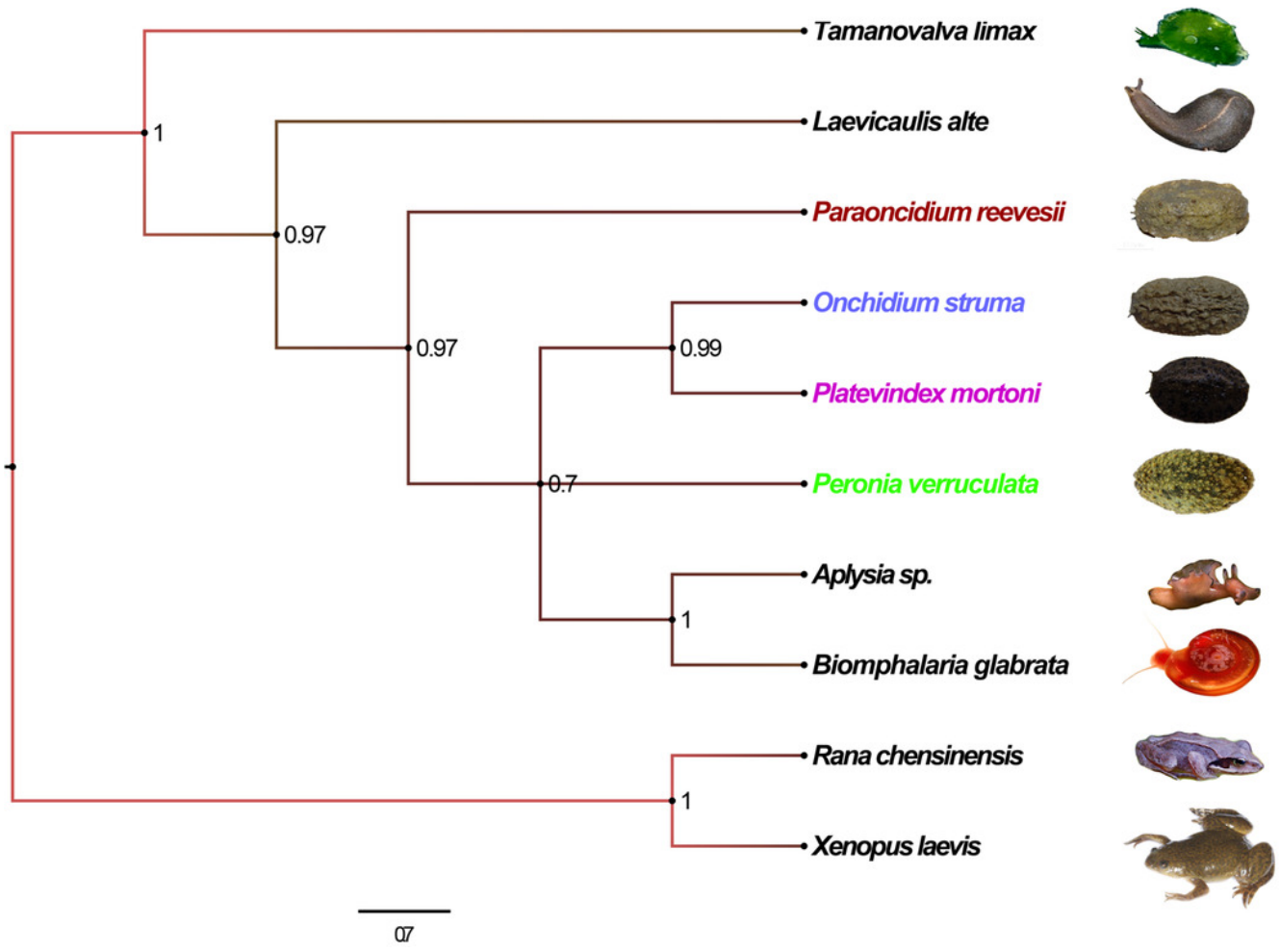

Figure 5

Expression levels of NMHC II gene in different tissues from four representative Onchidium.

S= *Onchidium struma*; M=*Platevindex mortoni*; R= *Paraoncidium reevesii*; V= *Peronia verrucula* (Almost did not express in tested tissues).

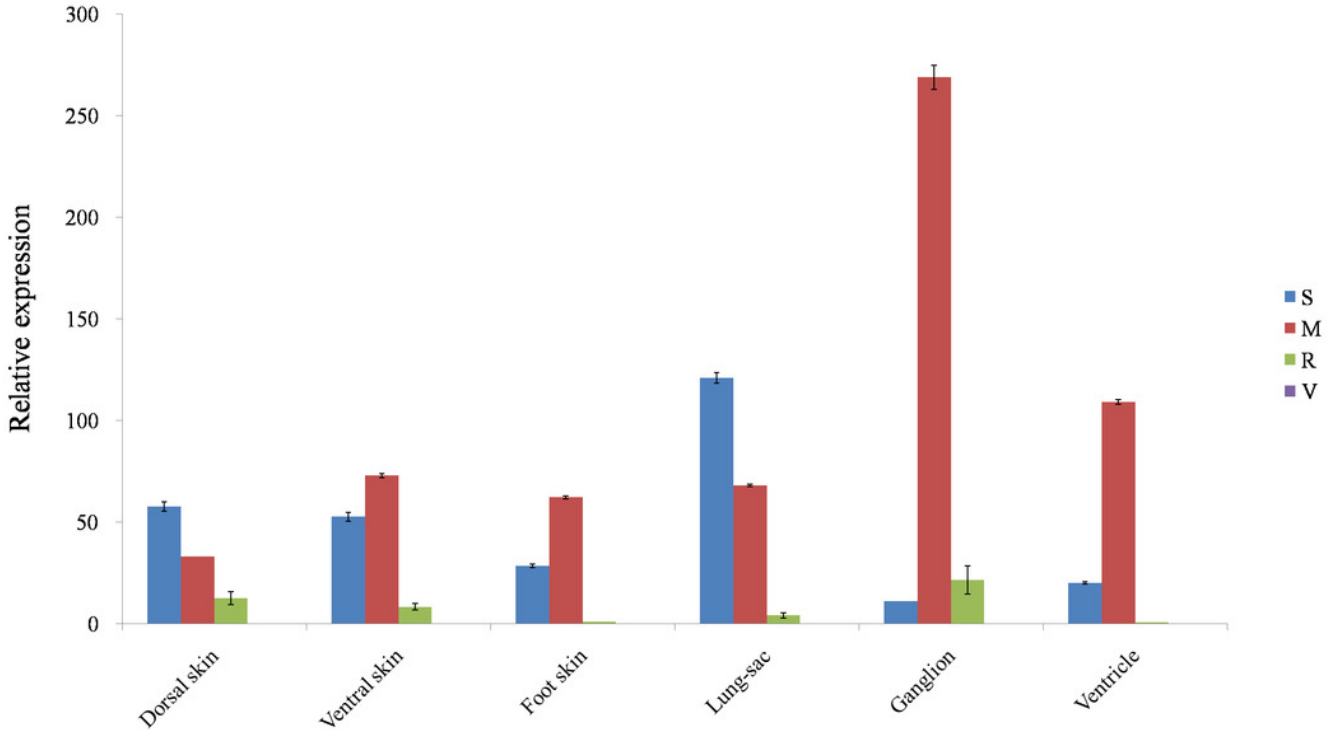

Figure 6

RT-qPCR analysis of the expression profiles of onchidiids *MyHC* in different tissues

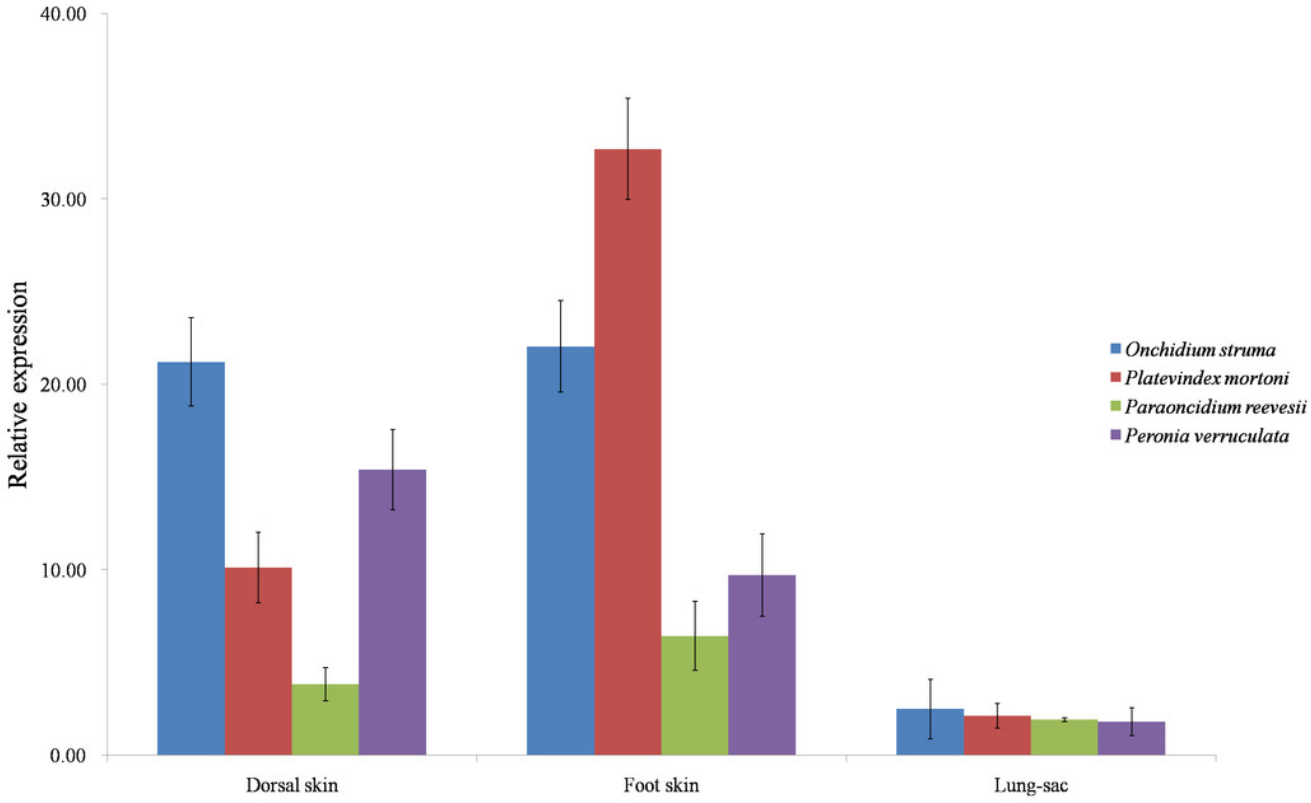

Table 1(on next page)

Dorsal skin thickness of four species in the family Onchidiidae(Unit: μm).

Statistical analysis of the thickness of epidermis, stratum spongiosum, stratum compactum and whole skin was done separately among the four species;* indicates significant difference($P<0.05$);** indicates extremely significant difference($P<0.01$).

1

Species	Epidermis		Stratum spongiosum		Stratum compactum		Whole skin	
	Min ~ Max	Mean ± SE	Min ~ Max	Mean ± SE	Min ~ Max	Mean ± SE	Min ~ Max	Mean ± SE
Onchidium struma	30.38 ~ 65.08	43.01 ± 5.07**	212.29 ~ 830.61	475.97 ± 103.45**	271.62 ~ 372.50	287.79 ± 20.37	548.20~11 56.77	816.74 ± 107.90*
Paraoncidium reevesii	20.54 ~ 30.35	26.17 ± 1.89	247.87 ~ 617.63	438.69 ± 67.23	208.16 ~ 364.28	293.30 ± 24.43	531.16 ~ 921.01	764.98 ± 62.65
Platevindex mortoni	27.39 ~ 36.02	32.78 ± 1.35*	224.59 ~ 483.42	358.12 ± 35.79	172.55 ~ 425.91	266.36 ± 37.36	473.84 ~ 885.09	662.29 ± 65.58
Peronia verruculata	51.77 ~ 110.06	72.06 ± 8.22**	173.19 ~ 409.18	345.05 ± 37.94	371.97 ~ 627.31	486.21 ± 47.24**	846.86 ~ 997.13	914.37 ± 23.1*
Peronia verruculata (gill)	26.93 ~ 53.83	37.16 ± 3.99	43.38 ~ 73.39	54.78 ± 4.78**	87.63 ~ 177.73	124.34 ± 14.75**	178.95 ~ 246.05	205.35 ± 12.38**

Table 2(on next page)

Primer sequences and characterization of 26 SNPs in *Onchidium struma*.

Observed heterozygosity (H_O), expected heterozygosity (H_E), the test for deviation fromHWE (*P*), and single nucleotide polymorphism (SNP).

1

Locus	PCR primers (F,R) and extension primer (P)sequences (5'–3')	SNP	H_O	H_E	*P*	Function
S_Unigene1402_c0_seq1_342	F:TGTCTGGCTATCCACTGA S:TTCAGGATTCCTTTTGC P:TTTTTTTTTTTTTTTTTTTTTTTTAAGTGAGCATACCACATGCC	G/A	0.5000	0.5000	0.0465	Hypothetical protein DAPPUDRAFT_228516
S_Unigene1402_c0_seq1_2685	F:TGTCCACTCCCAGCAGA S:GAGAATGCAGACAATACAAAA P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGATGTAAAGTAAGCAGTGGAGC	A/T	0.9818	0.0182	0.0000	Myosin VI
S_Unigene1402_c0_seq1_471	F:TCCGAGGTTCCCTTGCT S:GACAAAGAACAAGAAGAGGACA P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACCAGCGAGCTCCTCATTC	A/T	1.0000	0.0000	0.0096	Myosin-VI-like
S_Unigene508_c0_seq1_142	F:AGATGGACGCACCTTGT S:AAGTTTTCACAAAGATCTGCA P:TTTTTTTTTTTTTTTTTTTTAGTCTGAGCACCAAGTGGAG	T/A	0.9815	0.0185	0.0286	Ubc protein
S_Unigene1402_c0_seq1_1362	F:TTGGCTTGAACTTGCGA S:CAGTGGTGTACTCTGTCTGTGA P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTATCGTGCAGCCCA	C/T	0.9825	0.0175	1.0000	Hypothetical protein DAPPUDRAFT_228516
S_Unigene1402_c0_seq1_1053	F:CCTGGAGTTTACGCAGT S:CAAACATGGACGTCTTGA P:TTTTTTTTATGACCAAGAGGCTGGCAGA	C/A	1.0000	0.0000	0.0095	Myosin-VI
S_Unigene1402_c0_seq1_2178	F:TCCTTGTTGCGACTGTG S:GTGGTATCTTTGACCTCCT P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGTGAAGTGATCATACTTTGG	T/C	0.9032	0.0968	0.0000	Myosin-VI-like
S_Unigene1402_c0_seq1_99	F:GCCTACCCTTCCTCTACTT S:TGGACCAGCACTACTCAA P:TTTTTTTTTTTTTTTTTTTTTAGGCCCAGAAAGTGGCTTC	T/A	0.4717	0.5283	0.0113	Myosin-VI-like
S_Unigene685_c0_seq1_3534	F:GACCTCAAGGACCCACTG S:CCTCAATAGGTTGGTCATACT P:TTTTTTTTTTTTTTTTTTTTTTTTTGCCCTTGCCAGCATAGTT	C/T	0.9655	0.0345	0.0001	Col1a2
S_Unigene512_c0_seq1_971	F:AATCCTATTCTGGAAGCCT S:AATCAAAGTTGATGCGG P:TTTTTTTTTTTTTTTTTTTTGCCAAGACCATCAAGAATGA	T/C	0.9455	0.0545	0.0889	Myosin heavy chain, non-muscle isoform X7
S_Unigene512_c0_seq1_5524	F:GATGAACACACCAACACAGAG S:ACTGAGCGTTCAGAGGC P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATCTGCTCCACCTGGAGT	A/T	0.9649	0.0351	0.9243	Myosin-10 isoform X6
S_Unigene512_c0_seq1_5912	F:GAGTGAAGGCCCTGAAA S:TGCTCATCAAGTTCTCGC P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGATGAGGCTGAGGAAGA	G/A	0.5254	0.4746	0.0251	Myosin heavy chain
S_Unigene1402_c0_seq1_1359	F:TTGGCTTGAACTTGCGA S:CAGTGGTGTACTCTGTCTGTGA P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTATCGTGCAGCCCAGTT	A/G	0.9583	0.0417	0.0050	LOC443649 protein, partial
S_Unigene1402_c0_seq1_716	F:CTTGACGTGCGGCAACC S:CACAGGGACAGAGAACTGGC P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACCAGGTGGAAGATATCACC	A/G	0.9556	0.0444	0.0006	Myosin-VI isoform 1
S_Unigene11849_c0_seq1_804	F:CCAAGCCAAGAGGACTTA S:CATGGGACTTTTGGTTT P:TTTTTTTTTTTTTTTTTTGGCAGGGACTGTATGTAACC	G/A	0.4909	0.5091	0.5486	Mitogen-activated protein kinase
S_Unigene1402_c0_seq1_3336	F:CAGCACTCTGTCAGGTACTT S:GTAACCAAGACCAGCCA P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACTCGGTCTTCCCAGCTCC	T/C	0.9565	0.0435	0.0644	Hypothetical protein EGM_13779
S_Unigene3026_c0_seq1_3726	F:AGGTCTAAGGTGGATGATTC S:TCTGGATTCTGAGGTGCT P:TTTTTTTTTTTTTTTAGATCTGAGCCAGAGGGCAG	A/G	0.9825	0.0175	1.0000	Serine/arginine repetitive matrix protein 2-like

S_Unigene1402_c0_seq1_300	F:CAGCAACCATAAGAATAGGA S:GCACGGCATGTGGTATG P:TTTTTTTTTTTTTTTTTTTTTTTTTTTGATGGTCAGTGGATAGCCAG	T/C	0.5893	0.4107	0.0976	Myosin-VI
S_Unigene1402_c0_seq1_1908	F:CAGTTGTTTCCTGAATTTG S:CGAAGAATCCATTGTTGA P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTTCCAGGATACAGAATCCAC	T/G	1.0000	0.0000	0.0000	Myosin VI
S_Unigene1402_c0_seq1_2823	F:CCACCAGTGAATAGATACCTAA S:CGTGCTGGATGATGTCAA P:TTTTTTTTTTTGCTGTGTGCGATGCAGC	A/T	0.9483	0.0517	0.0873	Jaguar, isoform I
S_Unigene394_c0_seq1_418	F:ATGGATGGTACTGAAGGTCT S:ATGATTCTTCCGAGTGTCTT P:TTTTTTTTGGTGAGCCCTGTGTGGACAT	T/A	0.9000	0.1000	0.7108	H+ transporting ATP synthase beta subunit isoform 2
S_Unigene1402_c0_seq1_1570	F:CTCAGCAAACTTGCCCG S:CGATTGGATGCTAGGCTCT P:TTTTTTTTTTTTTTTTTGGTCAAACCAAACTTAAAGTCC	C/T	0.9211	0.0789	0.8371	CRE-SPE-15 protein
S_Unigene508_c0_seq1_283	F:TTGAGCCATCTGACACAAT S:GCCATCCTCCAACTGTTT P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGGACAAAGAGGGAATCCC	T/C	0.9245	0.0755	0.0180	Ubc protein
S_Unigene1402_c0_seq1_2789	F:CAAAAGCAACATTGCCCA S:CGATGCAGCTATGAAGCAC P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCCACCAGTGAATAGATACCTA	A/T	0.9818	0.0182	0.0011	Protein SPE-15
S_Unigene1402_c0_seq1_2601	F:TTTCAGTGGCACCTTGAT S:AAGGAGGAACTGAGGGA P:TTTTTTTTCTGGTCAACCGTGTCATGCA	C/T	0.2553	0.7447	0.0000	AGAP000776-PA
S_Unigene1402_c0_seq1_975	F:CTTTTTCGCTCCAGCTCT S:GACTGCGTAAACTCCAGG P:TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGAAACAGCGCCGAGCAGA	A/C	0.8000	0.2000	0.5133	Jaguar, isoform H

2

3

Table 3(on next page)

PCR primers used in gene cloning.

Note: We designed seven pairs of primers and three pairs of primers for reverse transcription PCR (RT-PCR) amplification of the coding region,as the length of *Os-NMHC* and *MyHC* are too long.

1

Usage	Primer's name	Primer sequence(5'-3')	Explanation
RT-PCR	Test-1F	CCAACCGCACCAGCCGTGAGT	To amplify one part of *Os-NMHC* fragment
	Test-1R	GCGGTCCAGAGATTTGTTGAT	
	Test-2F	TAAGAATAAGTATGAGGCAAT	To amplify one part of *Os-NMHC* fragment
	Test-2R	GCTCCACTGTCATATCGTCCA	
	Test-3F	GACTTCCTACAACTTCGAGCA	To amplify one part of *Os-NMHC* fragment
	Test-3R	CTCTTTCACTCTCTGCTTGTC	
	Test-4F	ACCGCACTAACCCAGGCATTC	To amplify one part of *Os-NMHC* fragment
	Test-4R	CTCTGGATGACACGGATAGCA	
	Test-5F	CTGTATCGCATTGGGCAGAGC	To amplify one part of *Os-NMHC* fragment
	Test-5R	GCTGTGGTGTCCAGGGAATCT	
	Test-6F	AGGAAGAGAACAAGAGAATCAG	To amplify one part of *Os-NMHC* fragment
	Test-6R	AGGAAGAGAACAAGAGAATCAG	
	Test-7F	CCAAGCGTAATGCTGAGTCTG	To amplify one part of *Os-NMHC* fragment
	Test-7R	CATCCTCTTCTCCATCTTTCT	
	Test-8F	TGCGTGGCTATCAACCCC	To amplify one part of *MyHC* fragment
	Test-8R	GCCCTCAAGCACACCGTT	
	Test-9F	AGACTGTGTCCCACTTGC	To amplify one part of *MyHC* fragment
	Test-9R	TGAGCGGACGGATGAGAT	
	Test-10F	GTCAAGAAATACCAGCAG	To amplify one part of *MyHC* fragment
	Test-10R	TAGTGATGATGATGGTGG	
RACE	3'RACE-F1	ATGTCGGATAAAGCCCGCAAAG	Gene-specific outer primer for *Os-NMHC*
	3'RACE-F2	GCACGCACAAAGGCAACC	Gene-specific inner primer for *Os-NMHC*
	3'RACE-F3	GCGGCACACCAAGTTTGACCACAT	Gene-specific outer primer for *MyHC*
	3'RACE-F4	AACGAGGGTGGAATCCGGACTATA	Gene-specific inner primer for *MyHC*
	3'RACE outer primer	TACCGTCGTTCCACTAGTGATTT	Primers from kit
	3'RACE inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
	5'RACE-R1	TTGGCTTGTAGCAGTTGGTTCTCA	Gene-specific outer primer for *Os-NMHC*
	5'RACE-R2	AAACCCATTGGATTCGTCTG	Gene-specific inner primer for *Os-NMHC*
	5'RACE-R3	GTAGGCATTGTCAGAGAT	Gene-specific outer primer for *MyHC*
	5'RACE-R4	AGGGGTTGATAGCCACGC	Gene-specific inner primer for *MyHC*

	5'RACE outer primer 5'RACE inner primer	CATGGCTACATGCTGACAGCCTA CGCGGATCCACAGCCTACTGATGATCAGTCGATG	Primers from kit
qRT-PCR	qRT-PCR primer F qRT-PCR primer R	AGACTGGTCCAAGTATGCCTA CCATAATGCTCATGGACTCG	Used to amplify *Os-NMHC* fragment for real-time PCR
	qRT-PCR primer F qRT-PCR primer R	GCCTCCTCATTTGTTCTCCA ATCTTCTTCTCGGCTCCCTC	Used to amplify *MyHC* fragment for real-time PCR
	18S primer F 18S primer R	CGGCTACCACATCCAAGGAA GCTGGAATTACCGCGGCT	Used to amplify 18S fragment for real-time PCR

2

Table 4(on next page)

Structural differences among lung sacs of four species in the family Onchidiidae.

1

	Paraoncidium reevesii	*Platevindex mortoni*	*Peronia verruculata*	*Onchidium struma*
Reticular septa	Small pore, thick wall	Big pore, thick wall	Big pore, thick wall	Big pore, thin wall
Secondary septa	Nothing	Developed	Developed	Developed
Third septa	Nothing	Nothing	Nothing	Developed
Diameter of sac rooms (μm)	0.5-1.5	0.8-5.0	4.5-6.6	5.1-12.7
Diameter of small room (μm)	Nothing	0.4-2.7	1.5-4.2	3.4-7.3
Diameter of subordinate rooms(μm)	Nothing	Nothing	Nothing	0.7-4.5

2

3

Table 5(on next page)

Annotated unigenes by gene ontology.

Table 4.Annotated unigenes by gene ontology.

	Platevindex mortoni	*Paraoncidium reevesii*	*Onchidium struma*	*Peronia verruculata*
Biological process	68918	124598	221660	137152
Molecular function	29298	51891	90183	60939
Cellular component	33109	57136	105005	65006

Table 6(on next page)

Data of four Onchidiidae

Sample_M, *Platevindex mortoni***; Sample_R,** *Paraoncidium reevesii*; **Sample_S,** *Onchidium struma*; **Sample_V,** *Peronia verruculata*.

1

Sample ID	SeqType	Raw Read length(bp)	Read Num.	Data Product	Effective read Num	Effective Data	Effective Rate(%)
Sample_M	Pair-End	101	61356624	6197019024bp (6.197Gb)	60219324	5841892655 bp (5.842Gb)	94.2694
Sample_R	Pair-End	101	90701864	9160888264bp (9.161Gb)	89062542	8617271978 bp (8.617Gb)	94.0659
Sample_S	Pair-End	101	63774300	6441204300bp (6.441Gb)	62624204	6073850713bp (6.074Gb)	94.29682
Sample_V	Pair-End	101	62832016	6346033616bp (6.346Gb)	61663900	5987378475 bp (5.987Gb)	94.34836

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