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Evolution of the tyrosinase gene family in bivalve molluscs: Independent expansion of the mantle gene repertoire ☆

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ABSTRACT

Tyrosinase is a copper-containing enzyme that mediates the hydroxylation of monophenols and oxidation of *o*-diphenols to *o*-quinones. This enzyme is involved in a variety of biological processes, including pigment production, innate immunity, wound healing, and exoskeleton fabrication and hardening (e.g. arthropod skeleton and mollusc shell). Here we show that the tyrosinase gene family has undergone large expansions in pearl oysters (*Pinctada* spp.) and the Pacific oyster (*Crassostrea gigas*). Phylogenetic analysis reveals that pearl oysters possess at least four tyrosinase genes that are not present in the Pacific oyster. Likewise, *C. gigas* has multiple tyrosinase genes that are not orthologous to the *Pinctada* genes, indicating that this gene family has expanded independently in these bivalve lineages. Many of the tyrosinase genes in these bivalves are expressed at relatively high levels in the mantle, the organ responsible for shell fabrication. Detailed comparisons of tyrosinase gene expression in different regions of the mantle in two closely related pearl oysters, *P. maxima* and *P. margaritifera*, reveals that recently evolved orthologous tyrosinase genes can have markedly different expression profiles. The expansion of tyrosinase genes in these oysters and their co-option into the mantle's gene regulatory network is consistent with mollusc shell formation being underpinned by a rapidly evolving transcriptome.

1. Introduction

Tyrosinases, tyrosinase-related proteins, hemocyanins and catechol oxidases are members of the type-3 copper protein superfamily. These enzymes possess a conserved pair of copper-binding domains, known as Cu(A) and Cu(B), each of which is coordinated by three conserved histidines [1,2]. Members of this superfamily are present in both eukaryotes and prokaryotes, and are involved in a wide array of biological processes, including pigmentation, innate immunity, oxygen transport, sclerotization and wound healing [3–6]. The type-3 copper protein superfamily can be classified into three subclasses based on domain architecture and conserved residues in the copper-binding sites—secreted (α), cytosolic (β) and membrane-bound (γ) subclasses—and is typified by multiple and independent lineage-specific gene expansions and gene losses [7].

Tyrosinases (EC 1.14.18.1) catalyse both the initial hydroxylation of monophenols (e.g. tyrosine) and the further oxidation of *o*-diphenols (e.g. DOPA and DHI) to *o*-quinones [8] to produce melanin. In vertebrates, tyrosinase and its related proteins regulate pigment synthesis [3,4]. In some invertebrates, melanin can physically encapsulate pathogens [5], and is therefore an important component of the immune system. Moreover, in insects other products of the melanin pathway participate in cuticle sclerotization and wound healing [6]. In molluscs, tyrosinase is secreted (α-subclass) and appears to contribute to shell pigmentation and formation by the cross-linking of *o*-diphenols and quinone-tanning to form the non-calcified periostracal layer [9–12]. Tyrosinase gene expression and spatial localization in the organ responsible for shell formation and patterning in molluscs, the mantle, is consistent with a role in shell fabrication [13].

In this paper, we reveal through comparative genomics and transcriptomics that the tyrosinase gene family has undergone substantial expansions in at least two bivalve lineages, and that the resulting gene duplicates have been co-opted into the mantle gene regulatory network. Unique expression profiles of orthologous, lineage-restricted tyrosinase genes in the mantles of two closely related pearl oysters, *Pinctada maxima* and *P. margartifera*, which are estimated to have diverged 8 million years ago [14], indicates that regulatory evolution further contributes to the neofunctionalization of these new tyrosinase genes in shell formation.

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2. Materials and methods

2.1. Genome- and transcriptome-wide surveys of tyrosinase genes

All potential tyrosinase genes were identified by HMMER searches using default parameters, an inclusive *E*-value of 0.05 and the tyrosinase domain (PF00264) as the profile HMM (www.hmmer.org). The analysed molluscan genomes included *Lottia gigantea* (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html) [15], *Crassostrea gigas* (http://oysterdb.cn/) [16] and *Pinctada fucata* (http://marinegenomics.oist.jp/pinctada_fucata) [17]; the non-redundant protein database at the NCBI (National Centre for Biotechnology Information) was also analysed. Additionally, publicly available mantle transcriptome data from *P. margaritifera* (NCBI SRA: SRR057743, [18]), *P. fucata* (DDBJ SRA: DRS000687 and DRS000688, [19]), *C. gigas* (http://gigadb.org/dataset/view/id/100030, [16]), *Mytilus edulis* (http://www.ebi.ac.uk/ena/data/view/PRJEB4516, [20], *Hyriopsis cumingii* (NCBI SRA: SRR530843, [21], *Laternula elliptica* (NCBI SRA: SRA011054, [22]), *L. gigantea* (NCBI EST: FC558616-FC635770), *Patella vulgata* [23], *Haliotis asinina* (NCBI EST: EZ420605-EZ421271, [24] and *H. rufescens* (http://datadryad.org/resource/doi:10.5061/dryad.85p80, [25] were downloaded. *P. maxima* mantle transcriptome was obtained using 454 GS-FLX Plus sequencer (F. Aguilera et al., 2013, unpublished data).

For transcriptome datasets, low-quality reads were removed and the remaining sequences de novo assembled using Trinity software [26] with default settings, followed by clustering of redundant contigs using CAP3 [27]. All transcripts from each species were translated into open reading frames and surveyed for tyrosinase sequence signatures using HMMER profiling. Tyrosinase sequences are available in the online Supplementary data File S1. *P. maxima* tyrosinase sequences have been submitted to NCBI (accession Nos. KJ533305–15). The derived protein sequences were BLASTP searched against the NCBI non-redundant protein database with an e-value of 1e-5 in order to corroborate tyrosinase as the best-hit matches.

2.2. Phylogenetic analyses

The retrieved protein sequences were aligned using the MAFFT algorithm [28] and then manually inspected to remove those hits fulfilling one of the following conditions: (1) not possessing all six conserved histidine residues in the copper-binding sites; (2) incomplete sequence with >99% sequence identity to a complete sequence from the same taxa; and (3) sequences that showed extremely long branches in the preliminary maximum likelihood trees. The final alignment was refined using the RASCAL webserver [29] and analysed with Gblocks 0.91b [30] to select conserved regions. Neighbor-joining (NJ) reconstructions were performed using MEGA 5.2.2 [31] using the JTT substitution model [32] (4 gamma categories) and 1,000 bootstrap replicates. Maximum-likelihood (ML) trees were constructed using RAxMLGUI v. 1.3 [33] and the WAG substitution model [34], gamma distribution ("PROTGAMMA" implementation), four discrete rate categories, starting from a random tree and 1,000 bootstrap replicates. Bayesian inferences (BIs) were performed using MrBayes v. 3.2 [35] and the WAG model [34] (4 gamma categories). The inference consisted of 1,500,000 generations with sampling every 100 generations, starting from a random starting tree and using four chains. Two runs were performed to confirm the convergence of the chains. Trees were visualised and edited using FigTree v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). All alignments are available upon request.

2.3. Gene architecture and synteny analysis

The draft assembly genomes of *L. gigantea*, *C. gigas* and *P. fucata* were downloaded from each genome portal mentioned above. In brief, the genomes were searched using the tyrosinase genes retrieved by HMM searches and the TBLASTN algorithm. Any identified scaffolds with similarity to tyrosinase genes were extracted for further analysis. Next, the exon-intron architectures of these genes were determined by alignment to the transcript. Each alignment was manually annotated with Geneious v. 6.0.5 (Biomatters Ltd.) and viewed using CLC Genomics Workbench v. 6.5.1 (CLC Bio).

To test whether the genes adjacent to the tyrosinase genes are shared across mollusc species (indicating syntenic conservation), scaffolds containing tyrosinase genes were analysed by Augustus v. 2.7 [36] to predict protein-coding sequences. All predicted sequences were BLASTX and BLASTP searched against the NCBI non-redundant protein database, using an *e*-value cut off of 1e-5, and the best-hit match was recorded. In a pairwise approach, predicted amino acid sequences for gene models adjacent to *P. fucata, C. gigas* and *L. gigantea* tyrosinase genes were reciprocally BLASTP searched and the genomic location of five genes upstream and downstream of each tyrosinase genes was compared. Due to the limited length of *P. fucata* scaffolds, additional TBLASTN searches were performed between the genes adjacent to *C. gigas* and *L. gigantea* tyrosinases against the *P. fucata* genome to identify the scaffolds of these neighbours within this species and determine synteny conservation.

2.4. Tissue sampling, total RNA extraction and cDNA synthesis

P. margaritifera were collected from the reef flat at Heron Island Reef, the Great Barrier Reef, Queensland, Australia, and *P. maxima* were provided by Clipper Pearls/Autore Pearling, Broome, WA, Australia. Four individuals of each pearl oyster species were sampled. The gill, foot, adductor muscle, mouth, labial palp, mantle edge and mantle pallial were dissected from these individuals. Additionally, a section of mantle from the outer edge to the centre of four individuals of both pearl oyster species was divided into four equal sections in order to evaluate tyrosinase gene expression across the mantle.

Total RNA was extracted from the tissues and mantle sections with Tri reagent (Sigma–Aldrich) following a protocol modified from Gao et al. [37] to remove inhibitory pigments. RNAs (500 ng) were treated with Amplification Grade DNase following the instructions of the manufacturer (Invitrogen). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

2.5. Transcriptome profile analysis and real-time quantitative-reverse transcription PCR (qPCR)

Tyrosinase transcript abundances were assessed for five bivalve species (*P. maxima, P. margaritifera*, *P. fucata*, *C. gigas* and *L. elliptica*) using the single- and pair-end read sequences retrieved from each species. All mantle transcriptomes were sequenced from adult animals [16,18,19,22], allowing for direct RNA–Seq comparisons.

Tyrosinase quantification from RNA–Seq data was conducted with RSEM v. 1.2.8 [38]. This allows for an assessment of transcript abundances based on the mapping of RNA–Seq reads to the assembled transcriptome. Gene-level expression was multiplied by 10^6 to obtain a measure given as transcripts per million (TPM) for each gene. Because gene length may vary between samples (isoforms) and species (orthologues), we prefer the use of TPM values over RPKM (read per kilobase per million) values. TPM is independent

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