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Mini-review an insect-specific system for terrestrialization: Laccase-mediated cuticle formation

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ABSTRACT

Insects are often regarded as the most successful group of animals in the terrestrial environment. Their success can be represented by their huge biomass and large impact on ecosystems. Among the factors suggested to be responsible for their success, we focus on the possibility that the cuticle might have affected the process of insects' evolution. The cuticle of insects, like that of other arthropods, is composed mainly of chitin and structural cuticle proteins. However, insects seem to have evolved a specific system for cuticle formation. Oxidation reaction of catecholamines catalyzed by a copper enzyme, laccase, is the key step in the metabolic pathway for hardening of the insect cuticle. Molecular phylogenetic analysis indicates that laccase functioning in cuticle sclerotization has evolved only in insects. In this review, we discuss a theory on how the insect-specific "laccase" function has been advantageous for establishing their current ecological position as terrestrial animals.

1. Introduction

The recent large-scale analysis of phylogenetic relationships within the arthropods indicates that the occurrence of Hexapoda or Insecta goes back to the early Ordovician (Misof et al., 2014), suggesting that they were one of the earliest animals to form the first terrestrial ecosystem with plants. The fossil records also indicate their early colonization into the terrestrial ecosystem (Engel and Grimaldi, 2004), as has been assumed in other arthropods, such as Chelicerata and Myriapoda (reviewed in Selden, 2005 or Kenrick et al., 2012). The body of arthropods is covered by a cuticle, which is thought to have been an advantageous factor when they started to move from the aqueous to the terrestrial environment. It serves as a barrier to maintain homeostasis in the body cavity. It also serves as an exoskeleton, providing mechanical support against gravity. Generally, the arthropod cuticle is an extracellular matrix composed mainly of the polysaccharide chitin and proteins. Structurally, the cuticle of the four arthropod sub-phyla (Chelicerata, Myriapoda, Crustacea and Insecta) has the common feature of being divided into epicuticle and procuticle. The procuticle is further divided into exocuticle and endocuticle (Neville, 1975; Roer and Dillaman, 1984; Dalingwater, 1987; Thorez et al., 1992). In the process of cuticle hardening, both exo- and endocuticle are stabilized through 1) cross-linking reactions (termed tanning or sclerotization), and 2) biomineralization, such as calcification (Neville, 1975; Andersen, 2010; Nagasawa, 2012; Luquet, 2012).

In the four arthropod subphyla, multiple-types of modes have been found as systems for cuticle hardening. Calcium ions accumulate when the cuticle hardens after molting in crustaceans and diplopods (Shaw, 1968; Barnes, 1982; Nagasawa, 2012; Luquet, 2012). In contrast, calcification is generally not observed in insect cuticles; exceptions include the puparium of certain dipterans (Fraenkel and Hsiao, 1967; Bodnaryk, 1972) and the exocuticle of certain lepidopterans and hymenopterans (Hackman, 1964). Although calcification seems to contribute to cuticle strength in some insects (Bodnaryk, 1972), hard cuticle, such as that in the mandible or ovipositor, contains high amounts of zinc or manganese (Fontaine et al., 1991; Quicke et al., 1998; Schofield, 2001; Schofield et al., 2002). In cheliceratan species, calcification in the cuticle is much lower, but zinc is incorporated into hard part of the cuticle structures including fangs, chelicerae, claws or stings (Schofield et al., 2003; Gallant et al., 2016; Politi et al., 2016). In several species, the sulfur levels are high, suggesting the formation of disulfide bonds (Lafon, 1943; Krishnan, 1953). The high levels of sulfur are in contrast to the case of insects, in which the majority of cuticular structural proteins are devoid of sulfur-containing residues and disulfide bonds are assumed to be rare. The recent molecular analyses and bio-informatic surveys have revealed that there are a few families of Cys-containing cuticular proteins, such as CPAP1, CPAP3, and CPCFC. CPAPs are characterized by the presence of six cysteines that are assumed to be linked by disulfide bonds to make sites for chitin binding (Jasrapuria et al., 2010; Willis et al., 2012). CPCFCs have repeats of 16 residues containing two cysteines in C-x(5)-C motif (Vannini et al., 2015), and lepidopteran insects have proteins with many C-x(4)-C motifs (Asano et al., 2013). Nonetheless, for the most abundant families of cuticular proteins, lack of cysteine residue is evident (Willis, 2010).

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Transglutaminase catalyzes cross-link formation between glutamate and lysine residues in proteins, and this enzyme is another factor possibly involved in stabilization of the cuticle of chelicerates, such as the horseshoe crab, *Tachypleus tridentatus* (Iijima et al., 2005). Functions of this enzyme in stabilizing the insect cuticle have also been discussed (Shibata et al., 2010). Chilopoda (centipede) is classified into Myriapoda, but unlike diplopods, it is proposed that the centipede body is stabilized through the process of “aromatic tanning”. Cuticular proteins might be covalently linked together with tyrosine hydroxyl radicals derived from free tyrosine or tyrosine residues on the proteins (Blower, 1950). The involvement of dual-oxidase (duox) can be one possible hypothesis that is based on the observations in the process during cuticle formation of nematodes. Duox is a membrane protein composed of two domains: extracellular peroxidase and inner cellular NADHP-oxidase. Cross-linking reactions through the formation of di-tyrosine is the key for duox-mediated cuticle stabilization (Edens et al., 2001). Also, in insects, it was shown that resilin, a chitin binding protein that is used for making rubber-like matrices, is cross-linked through the formation of di-tyrosine (Andersen, 1963). Cross-link reactions of cuticular proteins via di-tyrosine formation has been demonstrated using peroxidase as the catalyst in *in vitro* model systems (Suderman et al., 2010). The involvement of the gene for duox in stabilization of wing structures in the fruitfly, *Drosophila melanogaster*, has been demonstrated (Anh et al., 2011).

In insect cuticle, unlike the cases in other arthropods, it is thought that oxidation reactions of *ortho*-diphenols are mainly involved in cuticle stabilization (Fig. 1). Two catecholamines, *N*-acetyl-dopamine (NADA) and *N*-β-alanyl-dopamine (NBAD), have been identified as the main substrates used for cuticle hardening and also as quinone pigmentation (indicated with red-lined boxes in Fig. 1A) (Wright, 1987; Arakane et al., 2009; Andersen, 2010). After oxidation of NADA and NBAD, the corresponding quinones are thought to mediate cross-link formation between cuticular proteins or between proteins and chitin fibers (reviewed in Andersen, 2010, 2012). During oxidation of these catecholamines, co-existing L-dopa and dopamine (DA) are also oxidized to dopa-quinone and DA-quinone, respectively and enter the melanin synthesis pathway.

2. The importance of Laccase2, an insect specific protein

For catalyzing the oxidation reactions of these catecholic compounds in the insect cuticle, a copper enzyme, laccase, appears to play indispensable roles (Arakane et al., 2005, 2009). Laccase was originally found in the Japanese lacquer tree, *Rhus vemicifera* (Yoshida, 1883). Since then, laccases from plants, fungi, and bacteria have been extensively studied, and a huge amount of knowledge has been accumulated on its enzymatic properties, molecular structure and gene functions (Nakamura and Go, 2005; Sharma et al., 2007). In animals, however, until recently insect laccases are the only examples, the molecular properties or functions of which have been studied. Laccase can catalyze monoelectric oxidation of substrates such as *o*- and *p*-diphenols, polyphenols, aminophenols, aromatic amines and aliphatic amines. These reactions are associated with electron transfer from the substrate to molecular oxygen, resulting in the production of water molecules. Laccase belongs to the three-domain multi-copper oxidases (3dMCOs), which are composed of tandem repeats of three cupredoxin domains (Nakamura and Go, 2005; Sharma et al., 2007).

Insects generally have genes for two types of 3dMCOs, which are designated “laccase2” and “MCO1” (Dittmer et al., 2004; Lang et al., 2012). Loss-of-function analyses for laccase2 genes by knockdown in multiple orders, including the red flour beetle, *Tribolium castaneum*, have demonstrated that the genes for laccase2s are required for insect cuticle formation, especially its pigmentation, sclerotization and stabilization (see Fig. 1B) (Arakane et al., 2005, 2009; Niu et al., 2008; Elias-Neto et al., 2010; Futahashi et al. 2011; Ye et al., 2015). Laccase2-depleted individuals exhibit molting-defect mortality or lethality soon after molting, probably due to the incomplete formation of the new cuticle. The newly formed cuticle is soft, and the new cuticle becomes malformed which may be caused by incomplete hardening of the cuticle (Arakane et al., 2005; Ye et al., 2015; Aizawa et al., in preparation). The laccase2-depleted insects also show a severe cuticle pigmentation defect, since laccase2 is the key enzyme for both the cuticle hardening and pigmentation pathways (Fig. 1A). It has been reported that expression of laccase2s is prominent in pre-molt stages; after molting, however, the expression becomes very weak (Yatsu and Asano, 2009; Dittmer et al.,

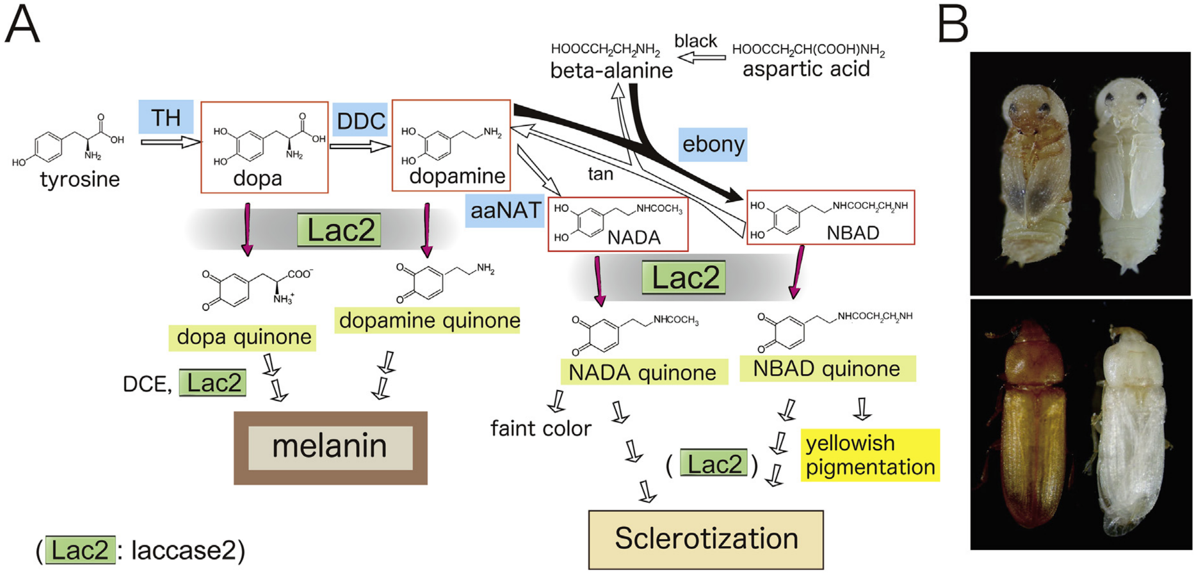

Fig. 1. (A) Tyrosine metabolic pathway for cuticle construction in insects. This pathway is drawn according to Arakane et al. (2009) with minor modifications. Four major substrates for laccase2 (Lac2) are indicated with red bordered boxes. Enzymes for substrate supply are indicated with sky-blue boxes. (B) The phenotypes of laccase2 knocked-down individuals of the red flour beetle, *T. castaneum*. dsRNA for Lac2 was injected into the pupae. In the pharate pupal stage (just before eclosion), no pigmentation occurs in legs, the tips of hind wings or the mandible (right pupa in upper photo). After eclosion, the formation of the adult cuticle was severely affected. At high concentrations, dsRNA for Lac2 causes a defect in eclosion (not shown). The right Lac2-depleted individual in the lower photo is a hypomorph from injecting a lower concentration of dsRNA (Arakane et al., 2005; Arakane et al., 2009) (the photo was supplied by courtesy of Dr. Yasuyuki Arakane). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2009; Futahashi et al., 2011: Masuoka et al., 2013). The expression before molting indicates that laccase2s mainly accumulate in the exocuticle that is secreted before ecdysis. This finding is consistent with the observation that the outer part (the part produced earlier than inner part) of the cuticle is heavily tanned or sclerotized (reviewed in Neville, 1975 and Andersen, 2010; Masuoka and Maekawa, 2016).

Regarding the enzymatic properties of the two 3dMCOs in insects, it has been shown that laccase2s (both endogenous and recombinant proteins) exhibit activity against the catecholic compounds found in insect cuticle (Yamazaki, 1972; Yatsu and Asano, 2009; Dittmer et al., 2009; Gorman et al., 2012). Also, the recombinant MCO1s can oxidize the catechols, but with low efficiency relative to laccase2s. One of the major differences between the enzymatic properties of laccase2s and those of MCO1s is that MCO1s exhibit activity towards both ferrous ions and ascorbate. Although the activity towards ferrous ions is lower relative to 3dMCOs that were previously identified as ferroxidases from other sources, insect MCO1s can oxidize ascorbate with high efficiency. In *melanogasterD*., and the malaria vector mosquito, *Anopheles gambiae*, MCO1s are expressed in the midgut epithelia, and iron homeostasis (possibly through ascorbate metabolism) may be one of critical roles of MCO1s *in vivo* (Lang et al., 2012; Peng et al., 2015). The genes for MCO1s do not seem to participate in hardening of the cuticle, which is supported by observations that MCO1 knockdown does not result in any visible phenotypic changes of cuticle formation (Arakane et al., 2005; Lang et al., 2012; Peng et al., 2015).

To discuss the evolution of laccase2, we constructed a molecular phylogenetic tree of 3dMCOs from insects and non-insect arthropods. To collect protein sequences, we searched the Ensemble Metazoa database (http://metazoa.ensembl.org/), which integrates genomic information of metazoan species. This database includes 38 insects and 7 non-insect arthropods (for details, see Appendix). For information from non-insect arthropods and primitive insects that are not included in the Ensemble Metazoa database, we used the i5k database (https://i5k.nal.usda.gov/webapp/blast/) and also the recently reported completed genomic information of the two crustaceans and two non-insect hexapods (Song et al., 2016; Kao et al., 2016; Faddeeva-Vakhrusheva et al., 2016, 2017). In our search, we collected 3dMCO sequences of insects from multiple orders including Odonata, Ephemeroptera, Isoptera, Homoptera, Hemiptera, Diptera, Coleoptera, and Lepidoptera that covered wide range of both hemi- and holo-metabolous species. We also collected all the available sequences from non-insect arthropods of five crustaceans, one myriapod, and three non-insect hexapods, but there appear to be no genes encoding 3dMCO proteins in the genomes of any chelicerates. We also added a 3dMCO sequence from the white leg shrimp, *Litopenaeus vannamei* that was reported recently (Shi et al., 2017).

Fig. 2 shows the molecular phylogenetic tree of 3dMCOs from arthropods. The proteins are separated mainly into two clusters (A and B, indicated with light blue and light green shades, respectively). All the MCO1s that have been characterized previously (*T. castaneum, D. melanogaster, A. gambiae* and the tobacco hornworm, *Manduca sexta*) are included in cluster A. The A cluster also includes proteins from non-insect arthropods (myriapod, crustaceans, and non-insect hexapods). On the other hand, laccase2 forms an independent sub-cluster (deep green shade supported by the bootstrap value of 100%) inside the B cluster that is also supported by high bootstrap value (98%). In previous reports, the high sequence identity of insect laccase2s has been discussed (Dittmer et al., 2004, 2009; Asano et al., 2014). The phylogenetic tree also shows the high sequence conservation of laccase2s with the very short branch lengths in the sub-cluster. Importantly, the B cluster includes only insect proteins. Laccase2 is an insect specific protein that evolved independently from the lineage of MCO1s or non-insect arthropods' MCOs. We prepared another phylogenetic tree with more insect species (see Supplement), and monophylogeny of laccases2 is supported by 100% bootstrap value as in the tree constructed with the representative insect species (Fig. 2) (hereafter, laccase2 is referred to simply as laccase unless otherwise required).

3. How could laccase-mediated cuticle formation (LMCF) evolve in insects?

Crustacea and Insecta together form the taxon of Pancrustacea, which is separated from the lineage of Myriapoda (Giribet et al., 2001) (Fig. 3). In marine environments, crustaceans are the dominant arthropods, whereas insects dominate on land and in the air. Crustaceans are among the most morphologically diverse of any taxa (Martin and Davice, 2001), and this diversity is often compared with the high diversity of insects in terrestrial environments. These situations imply the high potentials of both crustaceans and insects to adapt a variety of environments by diversification. When we focus on their physiology or metabolism, what are the differences between insects and crustaceans that might have affected their evolutional processes or their distribution (marine or terrestrial)? Here, we consider systems for cuticle hardening a point to be discussed. In marine systems, crustaceans seem to utilize the high availability of calcium ions in seawater. In contrast, in terrestrial environments, the availability of calcium ions is restricted relative to marine environments. The acquisition of systems that do not rely on calcium ions might have a strong influence on adaptation to land environments. Cuticle formation through cross-link formations catalyzed by laccase (we call this system "laccase-mediated cuticle formation (LMCF)") might be a good solution.

After divergence of the lineages of insects and crustaceans, by acquiring the gene for laccase, the ancestral insects might have evolved the LMCF system, but crustaceans relied mainly on calcification for cuticle hardening. As described below, crustaceans may partially use systems similar to insects' LMCF, though they do not have gene for laccase, and the contribution of calcification seems to be far from negligible. In the marine environments, calcium is supplied easily from the surrounding seawater (Deshimaru et al., 1978). However, crustaceans might have met difficulty in terrestrialization from seawater. The difficulties can be exemplified by the observations that terrestrial crustaceans recycle calcium by adsorbing it from the old cuticle to make gastroliths (crystalline calcium carbonate adjacent to the stomach) or by consuming calcium ions in exuvia (Ueno and Mizuhira, 1984; Steel, 1993; Neues et al., 2011; Luquet, 2012). Water fleas are crustaceans belonging to Branchiopoda that is characterized by the main habitat in fresh water. Previously the very close relationship between Branchiopoda and Insecta, and therefore the fresh water origin of insects has been discussed (Glenner et al., 2006). The recent study on the newly found primitive crustacean, Remipedia, argues that its closer relationship with Insecta and marine origin of insects, but Branchiopoda is still the important taxon when we discuss on the origin of insects (Lozano-Fernandez et al., 2016; Schwentner et al., 2017). Although it has not been shown that the calcium ion really is the structural component contributing to the strength of the cuticle in *Daphniapulex*, this species loses 40% of its total calcium content with the old exuvia in each moulting even at low calcium concentrations (Alstad et al., 1999). In addition, in low-calcium conditions (< 1.5 mg/L), *D. pulex* fails to strengthen its carapace, increase its body size, or extend its neck spine, all of which are defensive responses occurring after reception of chemical compounds from predators (Riessen, 2012). These observations of *D. pulex* indicate the high calcium content in their cuticle and a positive correlation between the calcium ion and cuticle formation. The early insects, being liberated from the requirement for high calcium storage, might have been able to move to inland areas much more freely than animals requiring the calcium ion for construction of their body structure. In addition to calcium requirement, there is one more important aspect related to the evolution of LMCF in insects. We think that the insects' LMCF system was selected as an adaptive trait in terrestrial environments in which plenty of atmospheric oxygen is available, since the oxidation reactions catalyzed by laccase are accompanied by reduction of molecular oxygen as an acceptor of electrons from

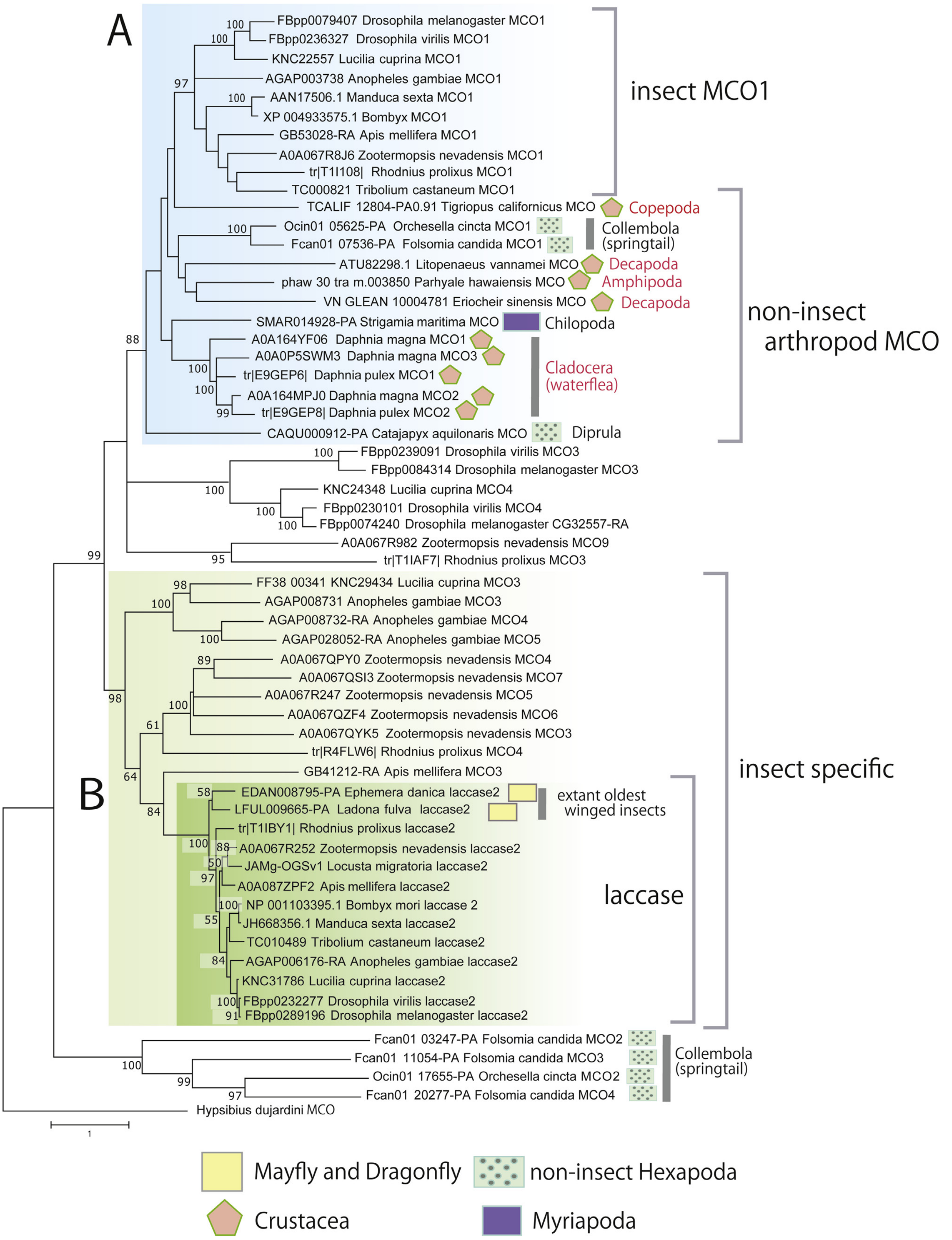

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Fig. 2. Molecular phylogenetic tree of multi-copper oxidases from arthropods. Bootstrap values lower than 50% are not shown. The main clusters with insect MCO1s (A) and laccases (B) are indicated in light blue and light green, respectively. Sub-cluster of laccases is indicated with deep green shade. MCOs from non-insect arthropods are individually indicated with distinct colors, according to their classifications. The sequences used are *S. maritima* MCO (SMAR014928-PA), *D. magna* MCO1 (A0A164YF06), *D. magna* MCO3 (A0A0P5SWM3), *D. magna* MCO2 (A0A164MPJ0), *D. pulex* MCO1 (E9GEP6), *D. pulex* MCO2 (E9GEP8), *L. cuprina* MCO3 (KNC29434), *L. cuprina* MCO4 (KNC24348), *L. cuprina* MCO1 (KNC22557), *L. cuprina* laccase2 (KNC31786), *Z. nevadensis* laccase2 (A0A067R252), *Z. nevadensis* MCO4 (|A0A067QPY0), *Z. nevadensis* MCO5 (A0A067R247), *Z. nevadensis* MCO6 (A0A067QZF4), *Z. nevadensis* MCO7 (A0A067QSI3), *Z. nevadensis* MCO3 (A0A067QYK5), *Z. nevadensis* MCO1 (A0A067R8J6), *Z. nevadensis* MCO9 (A0A067R982), *R prolixus* MCO1 (tr|T1I108|), *R. prolixus* laccase2 (tr|T1IBY1), *R. prolixus* MCO4 (tr|R4FLW6), *R. prolixus* MCO3 (tr|T1IAF7), *B. mori* MCO1 (XP_004933575.1), *B. mori* laccase2 (NP_001103395.1), *M. sexta* laccase2 (JH668356.1), *M. sexta* MOC1 (AAN17506.1), *A. mellifera* laccase2 (A0A087ZPF2), *A. mellifera* MCO3 (GB41212-RA), *A. mellifera* MCO1 (GB53028-RA), *T. castaneum* laccase2 (TC010489), *T. castaneum* MCO1 (TC000821), *A. gambiae* laccase2 (AGAP006176-RA), *A. gambiae* MCO4 (AGAP008732-RA), *A. gambiae* MCO3 (AGAP008731-RA), *A. gambiae* MCO1 (AGAP003738-RA), *A. gambiae* MCO5 (AGAP028052-RA), *D. melanogaster* laccase2 (FBpp0289196), *D. melanogaster* MCO1 (FBpp0079407), *D. melanogaster* CG32557 (FBpp0074240), *D. melanogaster* MCO3 (FBpp0084314), *D. virilis* MCO1 (FBpp0236327), *D. virilis* laccase2 (FBpp0232277), *D. virilis* MCO3 (FBpp0239091), *D. virilis* MCO4 (FBpp0230101), *E. Danica* laccase2 (EDAN008795-PA), *L. fulva* laccase2 (LFUL009665-PA), *L. migratoria* laccase2 (JAMg-OGSv1), *C. aquilonaris* MCO (CAQU000912-PA), P. *hawaiensis* MCO (phaw_30_tra_m.003850), *E. sinensis* MCO (VN_GLEAN_10004781), *T. californicus* MCO (TCALIF_12804-PA0.91), *O. cincta* MCO1 (Ocin01_05625-PA), *O. cincta* MCO2 (Ocin01_17655-PA), *F. candida* MCO1 (Fcan01_07536-PA), *F. candida* MCO2 (Fcan01_03247-PA), *F. candida* MCO3 (Fcan01_11054-PA), *F. candida* MCO4 (Fcan01_20277-PA), *L. vannamei* MCO (ATU82298) and *H. dujardini* (nHd.2.3.1.t17025-PA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

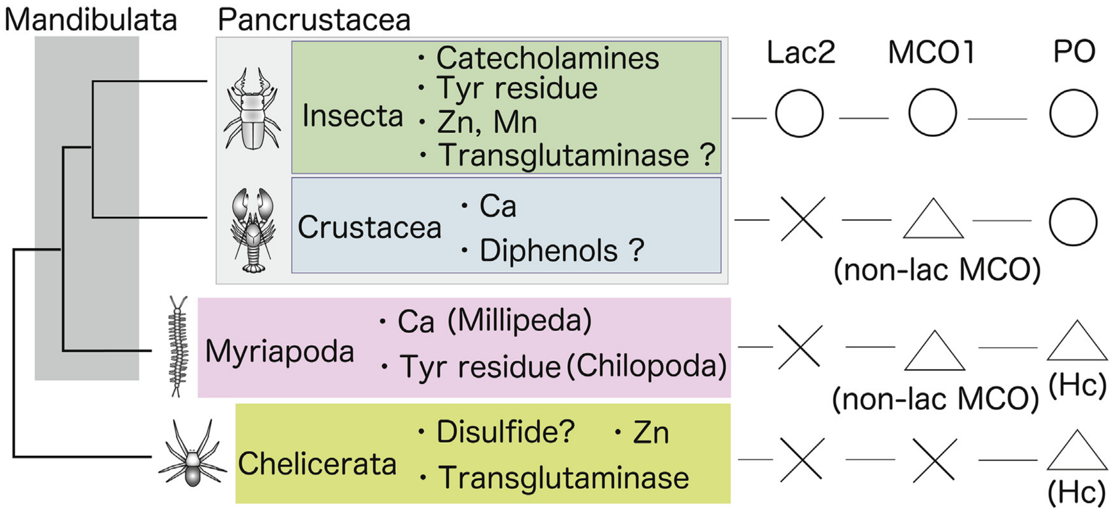

Fig. 3. Phylogenic relationship of arthropod sub-phyla. A figure (Fig. 1 in the reference (Hughes and Kaufman, 2002)) was modified. In the evolution of arthropods, ancestors of Chelicerata and Mandibulata were branched at first. Then, ancestors of Myriapoda and Pancrustacea were divided. From Pancrustacea, Crustacea and Insecta (Hexapoda) were derived (Giribet et al., 2001). In each sub-phylum, factors for cuticle hardening are indicated. The circles indicate the presence of each oxidase. The triangles indicate the presence of non-laccase MCO and Hc, that can substitute MCO1 and PO, respectively. The crosses indicate the absence of each oxidase (or its alternative).

substrates. In the period when arthropods began to colonize on land, the atmospheric oxygen had reached a level comparable to the current value (20%). In the atmosphere, the content of oxygen is much higher (30-fold) than it is in water. In addition, the density and viscosity of air are much less than those of water (1000- and 50-fold, respectively), making the absorption of oxygen much easier from the air (reviewed in Hsia et al., 2013). It is reasonable that LMCF accompanied with high amounts of oxygen consumption evolved in accordance with colonization into the atmospheric condition rather than in water.

Another unique property of the insect cuticle is their low weight, which can be achieved by the system without bio-mineralization (Borrell, 2004; Fernandez and Ingber, 2013). In the history of metazoan evolution, insects were the first animals to fly. Their light but strong cuticles could be one of the critical factors providing the insect the ability to fly. Acquisition of flight ability must have been a great advantage in survival competition. Currently, insect species number over half of all multicellular organisms including plants and fungi, and 99% of insect species are winged insects (Price, 2002). The merits and the impacts of their flight ability have long been discussed in relation to the wide distribution and diversification of insects (Grimaldi and Engel, 2005; Bradley et al., 2009). To further discuss about the possible relationship between LMCF and its impact on insect terrestrialization, we need to know more precisely the time point from which insect laccase originates. In our database search in i5k, we could obtain putative protein sequences (EDAN008795-PA and LFUL009665-PA) from the earliest extant winged insects (the mayfly, *Ephemera danica* and the dragonfly, *Ladona fulva*, respectively). In the phylogenetic tree (Fig. 2), these proteins fall into the clade of laccase2s. Based on our database search, all of the winged insects, at least, appear to have laccase genes. We have not obtained evidence for presence of laccase genes in wingless insects, because of the shortage in open bioinformatic information, but it is interesting to see whether the occurrence of insects and that of the laccase gene synchronized. As far as we searched, non-insect hexapods do not have genes for laccase. We are planning experiments to confirm the presence of laccase in the oldest extant insects, namely, jumping bristletail and silverfish.

4. Enzymes that can substitute for laccase

To understand how insects adapted to terrestrial environments, one of important questions may be how and why the system for catecholamine oxidation in the cuticle evolved in insects. Apart from the roles as substrates for oxidases, presence of catecholamines in body surfaces can be a protection against UV with their absorption capability or defense against microbes with the antibacterial activities (Schachter et al., 2007). Catecholamines used for such purposes might have been diverted as substrates for cuticle formation. Though insects are the only arthropod that acquired a gene for laccase specialized for cuticle hardening, there are multiple enzymes in non-insect arthropods that have potential to substitute the molecular functions of laccase. For instance, myriapods, non-insect hexapods and crustaceans have genes for non-laccase MCOs (Fig. 2). As described, it is assumed that insects' MCO1 is involved in iron homeostasis or ascorbate metabolism, but they have activity against catecholamines (Lang et al., 2012; Peng et al., 2015). It is possible that the MCOs of non-insect arthropods share the same molecular properties to oxidize wide ranges of substrates and can participate in cross-linking formation in their cuticles.

Other than MCOs, insects and crustaceans have another type of enzyme capable of catalyzing catechol oxidation. This enzyme, phenoloxidase (PO), is homologous to arthropod hemocyanin (Hc). PO is also a copper enzyme, but it belongs to the "type-3 copper protein" family (Decker et al., 2007), which is considerably different from 3dMCO. In earlier studies, PO had often been called tyrosinase-type phenoloxidase or simply tyrosinase, because the enzymological properties are similar to those of tyrosinases from other sources. In more recent researches, it was shown that PO does not belong to the tyrosinase family, though tyrosinases are also proteins belonging to the type-3 copper protein family (Fujimoto et al., 1995; Hall et al., 1995; Kawabata et al., 1995). Currently, "PO" or more generally the name of its precursor, "pro-phenoloxidase (proPO)," is widely used in insect science to indicate the protein with tyrosinase-like activity. Regarding the cuticular enzymes showing laccase-like activity, they were initially called laccase-type phenoloxidase or simply phenoloxidase. After confirmation that they have typical laccase-like sequences (Yatsu and Asano, 2009; Dittmer et al., 2009), the enzyme has been called laccase2 or laccase. It is well known that PO is involved in melanin formation as a defense reaction (reviewed in Ashida and Brey, 1998; Kanost et al., 2004; Iwanaga and Lee, 2005). PO can oxidize substrates for both melanin synthesis and sclerotization of insect cuticle (Aso et al., 1984; Asano and Ashida, 2001). It was confirmed in the silkworm, *Bombyx mori,* that PO is present in the cuticle as the inactive precursor (Ashida and Brey, 1995; Asano and Ashida, 2001), but double knockdown of the two *T. castaneum* PO genes (*Tyr-1, -2*) does not show any visible phenotypes in cuticle formation (Arakane et al., 2005). Similar results were obtained in *D. melanogaster*. Double deletion mutation for PO genes (*PPO1*, *-2*) does not affect cuticle tanning (Binggeli et al., 2014). Among the three PO genes (*PPO1-3*) in *D. melanogaster*, *PPO1* and *-2* are mainly expressed throughout the stages in contrast to the very weak expression of PPO3 in normal developmental conditions (Irving et al., 2005; Nam et al., 2008; Asano and Takebuchi, 2009). These observations indicate that PO is not used for cuticle formation in insects. A possible correlation between expression of PO and red coloration by oxidative cross-link of kynurenine and NBAD has been hypothesized (Nishikawa et al., 2013). This is an example that not laccase, but PO is involved in oxidative-conjugation of cuticular components, though its contribution to cuticle hardening is unknown.

Unlike insects or crustaceans, myriapods and chelicerates do not have PO but instead have a related protein, Hc, which can be converted to derivatives showing PO activity after certain modifications (Nagai et al., 2001; reviewed in Decker et al., 2007). Based on such characters, participation of Hc in cuticle hardening and pigmentation during development have been discussed (Adachi et al., 2005; Kuballa and Elizur, 2008), though this hypothesis requires experimental confirmation. In number of crustacean species such as *Cancer pagurus*, *Orconectes obscurus*, *Clibanarius olivaceous*, and *Carcinus maenas*, diphenol-oxidizing activity was found in the cuticles. Although it is not known whether they are categorized as PO or 3dMCO, it has been proposed that the enzymes with activity to diphenols are involved in tanning of epicuticle. In the fiddler crabs, *Uca pugnax* and *U. pagilator*, phenolic compounds like NADA and *N*-acetylnoradrenaline were identified. In the former species, it was demonstrated that NADA was synthesized in isolated epidermal cells (Dennell, 1947; Stevenson and Adomako, 1967; Summers, 1967; Summers, 1968; Chockalingam, 1971; Vacca and Fingerman, 1975a,b; reviewed in Stevenson, 1985). These observations suggest that there would be LMCF-like system or prototype of LMCF in crustaceans. Possibly the alternatives described (non-laccase MCOs, PO or Hc derivatives) can be candidate molecules catalyzing oxidative cross-link formation, though in the analysis of hydrolytic degradation products from the cuticles of non-insect arthropods, no catechol-amine derivatives typically found in sclerotized insect cuticle have been found (reviewed in Andersen, 2010). Detailed analyses of the genes for the alternatives are required to enhance our understanding of the origin of LMCF or relationship between LMCF of insects and the systems in non-insect arthropods. For further discussion, it is also important to study evolution of the system for substrate supply; dopamine-modifying enzymes, such as ebony or aaNAT (Fig. 1), and the presence of epithelially expressed isoforms of enzymes for dopamine synthesis, TH and DDC (Fig. 1), that have been reported in several insects (Shen et al., 1993; Vie et al., 1999; Ninomiya and Hayakawa, 2007).

Arthropods from all the four subphyla have oxidases (or precursors of oxidases) that can catalyze catecholamine oxidation. However, only insects evolved the efficient system utilizing catecholamine oxidation for cuticle hardening. We think that the acquisition of laccase gene is the key. Biochemical characterization revealed that the activity of insect MCO1s is less against substrates for cuticle hardening but can oxidize substrates in other metabolic pathways. The genes for MCO1s are involved in at least iron homeostasis, and in *D. melanogaster* strong knock down of the MCO1 gene leads to lethality at the pupal stage, indicating their important physiological roles (Lang et al., 2012; Peng et al., 2015). The ancestors of insects, by acquisition of multiple copies of 3dMCO genes, could modify the sequences of the newly acquired proteins independently from the molecular evolution of MCO1s. Finally, insects might have acquired the gene for laccase with the characters of MCO1s preserved, minimizing the risks by MCO1s' dysfunctions. Considering that laccases do not exhibit activity against either ferrous iron or ascorbate that MCO1s can oxidize (Lang et al., 2012; Peng et al., 2015), there may be a trade-off between the high activity to oxidize catecholamines and availability of a wide range of substrates. As described above, we are interested in the presence of laccase genes in primitive wingless insects. In such insects at the boundary with non-insect arthropods, it is possible that they have prototypes of LMCF mediated by non-laccase 3dMCO that could be intermediate molecules in the process of transition to laccase from ancestral proteins.

For the reason why laccase but not PO is used in cuticle sclerotization reaction in insects, we focus on a hypothesis on *para*-quinone methide (summarized by Andersen, 2010, 2012; Sugumaran, 2016). During cuticle sclerotization, *o*-quinones are readily cross-linked to their surrounding substances with their aromatic rings. On the other hand, from *o*-quinones, *p*-quinone methides can be produced by isomerization that is both enzymatic and non-enzymatic. It has been proposed that *p*-quinone methides and their downstream metabolites including dehydro-NADA and dehydro-NBAD are used for formation of cross-links with their side chains. Small amount of *p*-quinone methide production can be coupled with oxidation reactions of *o*-diphenols by tyrosinase-type enzyme. In contrast, *p*-quinone methide production is easily associated (up to 40% of the total products) with laccase-mediated oxidation as has been demonstrated with the enzyme from *M. sexta*, suggesting that no additional factors are needed for production of *p*-quinone methides in sufficient amounts for sclerotization (Thomas et al., 1989; Andersen, 2010, 2012). Differently from type-3 copper oxidases that catalyze two electron oxidation, free radicals are formed by one electron oxidation in laccase-mediated reactions. In *o*-diphenol oxidation, production of *o*-semiquinone radicals initially occurs, which can be vital for polymerization or cross-couplings (reviewed in Walker and Ferrar, 1998 and Kudanga et al., 2017). In plant physiology, it is thought that formation of free radicals from phenoic substrates is one of the key steps in biosynthesis of lignin, a material used to construct cell walls (reviewed in Ralph et al., 2009). Similar to insects, plants have both laccase and the type-3 copper enzyme that is called polyphenol oxidase (Pretzler and Rompel, 2018), and plants may use laccase for lignin production. Lignin is estimated to make up 20–35% of the dry weight of wood (Li, 2011), which is produced by oxidative polymerization of lignin monomers (phenyl propanes). It is thought that this oxidative process might have evolved when the level of atmospheric oxygen was high (Willis and McElwain, 2002). The recent phenotypic characterization of *Arabidopsis thaliana* mutants indicates the involvement of laccase genes in lignin polymerization (reviewed in Wang et al., 2015). In lignin biosynthesis, it is thought that quinone methides are produced from one electron oxidized products of lignin monomers (phenoxy radicals), and that they are implicated as one of the reaction intermediates. It is intriguing that both insects and plants adopted the same strategy to evolve laccase-mediated production of structural components in the conditions where they could utilize abundant molecular oxygen. This analogy could be an example of convergence in the evolution of molecular processes.

5. Adaptation to terrestrial environments by LMCF

Here we have discussed the possible involvement of LMCF in their success as terrestrial animals, but insects have evolved other traits and systems that could be advantageous for expanding their distribution and increasing their survivability and efficient use of natural resources. Flight ability, complete metamorphosis, symbiosis, eusociality and coevolution with plants have been the major topics of discussion on how insects have successfully expanded their territory and become so diverse (Grimaldi and Engel, 2005; Bradley et al., 2009). All the arthropod sub-phyla successfully adapted to terrestrial environments irrespective of the presence of the laccase2 gene, indicating that terrestrialization itself does not always require the system of LMCF. However, as described above, LMCF might have been a great advantage when insects or their ancestors were adapting to terrestrial environments. The adaptation-scheme can be summarized as in Fig. 4: 1) the ancestor of Hexapoda/Insecta colonized land, 2) On land, LMCF might have been selected as a system to use the high availability of molecular oxygen and to provide advantages for expansion into calcium-poor areas far from the seashore, LMCF without bio-mineralization could be one of the key factors providing a lightweight body suitable for flight, 3) flight ability might have accelerated the distribution of insects, creating open/new niches that could not be accessed by other arthropods. By implementing these hypothetical advantages associated with their earlier adaptation, insects currently retain the dominant position in terrestrial conditions. We hope that our new theory will lead to further discussion on the emergence/success of insects from this new point of view.

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Abbreviations

MCO	multi-copper oxidase
3dMCO	three domain MCO
PO	phenoloxidase
Hc	hemocyanin
NADA	*N*-acetyl-dopamine
NBAD	*N*-β-alanyl-dopamine
LMCF	Laccase-mediated cuticle formation

Appendix

Blast search and construction of phylogenetic tree

Sequences homologous to 3dMCO were collected from the EnsembleMetazoa database (http://metazoa.ensembl.org/), which integrates genomic information of metazoan species. The BLAST search program of this database includes seven non-insect arthropods (one myriapod (*Stigrimata maritima*), three crustaceans (*D. pulex*, *D. magna* and *Lepeophtheirus salmonis*) and four chelicerates (*Tetranychus urticae*, *Sarcoptes scabiei*, *Ixodes scapularis* and *Stegodyphus mimosarum*)). In all species, gene annotations have been reported (Altincicek et al., 2012; Colbourne et al., 2011; Grbic et al., 2011; Chipman et al., 2014; Sanggaard et al., 2014; Rider. Jr. et al., 2015; Gulia-Nuss et al., 2016; Økland et al., 2014). For query sequence, the catalytic domain of laccase2 of *B. mori* (NP_001103395.1) was used. 3dMCOs of insects have unique N-terminal extensions with cysteine-rich motif (Dittmer et al., 2004). As catalytic domains, we selected the 518 residues from the C-terminal residue of laccase2 of *B. mori* to remove the N-terminal extension (from the first Met to Cys223). After removal of the region, the catalytic domain has N-terminal 45 residues until the first histidine for copper binding. Among the proteins showing homology to the query sequence, we selected proteins by the presence of all the conserved amino acids involved in coordinating (the T1, T2, and T3 copper centers, see supplemental materials). In our search, we found over a hundred 3dMCOs from insects, one from a myriapod (*S. maritima*) and five

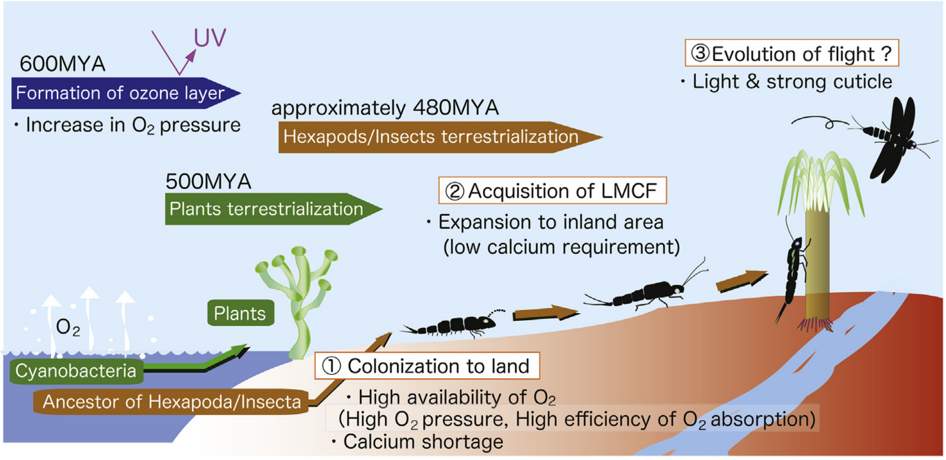

Fig. 4. A scheme of insects' adaptation to terrestrial environments. A figure in the website (https://www.brh.co.jp/seimeishi/journal/060/from_brh.html) was modified for easier understanding of our theory described here.

from two crustaceans (*D. pulex* and *D. magna*), but we could not find 3dMCO sequences in the remaining crustacean (*L. salmonis*) or any of the four chelicerates. In the i5k database, we collected laccase2-like sequences from insects including the mayfly, *Ephemera danica* and dragonfly, *Ladona fulva*. We collected one 3dMCO from the copepod, *Tigriopus californicus*, but could not find 3dMCO in the chelicerates (three spiders and one scorpion). We further checked for the presence of 3dMCO in the recently reported genomic information of two crustaceans, the Chinese mitten crab, *Eriocheir sinensis* (Song et al., 2016) and the amphipod, *Parhyale hawaiensis* (Kao et al., 2016), and the two springtails, *Orchesella cincta* and *Folsomia candida* (Faddeeva-Vakhrusheva et al., 2016, 2017). A sequence from the white leg shrimp, *Litopenaeus vannamei,* that have been reported recently was also included (Shi et al., 2017). For the phylogenetic tree in Fig. 2, we selected 13 representative insects, and all the non-insect arthropods that have 3dMCOs. The species used are: *Daphnia magna*, *Daphnia pulex*, *Eriocheir sinensis*, *Parhyale hawaiensis*, *Orchesella cincta*, *Folsomia candida*, *Tigriopus californicus*, *Catajapyx aquilonaris*, *Strigamia maritima*, *Manduca sexta*, *Bombyx mori*, *Drosophila melanogaster*, *Drosophila virilis*, *Anopheles gambiae*, *Tribolium castaneum*, *Apis mellifera*, *Bombus impatiens*, *Rhodnius prolixus*, *Zootermopsis nevadensis*, *Lucilia cuprina*, *Ephemera danica*, *Ladona fulva*, and *Locusta migratoria*. We also used an MCO from the tardigrade (*Hypsibius dujardini* (nHd.2.3.1.t17025-PA)) as the outgroup protein from non-arthropod species.

For inferring a phylogenetic tree, only the catalytic domains were used. For proteins with long N-terminal extensions, we defined that N-terminally located 45–50th residues from the first histidine for copper binding are the boundaries between N-terminal extensions and catalytic domains. If the proteins have shorter N-terminal sequences from the first histidine, they were used as they were (we prepared the FastA format sequences that were used for making the phylogenetic tree (see supplemental materials)). The catalytic domains were aligned with the MUSCLE program implemented in MEGA7 software (Edgar, 2004; Kumar et al., 2016). The phylogenetic tree was inferred using the maximum likelihood method based on the LG model (Le and Gascuel, 2008) with a discrete gamma distribution (5 categories) and the invariable site (LG + G + I) options using MEGA7 software (Kumar et al., 2016). Initial tree for the heuristic search were obtained automatically by applying neighbor-joining (Saitou and Nei, 1987) and BioNJ (Gascuel, 1997) algorithms with JTT model (Jones et al., 1992), and then searching the topology with superior log likelihood value by NNI (nearest neighbor Interchange) method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The values shown next to the branches are the statistical support assessed by 200 bootstrap replicates.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.03.007.

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