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The formation of the molluscan shell nacre is regulated to a large extent by a matrix of extracellular macromolecules that are secreted by the shell forming tissue, the mantle. This so called “calcifying matrix” is a complex mixture of proteins, glycoproteins and polysaccharides that is assembled and occluded within the mineral phase during the calcification process. The fine molecular basis that regulates nacre formation remains to be better characterized.

Noticeable advances in expressed tag sequencing of the freshwater mussels, such as *Elliptio complanata* and *Villosa lienosa*, provide a pre-requisite to further characterize the bivalve nacre proteins by a proteomic approach. In this study, we have identified a total of 48 different proteins from the nacre insoluble matrices, 31 of which are common of the two species. Few of these proteins, such as PIF, MSI60, CA, shematrin-like, Kunitz-like, LamG, chitin-binding containing proteins, together with A-, D-, G-, M- and Q-rich proteins, appear to be analogues, if not true homologs, of proteins previously described from the pearl oyster or the edible mussel nacre matrices, thus constituting a remarkable list of deeply-conserved nacre protein set. This work constitutes a comprehensive nacre proteomic study of non-pteriomorphid bivalves that concretely gives us the opportunity to describe the molecular basis of deeply conserved biomineralization toolkit among nacreous shell-bearing bivalves, with regard to proteins associated to other shell microstructures, to that of other mollusc classes (gastropods, cephalopods) and finally, to other lophotrochozoans (brachiopods).

1. Introduction

(a) Mollusc shell biomineralization

The molluscan shell is a unique model for biomineralization and constitutes a remarkable example of a natural composite biomaterial synthesized at ambient temperature from polar to tropical environment. It is composed of one or two polymorphs of calcium carbonate (*i.e.* calcite and aragonite), generally assembled in superimposed $CaCO_3$ layers of different microstructures [1]. These different shell layer textures are produced under the control of specific extracellular matrices that are secreted by different zones of the external mantle epithelium [2]. These 'cell-free' matrices, mainly constituted of proteins, glycoproteins and polysaccharides, surround nascent $CaCO_3$ crystals and are incorporated into the shell crystalline architecture in growth. Although the organic matrix is quantitatively a minor component of the shell (0.1 to 5 wt-%), it is thought to play a central role in the formation and shaping of these biomineral structures [3-4]. Indeed, these matrix components have been shown to be involved in the organization of a spatially prearranged scaffold, the nucleation, the orientation and growth of calcium carbonate crystals and the inhibition of their growth [5]. It has also been demonstrated that the nacre matrix controls the calcium carbonate polymorph [6] and that it exhibits cell-signalling properties [7-9].

(b) Nacre

Because of its extraordinary toughness, commercial value in jewelry and remarkable biocompatibility properties, nacre, so called the mother of pearl, is by far the most studied molluscan shell microstructure. Moreover, many authors consider it as the reference model for understanding how molluscs control the fine deposition of calcium carbonate layers [10]. From a structural viewpoint, nacre is constituted by a regular superimposition of polygonal flat aragonitic tablets of about 0.5 μm thickness, embedded in a peripheral thin organic matrix [11]. Nacre constitutes also a fascinating object for the investigation of evolutionary processes, as it appeared early in the fossil record, during the Cambrian times [12-13], and kept almost unchanged until now. At present it is distributed in four mollusc classes, comprising cephalopods, gastropods, monoplacophorans and bivalves. In this latter group, nacre is widespread in paleotaxodont, pteriomorphid, paleoheterodont and anomalodesmata clades [14]. One key question consists in determining if similar shell nacre textures, present in these different taxa, are produced by homologous sets of protein assemblages, or not. Such information would provide us a better appreciation on the recruitment of nacre matrix proteins

in the Cambrian, on the evolutionary constraints exerted on these proteins, and finally, on the open debate on the unique or multiple inventions of nacre among molluscs.

(c) Nacre proteins

Since the initial description of the primary structure of a mollusc shell protein in 1996 [15], numerous nacre associated-proteins have been described so far mainly from three principal models, the pearl oyster [2;16] and the edible mussel for bivalves [17-18], and the abalone for gastropods [19]. Comparison of their respective nacre biomineralization toolkits from the pearl mussel *Pinctada maxima* and the abalone *Haliotis asinina* underlined unexpectedly very few sequence similarities, suggesting either that the bivalves and gastropods acquired independently the capacity to synthesize nacre, or that these proteins present rapid evolution rate, especially in abalone, leading to functional conservation of respective nacre shell matrix protein (SMP) sets with only limited primary structure similarities [20]. In bivalves, most of the published data for nacre proteins comes from the Pteriomorphia sub-class [2;16-18]; only a limited amount of molecular data has been published so far on the other nacre-bearing bivalve groups [21-23]. To complete this patchy picture, we have developed a proteomic investigation on two nacreous bivalves that belong to the Palaeoheterodonta class, and to the Unionoida order, *Elliptio complanata* and *Villosa lienosa* [24]. Our molecular investigations are supported by the newly available large transcriptomic databases constructed in parallel from the two studied species [25-26], and are then providing strong support for the identification of SMP set of unionoid nacre. The present investigation gives us the opportunity to determine if bivalve nacres are made by molecular events that are controlled and conserved by the evolution processes, since the Phanerozoic times.

Our high-throughput high-output shotgun approach has allowed us to identify 48 different proteins from the nacre insoluble matrices, from which 31 are common to the two models. While recent data suggest a rapid evolution of shell matrix proteins leading to their extreme diversity [20], our work emphasizes on the contrary a deeply conserved biomineralization toolkit within nacre-bearing bivalves.

2. Results and Discussion

(a) Protein composition of unionoid nacre matrices

In order to investigate the nacre protein set in both *Elliptio complanata* and *Villosa lienosa*, two closely related freshwater bivalves (Unionoida; Unionidae), the non-fractionated insoluble matrices derived from respective shell nacre powder were analysed using a XL Orbitrap nanoLC-MS/MS, after digestion with trypsin enzyme (Figure S1). For both samples, the peak list generated from the MS/MS spectra was directly interrogated against the unionoids EST database, comprising 195,192 nucleotide sequences using MASCOT proteomic search engine (Version 2.1). Using this approach, we were able to enumerate 48 different proteins from the two nacre matrices (Figure 1; Supplementary table S1). More than 80% of these proteins (40 on the 48 identified in total) have been identified with high confidence, because they are present in both species or are identified by more than one peptide. Interestingly, 31 of the identified proteins have been detected in both *E. complanata* and *V. lienosa* testifying of the similarities of the nacre matrix compositions of these two species. We noticed that all conceptually-translated expressed sequence tag (EST) sequences that match to the MS/MS peptides present a signal peptide, when sequence exhibits complete N-terminus, suggesting that these proteins are all secreted by the mantle epithelia through classical cellular secretion pathway. This list of nacre proteins represents among the largest of nacre SMP sets described so far, comparable to published SMP set from *Pinctada* and *Mytilus* models, which count 35 and 42 proteins, respectively [16-18]. While the present results confirm the presence of previously described protein domains such as Pif, CA, Kunitz-like, WAP, M-rich, Q-rich, A-rich, or chitin-binding domains [21;23-24], in unionoid shell nacre matrix, they also contribute to consistently extend the list of proteins associated to nacre microstructure.

The proteomic analysis of these two unionoid nacre matrices reveals a diversity of SMP structures that can be broadly categorized into 5 main protein groups: ECM (ExtraCellular Matrix), RLCD-containing (Repetitive-Low Complexity Domain), enzyme, immunity-related, and 'uncharacterized'. Although our protein list may not be exhaustive - *i.e.* other minor protein matrix components remain to be characterized - we assume that it contains most of the abundant nacre SMPs: our approach indeed can identify most of the analogs/homologs of the main nacre SMP described in pteriomorphid models (Supplementary table S1). Many of these proteins, such as Pif, MSI60, CA, shematrin-like, Kunitz-like, LamG, chitin-binding containing proteins, together with A-, D-, G-, M- and Q-rich proteins, appear to be analogues, if not true homologs, of proteins previously described from the pearl oyster or the edible

mussel nacre matrices, and thus, constitute a remarkable set of deeply-conserved nacre proteins. Indeed, although only genetic information on the gene synteny would reliably support an hypothesis of homology relation between two proteins/genes of two organisms, we assume because of sequence of high sequence identities and also because of the specificity of some SMP domain architecture, that some of these proteins retrieved in these unionoid organisms would be true homologs of those observed in the pteriomorphid models.

(b) Extracellular matrix-related (ECM) proteins

Among the different unionoid nacre proteins that exhibits ECM protein signature (comprising collagen, fibronectin, laminin, chitin-binding and other protein- and polysaccharide-interacting domains), we identified different Pif-like isoforms, various chitin-binding containing proteins, a Ca-binding protein, a calcium-dependent (C-type) lectin, a mytilin-3 ortholog, together with previously unreported proteins for nacre such as a collagen-alpha-like, a MSP130-like, DMP-like, and a ANF_receptor (Figure 2; Supplementary table S1).

Pif-like and Pif-related proteins. The unionoid nacre matrix exhibits different forms of Pif-like proteins. Pif-177 (C7G0B5) was first described in the nacre of the pearl oyster [27], and homologous forms, BMSP-220 and BMSP-like, were subsequently detected in the shell matrices of the blue mussel *Mytilus galloprovincialis*, and of the giant limpet *Lottia gigantea*, respectively [28-29]. We have identified here at least 2 different Pif-like isoforms, called Pif-like 1 and 2, that exhibit both, from N- to C-terminus, the following succession of domains: one VWA, three CBD2, one RLCD and one LamG. This domain arrangement is characteristic of the Pif-177/BMSP-200/BMSP-like family [27-29]. Considering that the protein scores obtained for the Mascot identification of Pif-like 1 is among the highest obtained here, this supports the idea that the Pif/BSMP protein members are present in very high amount in the nacre matrix and that Pif/Pif-like proteins must certainly play a key function in mollusc shell formation [27-29].

Interestingly, three other proteins exhibit a domain organisation that partially resembles that of Pif-like proteins: UNP-2 (1 VWA, 2 long repeats and 1 LamG domains), UNP-3 (1 VWA, 16 CCP and 3 CBD domains), and UNP-5 (2 CBD2 and 1 LamG domains). Furthermore, various unionoid nacre proteins, called UNP-1, UNP-4, UNP-5, UNP-6 and UNP-7, bear characteristic chitin- or polysaccharide-binding domains (CBD2, Chitin_binding_3 or lectin C-type). The presence of these various VWA and chitin-binding domains suggest that these proteins might participate in structuring the chitin/protein nacre scaffold. In addition, we

identified other ECM-related proteins, such as DMP-like, MSP130-like, mytilin3-like, ANF-receptor containing or Ca-binding proteins, for which homologs have previously been described from other proteins associated with biomineralizations [30-32]. The involvement of these proteins in biomineralization processes is discussed below.

(c) Repeated low-complexity domain (RLCD) containing proteins

Our proteomic investigation also highlights the presence of a large set of 16 RLCD-containing proteins (supplementary table S1) for which similar proteins (that are genuine orthologous for some of them) were previously observed in *Pinctada* nacre, namely MSI60-like, shematrin, G- ,Q-, Y-, D- and M-rich proteins [2]. In addition, we identified new features such as a byssal protein-like, the homologs of which have been detected some years ago in the byssus of the blue mussel [33].

MIS60-like. This protein fragment presents high sequence similarities with MSI60, presenting characteristic repeats of G-rich and poly-A blocks (45% identity and E-value 3.1E-55), and initially described as a framework protein in the shell of *Pinctada fucata* [34]. MSI60 is a nacre-specific insoluble protein that exhibits poly-A and poly-G blocks giving MSI60 a certain similarity with hydrophobic spider and worm silk fibroins. More recently, another protein (has_CL10Contig2) with global similarities but no direct homology to MSI60 sequence has also been identified in the shell nacre of the abalone *Haliotis asinina* [19]. This suggests once more that such G- and A-rich proteins with hydrophobic silk-fibroin-like domains are key-players in nacre formation, in both bivalves and gastropods.

Shematrin-like. Only one protein corresponding to a shematrin-like protein has been identified in the two unionoids according to one peptide that gives a heterologous match with the EST sequence UTP_var00312 available from the brook floater *Alasmidonta vericosa,* another unionoid bivalve. This protein presents strong sequence similarity (72% identity and E-value 5E-155) with silkmapin a nacre protein from the unoinoid *Hyriopsis cumingii* [35], that present sequence similarity with shematrin proteins previously retrieved from *Pinctada* shell. The overall primary structure of shematrins is characterized by a signal peptide, G-G-Y-repeat motif, together with other more variable G- and Y-rich repeated low-complexity domains (RLCDs) and KKKY N-termini [36], that are also observed in this novel unionoid putative member. Shematrin-likes constitute one of the main SMP family, found in both nacre and prismatic layer of the shell of *Pinctada spp.*, and their corresponding transcripts are highly expressed in the mantle [2;20]. Surprisingly, their homologous sequences lack in the TSA

databases of *E. complanata* and *V. lienosa*. We assume that the matching peptide observed in the two unionoid models may correspond to a genuine shematrin-like protein, similar to the one derived from *A. vericosa* EST. The apparent absence of shematrin-like transcripts in the *E. complanata* and *V. lienosa* databases call for explanations at two levels: a) Restricted temporal expression [37], outside the 'window' of tissue sampling. Indeed, previous investigations have highlighted that key SMPs could be underrepresented or absent from a mantle EST database for such a reason [29]. b) Spatial expression of the shematrin transcripts restricted to the mantle; their absence in the transcriptomes of *E. complanata* and *V. lienosa* may be simply due to tissue sampling for EST construction, which was not particularly focused on the mantle [25;26].

Byssal protein-like, N-, T-, Q-, G- and M-rich proteins.

Our proteomic investigation has detected in the shell nacre of *V. lienosa* a sequence, which exhibits global similarities with a byssal protein-like from the mussel *Mytilus galloprovincialis* (33% identity). This protein exhibits repeat domains that are enriched in G, D, N and Y residues (Supplementary figure S2; supplementary table S1). Byssus is a silk-like acellular thread that is secreted by the foot of various bivalves, and that firmly anchors the organism to its substratum owing to strong adhesive distal plaques. A very recent proteomic investigation [38] has shown that several proteins constituting the byssal acellular structures (tyrosinase, A2M, EGF and VWA containing proteins) are common with those of the shell layers, claiming for similarities of the formation and the evolution of these two structures.

In addition, mollusc matrix proteins rich in Q have also been found in bivalves: for example MPN88 was previously characterized from the pearl oyster *Pinctada margaritifera* [39]. In the shell of the gastropod *Lottia*, at least 4 different matrix proteins present similar features [29]. Interestingly, vertebrate teeth contain various Q-rich proteins belonging to the secreted calcium-binding phosphoprotein family, which are believed to interact with calcium ions and regulate the tooth mineralization process [40]. In parallel, it has been proposed that the numerous Q residues of various silk-like fibroins, which also present numerous A- and G-rich domains, are implicated in the tensile mechanical properties of these proteins [41]. Few proteins with M- and G-rich composition were identified among the RLCD-containing shell proteins listed in this study. Proteins with similar enrichments in M and G residues have also been recently described in the matrix of *Pinctada* [42], of *Haliotis* [19] and of *Lottia* [29].

However, the functional characterization of these characteristic domains in mollusc SMPs remains to be done.

In the present case, in spite of overall sequence similarities observed within these several RLCD protein categories identified in different models, it remains difficult to clearly outline whether they are truly homolog considering limitation of standard Blastp tools for low-complexity sequences, the fast rate of evolution of these domains [41;43] and that fortuitous low-complexity sequence similarities can be obtained between non-homologous SMPs that have evolved completely independently.

(d) Other bivalve shell-conserved proteins

Carbonic anhydrase. Carbonic anhydrase (CA) is also a ubiquitous enzyme that is essential for calcium carbonate biomineral formation. It has been found in the organic matrices of various metazoan skeletons [44-46]. In molluscs, shell-specific form of CA, exhibiting G, N and D-rich LCDs, have been isolated first from the shell matrix of the pearl oysters *Pinctada* sp. [15]. In our study, the CAs detected in the two unionoid nacres do not possess similar supernumerary RLC domains, suggesting that matrix-associated CAs do not necessarily require such domains for being occluded in calcium carbonate microstructure or for exhibiting affinity with calcium carbonate mineral surfaces. At last, our results confirm the presence of CA in a non-pteriomorph bivalve shell as previously suggested [21;23]. Taken together, our findings reemphasize the central role played by extracellular CAs in $CaCO_3$ biomineralization processes.

Chitin-interacting enzymes. Among the SMPs, we identified two enzymes, namely chitinase 3 and chitobiase, which both exhibit glyco_hydro_18 and glyco_hydro_20 domains. These domains belong to the glycoside hydrolases family that hydrolyses the glycosidic bond between carbohydrates or between a carbohydrate and other components. Indeed, chitin is known to represent a major polysaccharide in mollusc shells. It is involved in constituting a 3-dimensional framework, in association with silk-like and acidic proteins in which nacre tablet crystals grow [10]. The identification of chitin-interacting enzymes, coupled with the detection of several chitin-binding domains in bivalve nacre SMPs strongly suggests an important role of chitin-cleaving enzymes in the fine molecular rearrangement of the chitin framework following the secretion of the nacre macromolecular ingredients at the vicinity of nascent growing mineral tablets.

Tyrosinase. The first mention of the involvement of a tyrosinase in bivalve shell formation

concerned the deposition of the periostracum and of the prismatic layer in the pearl oyster [47] In particular, it was shown by *in situ* hybridization that the zone of the highest expression of tyrosinase was the mantle edge from which these two layers are secreted. Recently, this enzyme has also been detected in lesser amount in nacre shell structure [16]. Tyrosinase is an oxidase that controls the production of protein melanogenesis *via* cross-linking by hydroxylation of the phenol groups present in tyrosine residues and their conversion to quinones. Biomineral-associated tyrosinases contribute to periostracum tanning [47]. They may be also involved in the maturation and hardening of the biomineral-hydrogel (that precedes crystallization) in prismatic as well as in nacreous shell layers [10].

Thioredoxin (TRX) and superoxide dismutase (SOD). Two enzymes involved in antioxidant processes, TRX and SOD, were also detected in the shell nacre of *V. lienosa*. Besides the identification of various isoforms of peroxidases in *Lottia* shell matrix [29], there are to date only a couple of examples of such redox enzymes in mollusc shell matrices [17;48]. In the present study, TRX and SOD were detected only via a limited number of peptides, suggesting that they may be minor component of the nacre matrix. Their putative function in shell biomineralization remains enigmatic. We cannot exclude the possibility that they are contaminants: SOD, for example, constitutes the main hemolymph protein, in charge of oxygen transport in bivalves [49]; its accidental occlusion in shell calcified tissues is not an unlikely scenario.

Immunity-related proteins. Finally, we detected, in both *Elliptio* and *Villosa*'s nacre matrices, four proteins with potential immunity-related functions. They exhibit indeed Kunitz-like (KU), or whey acidic protein (WAP) or macroglobulin protease inhibitor domains (Figure 1; supplementary table S1). The presence of such protease inhibitors in an acellular biomineral is, at first sight, puzzling. However our finding does not constitute a single observation, since the presence of such immunity-associated domains in shell proteins has been already noticed in different metazoan calcium carbonate biominerals: the shells of abalones [19;50], of pearl oysters [2;51-52], of the giant limpet [29] and, at last, the chicken egg shells [46]. One has proposed that these molecules are involved either in immunological defence mechanisms, or in the regulation of the matrix maturation, by controlling cleavage processes of other matrix proteins [53-54].

(e) Inherited set of nacre and shell matrix proteins in bivalves

One of the most striking results of our study was the detection, in *Elliptio and Villosa's* shell matrices, of various proteins that exhibit high sequence homologies with SMPs extracted from the nacreous shell layers of *Pinctada* and/or *Mytilus* bivalves. This constitutes a very consistent evidence of the existence of a set of ancient, *i.e.* Palaeozoic, nacre proteins shared between the various nacre-forming bivalve taxa (Figure 3).

More than two decades ago, when molluscan nacre matrices were solely analyzed for their amino acid compositions after hydrolysis of the bulk, the similarity of their compositions characteristically marked by high contents in glycine, alanine and aspartate residues, suggested identical molecular mechanisms for controlling the deposition of nacre tablets in the different nacre-bearing molluscs [55]. But, since the publication of the first full primary structure of a nacre protein, in 1996 [15], the increasing number of discovered SMPs has point out an extraordinary diversity of nacre-associated proteins among the different taxa [8;9]. This suggested on the contrary the use of different protein toolkits for nacre deposition. This view seemed to prevail, in particular with the finding that the bivalve *Pinctada* and the gastropod *Haliotis* possess absolutely dissimilar sets of shell-building genes [19;20]. This abrupt conclusion was however counterbalanced later by one of our studies that clearly demonstrated that the nacre of the edible mussel *Mytilus* and the non-nacreous shell microstructure of the limpet *Lottia*, contains number of proteins that are similar - if not homologous - to that of *Pinctada's* nacre [2;29;30]. The present set of data supports and reinforces the "deep origin scenario" by revealing a shared protein toolkit in the nacre of bivalve species that belong to three clades, Mytiloida, Pterioida (both belonging to Pteriomorphia subclass) and Unionoida (the main order of the Palaeoheterodonta subclass) that diverged in the Palaeozoic times [56].

The list of shared nacre proteins between the three main bivalve models comprises: MSI60, Pif, CA, macroglobulin, VWA-, LamG-, CBD2- and Kunitz-like-containing proteins, along with various RLCD protein exhibiting A-, D-, G-, Q- and M-rich domains. This short list potentially represents the inherited ancestral toolkit - or at least a part of it - required for nacre formation. Other proteins appear to be shared by only two species out of three, and are probably related with taxon specificity adaptations. One has to notice that some of the proteins listed on figure 3 have been also identified in the prismatic layers of the three bivalves, suggesting that their molecular functions might not be exclusively restricted to nacre deposition.

By identifying nacre proteins from two non-pteriomorph species and reporting a deeply conserved nacre protein set in bivalves this work adds new evidence that that the calcifying

matrix of molluscs is comprised of both deep conservations and lineage-specific protein novelties. The comparison of these bivalve-conserved protein sets to those previously detected in gastropod nacre is confirming the global dissimilarity of the nacre SMP sets of bivalves and of gastropods (at least those of the abalone) [19]. Bivalve and gastropod exhibit different kinds of nacres (respectively, sheet and columnar nacres), which homology still constitute an actual debate that still oppose parcimony principles [14], crystallographic and microstructural textures [11;57], along with paleontological and molecular evidences [12;13;20].

Figure 4 summarizes all available information on the occurrence of protein domains observed from shell proteome of various mollusc models, comprising nacre and prismatic shell layers of bivalves (Unionoids, *Pinctada* and *Mytilus*), cross-lamellar layers of gastropod (*Lottia* and *Cepaea*), and finally, fibrous layers of brachiopods, taken as an outgroup. This figure shows that several proteinaceous domains, which appear to be shared between the different nacre models, are also detected in bivalve prismatic layers or in the shell of cross-lamellar gastropods. This clearly suggests that these domains are not specifically involved in nacre formation, but that they display general function in shell formation.

The brachiopod shell proteomes, retrieved from the $CaCO_3$ shell of *Magellania venosa* [58] and the CaPO3 one of *Lingula anatina* [59], respectively, exhibit many similarities with those of molluscs in their respective protein and functional domain compositions. The list of shared domains comprises potentially key contributors of shell formation processes, such as D-, A- and G-rich, VWA, EGF, CCP, peroxidase, tyrosinase, chitinase, CA, KU, A2M and CBD2. Furthermore, Luo and co-workers [59] notice also that genes shared between *Lingula* and molluscs, such as calcium-dependent protein kinase and chitin synthase, exhibit high expression in larvae and mantle, indicating that they may also be involved in brachiopod shell formation. Taken together, these results indicate that a conserved molecular machinery exists for shell biomineralization in brachiopods and molluscs. This assumption needs however a further validation via functional analyses.

(h) Are metazoan SMPs deeply conserved?

The appealing scenario of an ancient and common origin of the biomineralization within the metazoan has been proposed at various occasions [3;60]. This scenario is based on the supposed existence of a shared ancestral metazoan toolkit that was implemented before the

main metazoan radiations. But to date, the data that support this view still remain extremely tenuous. For example, no one ever evidenced a universal molecular marker of the skeletal matrices of all calcifying metazoans. Many skeletal matrix proteins belong to families that are not necessarily specific of biomineralization processes, suggesting that many proteins could have been coopted secondarily for calcification purpose. For example, although CA has been observed in various biomineralization models, comprising egg-shell, echinoderm skeleton, mollusc shell or sponge spicules, it constitutes a broad and ubiquitous protein family presenting various members in all organisms, calcifying or not; their phylogenetic analysis shows a very complex evolutionary history, indicating that they could have been recruited several times, in independent manners, in biomineralization structure within the different metazoan clades [61].

At the opposite of the CA example, MSP-130 represents a remarkable illustration of a conserved molecular function that may be strictly restricted to biocalcification, but present in distinct metazoan lineages. MSP-130 is a protein, initially identified in the sea urchin *Strongylocentrotus purpuratus* [62], which expression is specific to primary mesenchyme cells [63] - the cells that initiate the skeletal tissues in echinoderms - and that represents one of the main constituent of the calcifying matrix of the different skeletal structures, including the teeth, the spicules, the test or the spines [64]. So far, no other function than those related to biomineralization has been described for this protein. Interestingly, during our previous investigation of the shell proteins of the Pacific oyster *Crassostrea gigas*, we detected a protein, called gigasin-3, which partial sequence exhibits noticeable similarity with *S. purpuratus* MSP-130 [31]. Even more recently, the msp130 gene was also identified in a calcified tube-forming polychaete [65]. With the present study, the recognition in nacre matrix of two unionoids of a similar MSP-130-like protein firmly supports the idea that this biomineralization-specific protein or protein family is shared between different metazoan calcifying lineages, reintroducing the scenario of common ancestral mechanism for metazoan biomineralization processes.

In addition, we have detected that the two unionoid proteomes contain both a dentin-matrix protein-like (DMP-like), exhibiting a remarkable FAM20C domain (Supplementary figure S3). Indeed, FAM20C is a kinase that phosphorylates the majority of the phosphoproteome in the extracellular matrices of bones and teeth, constituting a key player of vertebrate biomineralization process [66]. Other recent investigations have revealed the presence of FAM20C containing protein in the shell matrix of the giant limpet *Lottia gigantea* [48], and

of the brachiopod *Magellania venosa* [58]. The presence of such key-protein, regulating the biomineralization formation of vertebrates, in the shell matrices of molluscs and brachiopods strongly reinforces the idea of common molecular machinery, supporting the scenario of a conserved biomineralization toolkit between different metazoan lineages.

3. Conclusion

Our study presents proteomic results obtained from the nacre matrices of two unionoid (Paleoheterodonta) bivalves. Our approach is robust and powerful since it allows annotating genes encoding proteins that are undoubtedly shell matrix constituents, therefore, putatively important players in the nacre assembling process. It excludes genes that encode mantle-secreted proteins – recognizable by their signal peptide signature – but which are not occluded within the calcified tissue, *i.e.* proteins, which are not constituents of the shell organic matrix. We are fully conscious that some of these proteins, not detected by our approach, may be important players for calcification as well [67].

Our study has macro-evolutionary implications. Nacre is supposed to represent an ancestral shell microstructure, shared by different orders within the bivalve class. Because it remained almost unchanged until now, from a microstructural viewpoint, this suggests that at least a part of the molecular toolkit used for constructing nacre must have been kept conserved within these nacre-forming clades. Our proteomic approach focused on the unionoid bivalves has allowed detecting several genuine sequence homologies with known sequences of nacre proteins of pteriomorph bivalves, but also with other SMPs from gastropod and brachiopod models, unveiling a part of a deeply conserved shared legacy.

4. Material and methods

(a) Nacre matrix extraction

Fresh *Villosa lienosa* (6-8 cm in length) and *Elliptio complanata* (8-10 cm in length) shells were collected from Chewacla Creek near Auburn, Alabama (USA), in April 2012, and from Cree Lake near Cochrane, Ontario (Canada), in spring 2011, respectively. Among the paleoheterodont bivalves, both are representatives of unionoid order, the shell microstructures of which are characterized by the association of aragonitic prisms and nacre. Superficial organic contaminants as well as the periostracum were removed by incubating intact shells in

NaOCl (5%, v/v) for 24-48 h [68]. Shells were then thoroughly rinsed with water. The external prismatic layer was mechanically removed and the shell nacre broken into 1-mm large fragments before being ground to fine powder (> 200 µm) that was subsequently decalcified in cold acetic acid overnight (10%; 4 °C). The insoluble nacre matrix was then collected by centrifugation (15,000 g; 10 min; 4 °C), and the pellet rinsed six times with MilliQ water by series of resuspension-centrifugation before being freeze-dried and weighed.

(b) Peptide digest proteomic analyses

In-solution digestions of insoluble nacre matrix samples were performed. One mg of the matrices was reduced with 100 µL of 10 mM dithiothreitol (Sigma-Aldrich, France) in 500 mM triethylammonium bicarbonate pH 8.0 for 30 min at 57° C. Fifteen µL of iodoacetamine (50 mM, final concentration) was added and alkylation was performed for 30 min at room temperature in the dark, then digestion was performed by adding 10 µg of trypsin (T6567, proteomics grade, Sigma). The samples were incubated overnight at 37 °C, then centrifuged (30 min, 14,000 g) and 5 µL of the acidified supernatants was injected into the tandem mass spectrometer with an electrospray source coupled to a liquid chromatography system analysis (nanoLC-ESI-MS/MS). The protein digest concentration was prior estimated using a micro-BCA kit (Sigma-Aldrich, USA), with BSA as protein standard.

Protein digests were analyzed using a nano-LC (Ultimate 3000, Dionex) coupled to an ESI-LTQ-Orbitrap (LTQ Orbitrap XL, Thermo Scientific) mass spectrometer [69]. Above 10 µg of AIM digest protein digests solubilized in 10% ACN with 0.1% formic acid were injected in triplicates by the autosampler and concentrated on a trapping column (Pepmap, C_{18}, 300 µm x 5 mm, 5 µm 100 Å, Dionex) with water containing 10% ACN with 0.1% formic acid (solvent A). After 5 min, the peptides eluted onto a separation column (Pepmap, C_{18}, 75 µm x 500 mm, 2 µm 100 Å, Dionex) equilibrated with solvent A. Peptides were separated with a 2h-linear gradient of 80% ACN + 0.1% formic acid (solvent B) at flow rate of 200 $nL.min^{-1}$. The LTQ-Orbitrap mass spectrometer is outfitted with a nano-ESI electrospray source. Spectra of multiple charged precursor ions (+2 to +4) were collected from *m/z* 300-2000 at a resolution of 30,000 in the profile mode followed by data dependent CID and/or HCD spectra of the ten most intense ions. A dynamic exclusion window of 60 s was used to discriminate against previously analyzed ions.

(c) Bioinformatics

The MS/MS data were used for database searches using an in house version of the MASCOT search engine (Matrix Science, London, UK; version 2.1) against 138,345 and 46,051 TSA nucleotide sequences derived from *E. complanata* and *V. lienosa* EST libraries [25;26], respectively (plus few hundreds *Alasmidonta* spp. sequences), downloaded in December 2015 from the NCBI server (http://www.ncbi.nlm.nih.gov). LC-MS/MS data generated by each shell sample and protein band were searched separately, using carbamido-methylation as a fixed modification and methionine oxidation as variable modification. The peptide MS tolerance was set to 10 ppm and the MS/MS tolerance was set to 20 mDa. Proteins with at least two different peptides or identified in both species were considered to be fully valid.
Protein identification was performed using BLAST searches against the UniProtKB/Swiss-Prot protein sequence database (www.uniprot.org), retrieving only hit with e-value < 7E-8, and without filtering low complexity region. Signal peptides were predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP) and conserved domains from database models were predicted using external source database SMART (http://smart.embl-heidelberg.de). 3-D protein structures were predicted according to sequence homologies using the Phyre 2.0 online tool (http://www.sbg.bio.ic.ac.uk/phyre2) [70].

Data accessibility. Supplementary table S1 containing the complete list and description of all proteins identified in *E. complanata* and *V. Lienosa* nacre matrices by shotgun proteomics has been uploaded as electronic supplementary materials.

Abbreviations: A2M, alpha-2 macroglobulin; APase, alkaline phosphatase; ANF, extracellular ligand binding; BMSP, blue mussel shell protein; C1q, complement complex protein 1; CA, carbonic anhydrase; CBD, peritrophin-A chitin-binding domain; CCP/SUSHI, complement control protein; Chit_bind, chitin-binding domains; CLECT, C-type lectin; Copamox, copper amine oxidase; DMP, dentin matrix protein; EGF, epidermal growth factor; FAM20C, family with sequence similarity 20 - member C; FN3, fibronectin 3; IGF-BP, insulin growth factor-binding protein; KU, Kunitz-like domain; LamG, laminin G; MSP, mesenchym-specific protein; MSI60, matrix silk-like insoluble protein 60; PIF/Pif177, *Pinctada fucata* 177-kDa protein, RLCD, repetitive low-complexity domain; SMP, shell matrix protein; SCP, Cysteine-rich secretory protein; SOD, superoxide dismutase; TIMP, tissue inhibitor of metalloprotease; TRX, thioredoxin; UNP, uncharacterized nacre protein; VWA, von Willebrand factor type A; WAP, whey acidic protein; ZP, zona pellucida.

Author's contribution. B.M., G.B., S.B. & F.M. conception, design and coordination; J.A., A.M., L.M. preparation of the sample, analysis and interpretation of the data. All author contribute to the preparation and the revision of the manuscript.

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Figure legends

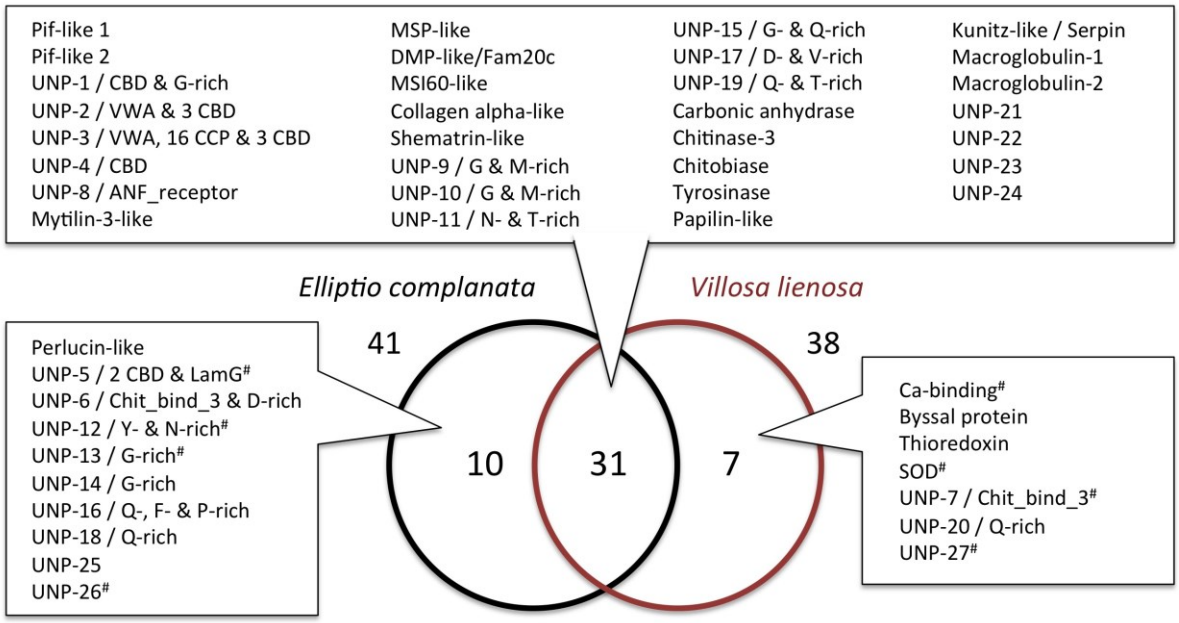
Figure 1. Identification of the nacre matrix proteins from *Elliptio complanata* and *Villosa lienosa* shells using MS/MS analyses. Venn diagram of 41 and 38 proteins identified in both nacre matrices. The hashtag symbol (#) beside some protein names represents cases where the identification is not 100% certain, since it is based only on one peptide and only in one of the two species.

Figure 2. Schematic representation of the primary structure of unionoid extracellular matrix (ECM) nacre proteins retrieved from *Elliptio* complanata and/or *Villosa lienosa*. Each complete protein sequence possesses a signal peptide indicated in red. Typical ECM domains are indicated as boxes on the protein structure. The list of ECM protein domains comprises VWA, CBD2, LamG, Chitin_binding_3, EFh, CLECT, ANF_receptor and FAM20C/DUF1193, together with few RLCDs or repeated domains. The "?" symbol indicates missing sequence information.

Figure 3. Nacre SMPs from bivalve models. In total, 48, 35 and 42 various matrix proteins have already been detected in the nacre of the three bivalve models that belong to the Unionidae, Pteriidae and Mytilidae families, respectively. Names correspond to proteins and/or proteinaceous domains identified in the different nacre SMPs. ^{p} and ^{m} exponents indicate that the protein was also detected in the prismatic shell layer of either *Pinctada spp.* or *Mytilus spp.*, respectively [2;15;17;18].

Figure 4. Comparison of SMP domain distribution in the shells of various lophotrochozoan models, focused on molluscs and brachiopods. The domains are sorted according to main categories detected in SMPs. * indicates that only genuine abundant SMPs presenting high confidence for their specific identification in the shell matrix, and not being potential considered as contaminant have been mentioned here.

738 Fig. 1



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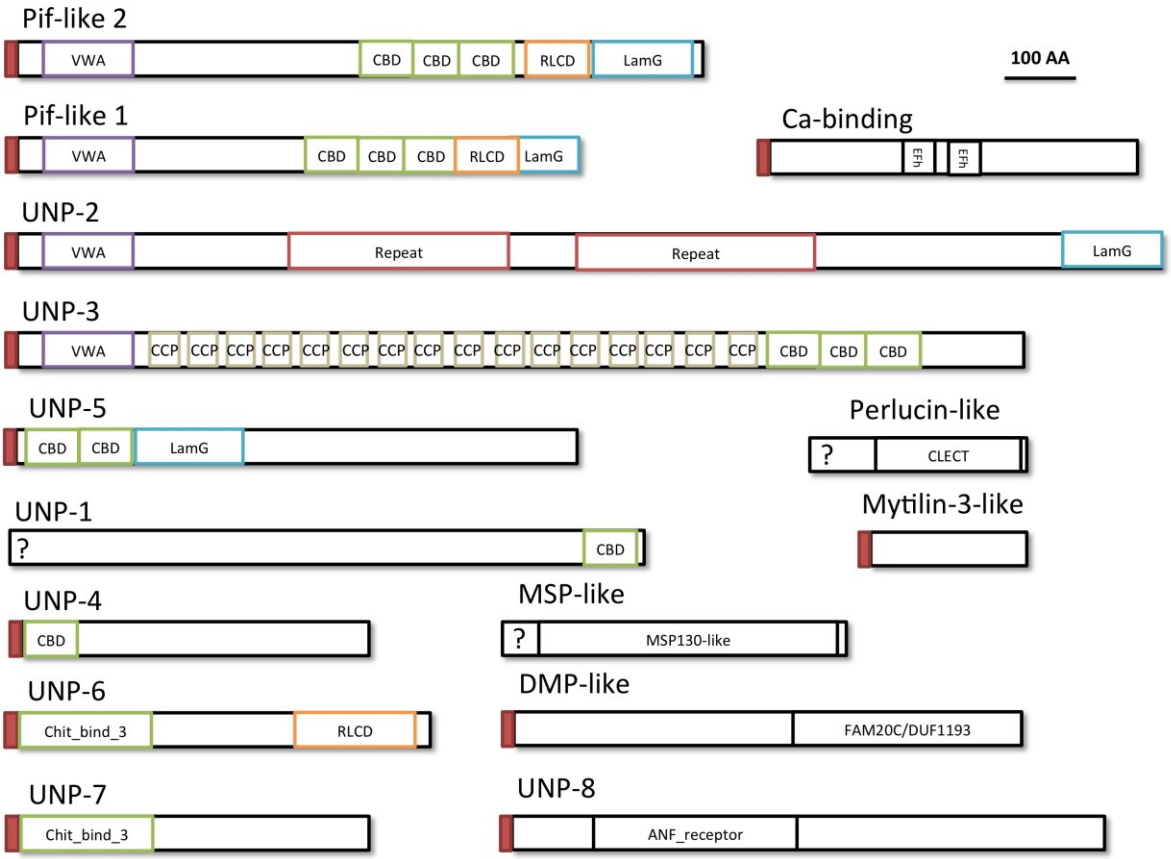
741

742 Fig. 2



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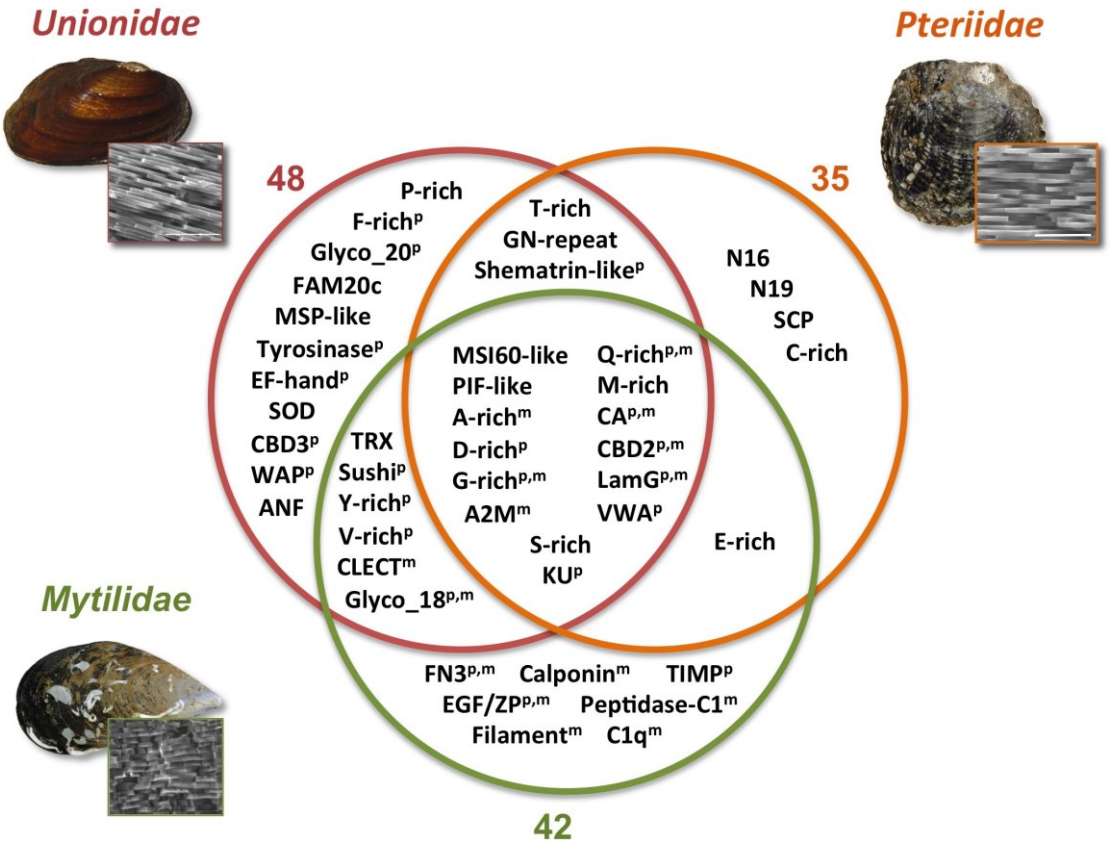
745

746 Fig. 3



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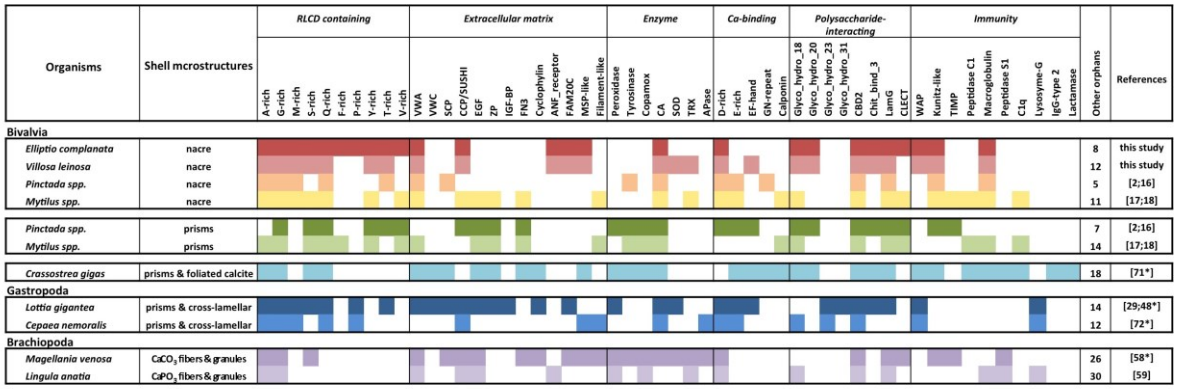
750 Fig. 4


Organisms
Shell mcrostructures
RLCD containing
A-rich
G-rich
M-rich
S-rich
Q-rich
F-rich
P-rich
Y-rich
T-rich
V-rich
Extracellular matrix
VWA
VWC
SCP
CCP/SUSHI
EGF
ZP
IGF-BP
FN3
Cyclophylin
ANF_receptor
FAM20C
MSP-like
Filament-like
Enzyme
Peroxidase
Tyrosinase
Copamox
CA
SOD
TRX
APase
Ca-binding
D-rich
E-rich
EF-hand
GN-repeat
Calponin
Polysaccharide-interacting
Glyco_hydro_18
Glyco_hydro_20
Glyco_hydro_23
Glyco_hydro_31
CBD2
Chit_bind_3
LamG
CLECT
Immunity
WAP
Kunitz-like
TIMP
Peptidase C1
Macroglobulin
Peptidase S1
C1q
Lysosyme-G
IgG-type 2
Lactamase
Other orphans
References
Bivalvia
Elliptio complanata
nacre
8
this study
Villosa leinosa
nacre
12
this study
Pinctada spp.
nacre
5
[2;16]
Mytilus spp.
nacre
11
[17;18]
Pinctada spp.
prisms
7
[2;16]
Mytilus spp.
prisms
14
[17;18]
Crassostrea gigas
prisms & foliated calcite
18
[71*]
Gastropoda
Lottia gigantea
prisms & cross-lamellar
14
[29;48*]
Cepaea nemoralis
prisms & cross-lamellar
12
[72*]
Brachiopoda
Magellania venosa
CaCO3 fibers & granules
26
[58*]
Lingula anatia
CaPO4 fibers & granules
30
[59]

751