

Differential expression of duplicated opsin genes in two eye-types of ostracod crustaceans

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Abstract: In the first molecular study of ostracod (Crustacea) vision, we present partial cDNA sequences of ostracod visual pigment genes (opsins). We found strong support for differential expression of opsins in ostracod median and compound eyes and suggest that photoreceptor specific expression may be a general phenomenon in organisms with multiple receptors. We infer that eye-specific expression predates the divergence of the two species examined, *Skogsbergia leneri* and *Vargula hilgendorffii*, because eye-specific opsin orthologs are present in both species. We found multiple opsin loci in ostracods, estimating that at least eight are present in *Skogsbergia leneri*. All opsins from both ostracod species examined are more closely related to each other than to any other known opsin sequences. Because we find no evidence for gene conversion or alternative splicing, we suggest the occurrence of many recent gene duplications. Why ostracods may have retained multiple recent opsin gene duplicates is unknown, but we discuss several possible hypotheses.

Introduction:

The Ostracoda are an ancient group of bivalved crustaceans that are well suited for studies of the evolution of vision for several reasons. First, many species require good vision. Some are predators, while others use flashing bioluminescent signals or reflected light for courtship (Cohen and Morin 1990; Morin and Cohen 1991; Parker 1995). Second, ostracods inhabit almost any conceivable aquatic niche; so related species have evolved in drastically different light environments from full spectrum shallow waters to lightless deep seas and caves. Third, one group of ostracods, Myodocopida, may have evolved compound eyes independently of other arthropods (Fryer 1996; Oakley In Press; Oakley and Cunningham 2002; Parker 1995). More specifically, phylogenetic evidence suggests that the ancestral ostracod lacked compound eyes (Oakley and Cunningham 2002). Finally, ostracods are amenable to the study of eye development and genetics because many species can be easily collected in large numbers, have short generation times and are easily maintained in the laboratory (Cohen 1983; Ikeya and Kato 2000).

Perhaps the biggest advantage of ostracods for studying visual evolution is the diversity of visual systems among species. Although some ostracods lack eyes altogether, others have as many as two distinct visual systems. Most ostracods have a median eye, which is a non-image-forming eye located on the anterodorsal surface of the body. Ostracod median eyes are considered to be of the “Ostracod-Maxillopod” morphological type, having a diagnostic three-cup structure, tapetal cells between pigment and sensory cells, and lens cells outside the pigment cups (Elofsson 1992). In two myodocopids examined previously, the two distal photoreceptive cups each contained about 35-40 reticular cells and the ventral cup contained about 25 such cells (Andersson 1979).

Some ostracods have a second visual system in the form of paired lateral eyes that are simple eyes in some species but compound eyes in most. Lateral eyes are present only in the Myodocopida, which account for about 10% of the roughly 8,000 described extant ostracod species. The individual facets (ommatidia) of ostracod compound eyes have a unique arrangement among arthropods. While many arthropod ommatidia have eight reticular cells and four crystalline cone cells (Melzer et al. 1997), ostracod ommatidia have six and two respectively (Andersson 1979). Typical shallow-water myodocopids have about 10-30 ommatidia per eye (Kornicker 1992), a small number compared to many insects (e.g. *Drosophila* has ~750). We are unaware of any study that examined the possibility of regional specialization of ommatidia, although size variation of ommatidia has been reported in individual myodocopid eyes, for example in *Philomedes lilljeborgii* (Kornicker 1989: pg 74)

In addition to median and lateral eyes, ostracods possess a Bellonci organ, which extends anteriorly from the ventral cup of the median eye (Andersson 1979). Some authors have suggested a visual function for the Bellonci organ, while others maintain that it has chemosensory function, or both visual and chemosensory function (Andersson 1979). To our knowledge, no other extra-ocular photoreceptors have been described in ostracods.

Although ostracods have tremendous potential to inform us about eye evolution, few evolutionary studies and no molecular studies on ostracod eyes are published. A major goal of this study is to characterize genes involved in ostracod vision. An obvious starting point is the well-studied family of opsin genes present in all organisms with photoreceptors. The evolutionarily conservative nature of opsins has allowed their characterization from numerous species. However, in contrast to the many vertebrates and

insects studied, the opsins of only two crustacean groups have been examined, including several crayfish and a single crab (Crandall and Cronin 1997; Hariyama et al. 1993; Sakamoto et al. 1996). Therefore, our ostracod data also provide an important contribution to the opsin database.

The main hypothesis we set out to test was that median and compound eyes of ostracods express different opsins. Although many arthropods besides ostracods have multiple photoreceptor types, only two have been examined previously for differential expression. *Drosophila* species express different opsins in compound eyes and their simple photoreceptors called ocelli (Pollock and Benzer 1988). Similarly, the horseshoe crab *Limulus polyphemus* expresses different opsins in compound eyes and ocelli (Smith et al. 1993). Here, we report strong evidence that the ostracod *Skogsbergia leneri* also exhibits differential expression of opsins in median and compound eyes. We also report the unexpected result of multiple closely related opsin loci, suggesting several recent gene duplication events. The reason why myodocopids apparently have maintained many recent opsin gene duplicates remains a mystery, however we discuss some potential explanations.

Methods

The Ostracoda are often divided into three major groups: Podocopa, Palaeocopa, and Myodocopa (Cohen et al. 1998). We present opsin genes from two ostracod species of the family Cypridinidae (Myodocopa: Myodocopida). We first report opsin fragments from *Skogsbergia leneri*. In this species, we performed a test of eye-specific gene expression, estimated the number of opsin loci indicated by our data, and performed two tests for gene conversion or alternative splicing. Next, we added opsin sequence data from the ostracod *Vargula hilgendorfii* and performed a phylogenetic analysis including data from other known opsins.

Collection

We collected both ostracod species using baited traps as previously described (Cohen 1983; Vannier and Abe 1992). We collected *Skogsbergia leneri* specimens at the Glover's Reef Marine Research Station pier in Belize (16° 45' N 87° 46' W), and *Vargula hilgendorfii* at Hojo pier in Tateyama, Chiba prefecture, Japan (35° 00' N, 139° 51' E) (Vannier and Abe 1995). *Skogsbergia leneri* collections were preserved directly in RNALater (Ambion, Austin, TX) and stored at -20°C. *Vargula hilgendorfii* specimens were frozen directly at -80°C.

Opsin characterization

We prepared total RNA from ostracods using a standard guanidinium-based protocol similar to that described elsewhere (Chomczynski and Sacchi 1987). First-strand cDNA synthesis was performed using SuperScript II Reverse Transcriptase according to manufacturer's instructions (Gibco) and using a poly-T primer with an additional arbitrary sequence (Table 1) for 3' Rapid Amplification of cDNA Ends (RACE) (Frohman et al. 1988; Sakamoto et al. 1996).

We initially amplified opsin fragments using PCR with degenerate primers designed from conserved regions of previously known crustacean opsins (primers 1080F and 1350R; Table 1). This reaction was cycled 45 times at 94°C for 30s, 30°C for 1 min, 72°C for 30s. We cloned these amplified fragments using the topo TA kit (Invitrogen) and sequenced multiple clones with vector primers, ABI Big Dyes sequencing chemistry

(Perkin Elmer) and an ABI 373, 377, or 3700 sequencer. Next, we designed ostracod specific primers for 3' RACE using the SLF primer for *S. leneri* and GapF, DelgapF or APF primers for *V. hilgendorfi* (Table 1) using 45 PCR cycles of 94°C for 30s, 50°C for 30s, and 72°C for 30s. Amplified RACE products were cloned and sequenced as above, yielding partial opsin sequences including the 3' untranslated region (UTR) of the genes.

We named clones as follows: The first two letters represent the species name, Sl for *Skogsbergia leneri* and Vh for *Vargula hilgendorfi*. The next part of the name is either the individual plus sex (e.g. M1 represents Male 1), or is the letter 'P' for multiple, 'pooled' individuals (in a few cases because of preservation methods, we were forced to pool multiple whole ostracods together to extract sufficient cDNA). The next part of the clone name has either CE for compound eye or ME for median eye if the clone is amplified from eye-specific cDNA. The final number(s) or letter(s) represent the specific clones. Because errors introduced by taq polymerase cannot easily be distinguished from true mutations, and because we are reporting an unexpectedly large number of opsin loci, we took a very conservative approach with respect to variation, which would lead to lower estimates of the number of loci present. Namely, we pooled together into a single sequence clones from the same individual that differed by less than 2%, a high estimate of potential taq polymerase error given our amplification conditions (Bracho et al. 1998). If more than five clones were pooled together, the last number is the number of clones followed by either 'sim' indicating that all clones are similar or 'id' indicating that all clones are identical. For example SIM1_ME_9sim is the consensus of nine similar clones (i.e. less than 2% sequence differences) from the median eye of Male 1 of *S. leneri*.

Test for eye-specific expression of opsins and allele number in S. leneri

We tested the hypothesis that one clade of opsins is expressed in *S. leneri* compound eye tissue while another clade of opsins is expressed in *S. leneri* median eye tissue. We sequenced cDNA prepared separately from each eye type of *S. leneri*. To prepare cDNA from single eyes, we dissected individual adult ostracods under a dissecting scope using insect pins (Carolina Biological) inserted into pencil erasers, and prepared total RNA and cDNA as described above. Dissections were repeated for both eye types from five *S. leneri* (3 males and 2 females). We sequenced 8-13 clones from each eye type from each of the five ostracods. Each amplification was accompanied by a negative control reaction and we liberally discarded reactions and reagents at any sign of contamination.

We also used the same amplification protocol on cDNA from pooled tissue as a sequencing control. If opsins from two different clades can be sequenced from pooled cDNA, then opsins from two different clades could also be sequenced from eye-specific cDNA, unless there is eye-specific expression. If differences in expression exist, then sequencing compound eye-specific cDNA should amplify opsins from one clade and median eye specific cDNA should amplify a separate clade of opsins.

For the expression test, we aligned nucleotides of all *S. leneri* clones by aligning inferred amino acids with default parameters of Clustal (Higgins et al. 1992) and back translating to nucleotides. We determined the best-fit likelihood model using likelihood ratio tests (Goldman 1993) implemented with ModelTest 3.04 (Posada and Crandall 1998). We estimated model parameters with likelihood and set these parameters for a heuristic search for the most likely tree in PAUP* 4.0b6 (Swofford 1999).

In a separate analysis we performed 10000 bootstrap pseudoreplicates using neighbor-joining on distances estimated from our best-fit likelihood model in PAUP* 4.0b6 (Swofford 1999). We chose this distance-based approach for the bootstrapping analysis because an equivalent ML analysis was too computer time intensive. Parsimony bootstrapping gave similar results that are not presented.

To determine the minimum number of opsin loci that exist in *S. leneri*, we assume that the species is diploid, so a maximum of two alleles per locus can exist in an individual. Note that we only determined locus number in one of the two species examined. We could neither count alleles nor show eye-specific expression in *V. hilgendorffii* because the majority of sequence data is from amplifications of multiple pooled individuals. Our preservation technique of *V. hilgendorffii* (many individuals frozen together at -80°C) made amplifications from single individuals of that species difficult (although some compound-eye specific sequences were successfully amplified).

Synonymous and Non-Synonymous Substitutions

We examined the ratio of synonymous to non-synonymous substitutions in the coding region of myodocopid opsins for all pair-wise comparisons. We used the program Syn-SCAN (Gonzales et al. 2002), which implements the method of (Nei and Gojobori 1986) while incorporating information on ambiguous nucleotides. Accounting for ambiguity was important because we pooled similar sequences, considering them as single alleles.

Tests for Gene Conversion or Alternative Splicing

Gene conversion is a consideration when studying a multi-gene family such as opsin. Although not reported in invertebrate opsins (Briscoe 2000), gene conversion was detected in primate opsin genes (Zhao et al. 1998; Zhou and Li 1996) and can obscure phylogenetic analyses. We therefore tested for the possibility that the process has occurred in myodocopid opsin genes. In addition, we also consider the possibility of alternative splicing because our data are cDNA sequences. Alternative splicing occurs during transcription of genomic DNA into mRNA and results in alternative sequences of a certain gene region being spliced together with the rest of the gene. Transcripts of the same gene resulting from different alternative splicing events could be incorrectly interpreted as transcripts of different genes. Since we are reporting an unexpectedly large number of opsin loci, we consider the possibility that some of this variation is introduced by alternative splicing.

If present, both gene conversion and alternative splicing would result in different regions of a DNA sequence having significantly different inferred phylogenetic history, i.e. the sequence would be a mosaic with different regions having different phylogenetic histories. Gene conversion involves recombination resulting in a gene region with different phylogenetic history. Alternative splicing involves splicing different gene regions in different transcripts of the same gene. Phylogenetic analysis of alternatively spliced or recombined regions of a gene would give significantly different results compared to the rest of the gene. To test for incongruent phylogenetic histories within ostracod opsins, we performed two separate analyses.

First, we used the program PLATO to test for phylogenetically anomalous gene regions (Grassly and Holmes 1997). The program assumes a model of evolution and a phylogenetic tree for the sequence data at hand. It then compares the maximum likelihood of different sized, contiguous portions of the sequence alignment to the likelihood of the entire sequence alignment. Regions of the sequence that have a significantly low

likelihood value are determined by Monte Carlo simulation. A low local likelihood can be attributed to either a slower rate of evolution or to different phylogenetic histories for the anomalous regions. A local difference in phylogenetic history is evidence for a recombination event such as gene conversion. In contrast, the absence of regions with significantly low likelihood is consistent with the null hypothesis of no recombination and similar rates of evolution in different regions. An analysis of our entire opsin data set with ML is computationally impractical, so we used a subset, namely the *S. lernerii* opsins. We used estimates of the best-fit model, parameter values, and ML tree (as described above under eye-specific expression) in PLATO using 1000 Monte Carlo replications to search for phylogenetically anomalous regions in *S. lernerii* opsin.

A second way we tested for anomalous regions of opsin sequences was to examine phylogenetic congruence between the 3' UTR and the coding portion of the gene. Gene conversion or alternative splicing could cause incongruence between UTR-type and gene phylogeny. Because these sequences were too divergent to align across all opsin sequences, we could not include the UTRs in a conventional phylogenetic analysis. Instead, UTR sequences were divided into six distinct types. The UTR types were compared to the opsin gene phylogeny based on only coding sequences.

Phylogenetic analysis of ostracod and arthropod opsins

To determine the relationship of myodocopid opsins to other known opsins, we used portions of published amino acid sequences assumed to be homologous to our myodocopid opsin fragments. We selected two divergent opsins from each major clade of the analysis performed by Briscoe (2000). We removed 16 *S. lernerii* sequences that were very similar to other sequences, in order to reduce the total number of sequences analyzed making full ML analysis possible. These sequences probably represent alleles of the same locus, and in many cases were identical sequences from different individuals. We used ClustalW with default parameters for alignment of inferred amino acids, and back translated to nucleotides. We again set parameters of the best-fit model to their ML estimates for a heuristic search in PAUP*. We also used PAUP* for bootstrapping with neighbor-joining and ML distances as above.

Results

We examined nucleotide sequences from a total of 156 clones, 44 of which were unique (i.e. contain greater than 2% difference from its closest neighbor). 36 of the unique sequences were from *S. lernerii*, and 8 from *V. hilgendorffii*. Of the *S. lernerii* sequences, 10 were from cDNA derived from multiple individuals pooled together (used as a PCR control in our expression experiment) and 26 were amplified from cDNA derived from individual eye types of five separate individuals. One *S. lernerii* clone, SIF2CE_5_8_9, was excluded from further analysis because it was apparently a PCR-induced chimera (in phylogenetic analyses, the clone was sister to all other myodocopid opsins, and phylogenetic analyses of different regions of the sequence placed the clone in different clades). Although most *S. lernerii* clones were derived from cDNA of a single eye type from a single individual, most *V. hilgendorffii* sequences were derived from the cDNA of many pooled individuals. All clones are putative partial opsin sequences that include the nucleotides coding for a portion of the third cytoplasmic loop, the entire the sixth and seventh transmembrane domains, the entire third extracellular loop, the entire c-terminal region, and most clones contain a 3' untranslated region. All sequences were deposited in GenBank with accession numbers AF353331- AF353374.

Our cloned sequences are similar in many respects to other known opsins. First, putative opsin fragments from both ostracod species showed significant similarity to other invertebrate opsins using BLAST searches of GenBank (Altschul et al. 1997). Second, several amino acids that are highly conserved in opsin genes are also conserved in all clones from both myodocopid species. A highly conserved lysine residue, which is the site of retinal binding in opsins (Sakamoto et al. 1996; Wang et al. 1980) is present in all clones examined (Fig. 1). Five amino acids of the cloned *S. leneri* gene fragments are identical to a series of residues believed responsible for binding and activation of the G-protein (Fig. 1; Franke et al. 1990; Franke et al. 1992; Sakamoto et al. 1996). Finally, the c-terminal regions of the cloned ostracod fragments are rich in serine and threonine residues, which may serve as phosphorylation sites for rhodopsin kinase (Sakamoto et al. 1996; Wilden and Kuhn 1982). Based on these similarities with other opsin genes and the fact that the sequences are derived from cDNA and therefore transcribed, we conclude that the cloned ostracod fragments are functional visual pigment genes. Although several features common to all opsins are conserved in these ostracod opsins, a surprising number of different sequences were present in ostracod opsins.

Test for eye-specific expression and locus number in S. leneri

The best-fit ML model for the *S. leneri* data, as determined by likelihood ratio tests using Modeltest 3.04 (Posada and Crandall 1998), was HKY85 + gamma (Hasegawa et al. 1985). Parameter estimates were base frequencies (a c g) = (.2581 .2925 .1842), transition/transversion ratio = 1.3091, gamma shape= 0.3155.

All 65 clones from median eye-specific RT-PCR clustered in one clade. In addition, 55 of 58 clones from compound eye RT-PCR clustered in the opposite clade (Fig. 2). Clearly, our amplification and sequencing protocol could identify opsins from both clades because it did so when using pooled cDNA as a template. Yet sequencing median eye-derived cDNA from single individuals yielded opsins from only one clade and virtually all compound-eye-derived clones clustered in a different clade.

We suspect two different causes to explain why the three clones sequenced from compound eye cDNA grouped in the median eye clade. First, a minor contamination could have occurred, including the possibility of amplified genomic DNA (rather than cDNA). Second, there could be minor “leaky” expression of opsins from the compound eye clade in the median eye. Regardless, the overwhelming majority of clones from each eye type clustered in opposite clades. Therefore, there exists a significant expression difference of opsins between median and compound eyes in *S. leneri*.

By counting the number of alleles from a single individual in a clade of opsins, we determined a minimum number of opsin loci in *S. leneri*. Since myodocopids are diploid (Moguilevsky 1985; Moguilevsky 1990), a maximum of two alleles should be present per locus. Therefore, if three alleles from the same individual occur in one clade, we conclude that at least two different loci must be present in that clade. Based on this consideration, we estimate that at least eight loci exist in *S. leneri*. At least six loci are found in the compound eye clade compared to two in the median eye clade (Fig. 2). The number of different sequences detected from a single individual ranges from three in SIM1 to seven in SIF1 (Fig. 2).

Synonymous (K_s) and Non-Synonymous (K_a) Substitutions

The rate of synonymous substitution was higher than non-synonymous substitutions in every possible pair-wise comparison between all myodocopid opsin

sequences. The average ratio of K_s to K_a was 12.16, offering no evidence for positive selection. In addition to measuring the ratio K_s to K_a with these analyses, we also identified identical alleles present in different individuals. These include SIM1_CE1_2_3b with SIM3_CE4; SIM2_CE12id with SIM1_CE2_4_6_8_10; SIM2_ME3_7_8_12 with SIF1_ME16_17_19_24; SIM1_ME6id with SIM2_ME9sim and with SIM3_ME10sim; and SIF2_ME13sim with SIP_11id. (See also branch lengths of figure 2).

Tests for gene conversion and Alternative splicing in Skogsbergia lernerii opsins

No phylogenetically anomalous regions were detected with PLATO, providing no evidence of gene conversion within the portion of opsin sequenced. Furthermore, the 3' UTR sequence variation was generally congruent with coding sequence variation (Fig. 3) as the six UTR types were associated with five major clades found by analyzing the coding portion of the sequence (Fig. 3). One major clade had two different UTR types: the basal *S. lernerii* compound eye clade had both type 3 and type 4 UTR sequences. Of possible concern is that the presence of only four UTR types in *S. lernerii* (besides the two in *V. hilgendorffii*) is inconsistent with our claim of at least eight different loci in that species. However, for two reasons, we argue this is not a concern. First, although UTRs within a type are similar enough to align, sequences of a given type are in fact variable. Figure 4 illustrates type 2 UTRs (associated with median eye opsins) and type 6 UTRs. Second, there is ample evidence that 3' UTR's can be highly conserved because they are often used in post-transcriptional regulation (e.g. Ch'ng and Badr 1994; Haeussler et al. 2000; Kohn et al. 1996; Lee et al. 1998). Therefore even if we hadn't found substantial variation, multiple loci could have very similar 3' UTR sequences.

Phylogenetic analysis of all ostracod opsins

The best-fit model for the data set including myodocopid and outgroup opsins was Kimura-two-parameter with invariant sites and gamma distributed rate heterogeneity (K80 + I + G). Parameter estimates were transition/transversion ratio = 1.0915, gamma shape = 1.1336, proportion of invariant sites = 0.0987.

The relationships of invertebrate opsins are presented in figure 3. In our analysis, all myodocopid opsins formed a well-supported (Fig. 3; 84%), monophyletic clade. Myodocopid compound eye opsins formed a well-supported clade (82%) that was reciprocally monophyletic to a myodocopid median eye clade. The median eye clade was not well supported (50%). Our analyses, including repeating analyses with different outgroups, which changed level of support significantly (not shown), indicate this ambiguity is in large part caused by the absence of close outgroups to the myodocopid opsins. The sister clade of myodocopid opsins is insect blue and UV opsins. Relationships of non-ostracod opsins in figure 3 are very similar to those presented by previous authors (Briscoe 2000; Chang et al. 1995; Pichaud et al. 1999). One apparent difference in our analysis is the placement of crab opsins as sister to all other arthropod opsins. However, this placement is found in fewer than 50% of our bootstrap replicates indicating substantial ambiguity. Previous analyses found similar ambiguity in the placement of these two opsins (Briscoe 2000; Pichaud et al. 1999).

Discussion

We report strong experimental evidence for differential, eye-specific expression of opsin in the myodocopid ostracod *Skogsbergia lernerii*. First, we found that all clones sequenced from compound eye cDNA grouped in one clade and virtually all clones sequenced from median eye cDNA grouped in another clade (Fig. 2). This highly non-random result strongly implies a difference in opsin expression in two ostracod eye types. This experimental approach was taken in order to lower the potential of contamination when using RT-PCR to establish tissue-specific expression: Instead of relying on presence or absence of RT-PCR amplification to determine expression, we took the extra step to sequence multiple clones from the pool of DNA amplified from different tissue-specific cDNAs. In addition to our results from *S. lernerii*, sequences from *V. hilgendorffii* were detected that grouped in the median and compound eye opsin clades, supporting the notion that gene duplication and specialization of expression preceded the separation of these two ostracod species. These two species are members of the same family, but we predict that the other four myodocopid families may show similar differential expression patterns, a hypothesis that remains to be tested.

Our results of eye-specific opsin expression are the first such report in a crustacean but have been reported previously in chelicerates and insects. In addition, vertebrates express different opsins in pineal glands (which often have photoreceptive function) and retinas (Blackshaw and Snyder 1999; Kawamura and Yokoyama 1996; Kawamura and Yokoyama 1997; Kawamura and Yokoyama 1998; Kojima and Fukada 1999; Yokoyama 1996). Similar results from such widely divergent taxa suggest that specialization of expression may be generally true in organisms with more than one photoreceptor type.

The ability to express different opsins in different eye types may be advantageous. Ostracod median and compound eyes are structurally and functionally very different and may therefore have different biochemical demands for opsin. As a first example, different eye functions may require sensitivity to different wavelengths of light. Indeed different eye types of *Drosophila* and *Limulus* are maximally sensitive to different wavelengths (Carulli et al. 1994; Smith et al. 1993). Second, the different eye types may also have different demands for the amount of opsin expression, which is known to fluctuate on daily cycles in crab compound eyes (Arikawa et al. 1987; Arikawa et al. 1988). Having two different genes with different regulatory regions might allow for separate regulation of opsin in each eye type.

Differential expression of opsins seems common across different taxonomic groups; nevertheless we are aware of one exception. In addition to compound eyes and ocelli, *Drosophila* and many other insects have larval photoreceptors. The larval photoreceptors in *Drosophila melanogaster* express the same opsins (Rh5 and Rh6) as R8, one of the photoreceptive cell types in each facet of the compound eye (Malpel et al. 2002). Interestingly, developmental evidence suggests that larval eyes were recently derived from compound eyes (e.g. Green et al. 1993), perhaps too recently to evolve differential opsin expression between the organs.

Although differential expression in two eye types clearly explains part of the opsin variation that we have found, we also report the presence of multiple opsin sequences from both species studied. We suggest that these different sequences represent multiple loci. Of primary importance is to consider possible experimental artifacts that could lead to the same result. For example, taq polymerase errors could be interpreted as multiple different sequences. However, we argue polymerase error could not have caused the

variation in opsin genes that we observed. First, we used a conservative approach when pooling different clones into the same sequence, considering only those that differed by more than 2% to be different sequences. Second, many of the different opsin sequences that we present have different 3' UTRs, some so different that they cannot be aligned reliably (Fig. 4). Taq polymerase error cannot account for such differences in UTRs and the presence of different UTR's is direct evidence for multiple different opsin genes. Third, we observed a non-random distribution of nucleotide substitutions; most observed substitutions occur at silent sites. Taq polymerase error would have introduced a random distribution of substitutions instead of clustering them at synonymous sites.

Assuming that our results are not caused by experimental artifact, the presence of a monophyletic clade of multiple opsin loci suggests that many recent gene duplications have occurred in ostracods. Monophyly indicates that the duplications occurred more recently than the origin of ostracods. While this is potentially a considerable amount of time, the duplications are recent compared to many documented opsin duplications in other arthropods. However we must consider that the result of monophyly could also be caused by alternative splicing or gene conversion between loci, but we did not detect any evidence of either. First, we were unable to find any evidence of gene conversion. Our analysis seeking gene regions with significantly low maximum likelihood values (assuming the tree estimated from all data) failed to detect a single anomalous region in the coding sequence. Furthermore, the 3' UTR sequences track the phylogeny of the coding sequence, suggesting a concordance in phylogenetic history between the coding and non-coding portions of the gene. Gene conversion is therefore a very unlikely hypothesis for explaining the pattern of molecular evolution described. These same analyses that failed to detect evidence of gene conversion also cast doubt on the possibility of alternative splicing as an explanation for opsin variation, because alternative splicing would splice together phylogenetically incongruous sequences, for which we find no evidence.

Color vision is the most obvious functional explanation for multiple opsins, but currently there is no decisive evidence for or against ostracod color vision. For example, a physiological study failed to find evidence for color vision. Huvad (1993) measured the spectral sensitivity of the eyes of *S. lernerii* and two *Vargula* species with spectrophotometry. She concluded that a single peak of maximum sensitivity in the short-wavelength, blue light range (460 nm) exists in ostracod eyes. Unfortunately, performing spectrophotometry on pooled extracts can fail to detect minority or labile visual pigments with different sensitivity. Given these ambiguities and the fact that no behavioral studies have been performed, the question of color vision in ostracods remains open.

Another possible explanation for multiple ostracod opsins is that they are expressed in the same photoreceptor cells and are maximally sensitive to similar wavelengths. Two such situations are already known in arthropods. Sakamoto et al. (1996) found two closely related opsins with overlapping expression in the same reticular cells of the crab *Hemigrapsus sanguinus*. They found only one wavelength sensitivity peak in those cells, suggesting that the two opsins have the same spectral sensitivity. The butterfly *Papilio xuthus* also expresses two very similar opsins in the same photoreceptor cells (Kitamoto et al. 1998). These authors suggested the two loci might have slightly different wavelength sensitivities, serving to broaden the spectral sensitivity of the photoreceptors.

Besides color vision, at least three other functional possibilities exist. One possibility is brightness range fractionation, the sensitivity of different photoreceptors (presumably expressing different opsins) to different light intensities. Such is the case in vertebrates with rod and cone photoreceptors. Range fractionation in *S. lernerii* might be useful since they are most active just before, during and after sunset (Cohen 1983), a period of transition in environmental light intensity. Second, multiple opsins could be expressed in extraocular photoreceptors, such as the Bellonci organ or as yet unknown structures - perhaps located near the compound eye - as in *Drosophila* (Yasuyama and Meinertzhagen 1999). Finally, selective expression of different subsets of opsins could lead to differences in visual response as described in Cichlid fishes (Carleton and Kocher 2001).

In summary, the first molecular foray into the study of ostracod vision has illustrated an example of differential eye-specific opsin expression, the first from any crustacean. Coupled with data on vertebrates and other arthropods, we suggest this may be a general phenomenon in taxa with multiple photoreceptors. In addition, we report the unexpected pattern of several recent opsin gene duplication events in ostracods. There are many potential reasons that are not mutually exclusive, which could explain the presence of multiple opsin loci, including color vision, increased spectral coverage, differential expression, brightness range fractionation and local DNA duplication unrelated to opsin function. Clearly, ostracod vision is worthy of future research.

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Table 1 – Primers used for amplification of ostracod opsin fragments.

Function	Name	Sequence
Reverse Transcription 3' RACE	DT-17-Adaptor RACE-Adaptor	GACTCGAGTCGACATCGTTTTTTTTTTTTTTTTTTT GACTCGAGTCGACATCG
Crustacean degenerate forward	1080F	TGCGYGMWCAGGCCAAGAAGATG
Crustacean degenerate reverse	1350R	GGCTGATGGCRTACACAATAGGG
<i>Skogsbergia leneri</i> forward	SLF	TGAGCGCWGAATGCCGCATTGC
<i>Vargula hilgendorfii</i> forward	DelgapF	CAGGCCAAGAAGATGAGCGC
<i>Vargula hilgendorfii</i> forward	GapF	GAACGTGGAGAGTCTCAGGAACGAGG
Conserved forward	APF	CAGGCCAAGAAGATGAACGTC

Figure Legends

FIG. 1. – Putative ostracod opsins share many features with known opsins. The G-protein binding site is conserved in G-Protein Coupled Receptors (like opsin) and the Retinal Binding site is conserved in opsins. Illustrated are aligned amino acid sequences from three previously published opsins, honeybee blue (#AF004168), honeybee UV (#AF004169) and ten selected ostracod clones. ‘ME’ refers to median eye and ‘CE’ refers to compound eye-expressed sequence. Illustrated are the two most divergent *Skogsbergia lernerii* opsins from each clade thought to contain multiple loci (see Fig. 2). Several conserved features of opsins are found in all ostracod opsins and are darkly shaded. These features are discussed in detail in the text, and include the labeled putative retinal-binding site. Amino acids conserved across all illustrated sequences are lightly shaded. Trans-membrane domains VI and VII from the crab sequence and aligned ostracod sequences are within unshaded boxes. The specific ostracod clones illustrated are (in order) as follows: VhP_4_7_10, VhP_A_B_G_G1, SIF1CE15, SIM1CE3_15, SIF2CE11_13, SIM3CE3_6_7_19, SIP5, SIF1CE6_7_8_9_12, SIM2ME3_7_8_12, SIF2ME13sim.

FIG. 2. - A test for eye-specific expression of opsin. We performed RT-PCR on compound and median eye tissue separately, and used pooled tissue (containing both eye types) as a RT-PCR control. Illustrated is the ML tree of *Skogsbergia* opsins using the best-fit model. See text for model, parameters, and details of the clone-naming scheme. A bootstrap analysis using 10000 replicates of neighbor-joining with ML distances found 100% support for two separate clades. All clones derived from median eye opsins cluster in one clade, while most clones from compound eyes cluster in a separate clade. As discussed in the text, three compound eye clones were identical to median eye clones. The sequences marked with ‘*’, although labeled with ME, each also contain a single clone derived from compound eye cDNA. The tree is rooted based on the broader phylogenetic analysis of Fig. 3. Because we can amplify opsins from both clades when using pooled cDNA, and because we used the same PCR protocol for all amplifications, we know a difference in expression must exist to explain the clade-specific amplification from cDNA derived from single eyes. Bootstrap values for all nodes are available upon request from THO.

FIG. 3. – A phylogenetic analysis of myodocopid and previously published opsins. Illustrated is the ML tree using the best-fit model. See text for model and parameter estimates. Nodes with greater than 90% bootstrap from 10000 replicates using neighbor-joining distances calculated using maximum likelihood are indicated with symbols. Some nodes of interest are labeled with bootstrap percentages. The tree is rooted with the mollusk *Sepia* as an outgroup. GenBank accession numbers for outgroups are as follows: *Sepia* = AF000947; Crab (1=D50583, 2 = D50584); Fruit Fly (Rh1=P06002, Rh2 = P08099, Rh3 = P04950, Rh4 = P29404, Rh5 = U67905, Rh6 = Z86118); Crayfish = S534941; *Limulus* = L037921; Butterfly (Pgl Rh1 = AF077189, Pgl Rh2= AF077190, Pgl Rh3= AF067080, Pgl Rh4= AF077193, Pgl Rh5= AF077191, Pgl Rh6= AF077192); (Bee UV = AF004169, blue = AF004168, LW = U26026).

FIG. 4. – Variation in 3' UTR sequences. A. Type-2 UTRs are associated with median eye-expressed opsins of *Skogsbergia leneri*, and are the most conserved of any UTR type from that species. B. Type-6 UTRs are associated with compound eye expressed opsins of *S. leneri*. Three type-6 sequences are not shown because they were truncated at the 3' end of the sequence. Other UTR-types are not illustrated, but show similar or more variation than the types illustrated here.

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