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# Positive selection on sperm ion channels in a brooding brittle star: consequence of life-history traits evolution

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## Abstract

Closely related species are key models to investigate mechanisms leading to reproductive isolation and early stages of diversification, also at the genomic level. The brittle star cryptic species complex *Ophioderma longicauda* encompasses the sympatric broadcast-spawning species C3 and the internal brooding species C5. Here, we used de novo transcriptome sequencing and assembly in two closely related species displaying contrasting reproductive modes to compare their genetic diversity and to investigate the role of natural selection in reproductive isolation. We reconstructed 20 146 and 22 123 genes for C3 and C5, respectively, and characterized a set of 12 229 orthologs. Genetic diversity was 1.5-2 times higher in C3 compared to C5, confirming that species with low parental investment display higher levels of genetic diversity. Forty-eight genes were the targets of positive diversifying selection during the evolution of the two species. Notably, two genes (NHE and TetraKCNG) are sperm-specific ion channels involved in sperm motility. Ancestral sequence reconstructions show that natural selection targeted the two genes in the brooding species. This may result from an adaptation to the novel environmental conditions surrounding sperm in the brooding species, either directly affecting sperm or via an increase in male/female conflict. This phenomenon could have promoted prezygotic reproductive isolation between C3 and C5. Finally, the sperm receptors to egg chemoattractants differed between C3 and C5 in the ligand-binding region. We propose that mechanisms of species-specific gamete recognition in brittle stars occur during sperm chemotaxis (sperm attraction towards the eggs), contrary to other marine invertebrates where prezygotic barriers to inter-specific hybridization typically occur before sperm-egg fusion.

*Keywords:* ion channel, marine invertebrate, reproductive isolation, sperm chemotaxis, sperm competition, transcriptome

## Introduction

Understanding mechanisms leading to the formation of new species is a fundamental question in evolutionary biology. In particular, how reproductive barriers evolve,

and what the genetic basis of barriers to gene flow is, remain enigmatic for many nonmodel organisms (Noor & Feder 2006; Nosil & Schluter 2011; Ellegren 2014). Thanks to their recent divergence, closely related species are fascinating models to study the mechanisms leading to reproductive isolation and ultimately speciation (Seehausen *et al.* 2014). It is generally expected that while global genetic divergence between recently

diverged species will be low, genes involved in reproductive isolation, also called ‘speciation genes’, will display higher levels of differentiation (Nosil & Schluter 2011; Nosil & Feder 2012).

When different species live in the same geographic location (i.e. in sympatry), mechanisms preventing genetic exchange between species can occur before or after the fusion of gametes. Therefore, these mechanisms are classified as prezygotic or postzygotic factors of reproductive isolation, respectively. While postzygotic isolation leads to death or sterility of the newly formed embryos due to genomic incompatibilities (i.e. Bateson–Dobzhansky–Muller incompatibilities), prezygotic isolation can include, for instance, temporal prezygotic isolation, where reproduction periods are not overlapping. In marine invertebrates, where the sense of sight is limited to photoreception, mechanisms of species recognition are mostly mediated through chemical signals (Palumbi 1994). For instance, there can be active avoidance of non-conspecific males and females, via chemical signals, to avoid interspecific mating (Hendler 1991; Bickford *et al.* 2007; Mercier & Hamel 2009). Finally, when closely related species occur in sympatry, genes involved in gamete recognition can evolve under positive diversifying selection and display increased genetic differentiation, therefore preventing the recognition of gametes belonging to different species and their fusion (Lessios 2011; Vacquier & Swanson 2011).

The most famous examples of gamete recognition proteins are the sperm protein bindin and its egg receptor EBR1 in sea urchins, and the sperm protein lysin and its egg receptor VERL in gastropods. The bindin is a protein involved in the recognition between the sperm and the egg vitelline envelope, as well as in the fusion of sperm and egg membranes. In contrast, the lysin is only involved in recognition and dissolution of the vitelline envelope, the fusion of sperm and egg membrane being mediated by the protein sp18 (Lessios 2011; Vacquier & Swanson 2011). Positive selection frequently targets these systems, as was shown for the bindin protein in sea urchins of the genus *Strongylocentrotus*, *Paracentrotus*, *Heliocidaris* and *Echinometra* (Lessios 2011), as well as in sea stars (Sunday & Hart 2013; Patiño *et al.* 2016), and for the egg receptor for sperm Obi1 (Hart 2013; Hart *et al.* 2014). Similarly, the proteins involved at the level of egg jelly and in particular their carbohydrate-recognition domains (suREJ) were found to be under positive diversifying selection in six species of sea urchins (Mah *et al.* 2005). This receptor interacts with sulphated polysaccharides to induce the acrosome reaction, polysaccharides which also display structural changes between closely related species of *Strongylocentrotus* sea urchins (Biermann *et al.* 2004). Prezygotic isolation through gamete recognition therefore seems to be

the rule in sea urchins and likely sea stars, but whether this mechanism is general to echinoderms, and more generally to marine invertebrates, is still unclear.

With more than 2000 extant species, brittle stars (Ophiuroidea) form the largest class of echinoderms. Brittle stars occur in virtually every marine environment, from very shallow to abyssal depths, and from tropical to Arctic and Antarctic areas (Stöhr *et al.* 2012). Interestingly, this class includes numerous complexes of cryptic species, among which morphological characters are poorly or not (yet) differentiated (Baric & Sturmbauer 1999; Spöner & Roy 2002; O’Hara *et al.* 2004; Muths *et al.* 2006, 2009; Boissin *et al.* 2008, 2011; Hunter & Halanych 2008; Stöhr *et al.* 2009; Heimeier *et al.* 2010; Stöhr & Muths 2010; Hoareau *et al.* 2013; Pérez-Portela *et al.* 2013; Naughton *et al.* 2014; de Moura Barboza *et al.* 2015; Taboada & Pérez-Portela 2016).

The brittle star cryptic species complex *Ophioderma longicauda* is composed of six divergent mitochondrial lineages displaying contrasting reproductive strategies (broadcast spawners and internal brooders; Stöhr *et al.* 2009; Boissin *et al.* 2011). This species complex, encompassing at least three distinct biological species, occurs in the East Atlantic Ocean and in the Mediterranean Sea. In the so-called broadcast spawners (the ancestral strategy), reproduction occurs once a year, fertilization is external and larvae experience a dispersive pelagic phase of about 6 days (Fenaux 1969, 1972). In contrast, in the brooding species (the derived strategy), fertilization is presumably internal, embryos develop for several weeks in female-specific pouches (bursae; Stöhr *et al.* 2009), and dispersal occurs only at the adult stage. The two reproductive systems presumably imply contrasted selective pressures at the gametic stage, which might impact the mechanism and evolution of prezygotic isolation. Here, we focused on two sympatric species from the *O. longicauda* complex, named temporarily C3 (broadcast spawner) and C5 (internal brooder) until formal description is completed (S. Stöhr, A.A.-T. Weber, E. Boissin, A. Chenuil, in preparation). These two species, in addition to displaying different reproductive strategies, do not reproduce at the same time of the year and display genetic, ecological, physiological and morphological differences (Weber *et al.* 2013, 2014, 2015). There is currently no gene flow between C3 and C5, and their divergence time is estimated to about 550 000 years (Weber 2015). Thus, this pair of closely related species provides an interesting model system to investigate reproductive isolation and genome differentiation. We used transcriptome sequencing and de novo assembly to compare the genetic diversity and investigate the role of natural selection in the reproductive isolation between *O. longicauda* C3 and C5. In contrast with sea urchins, we found

that in *O. longicauda*, proteins associated to gamete recognition are not particularly fast evolving. Yet, we identified two genes associated to sperm motility, which were positively selected in the brooding species.

## Material and methods

### *Individual sampling, lineage typing and RNA extraction*

Sampling was performed in four localities across the Mediterranean and the Atlantic (Madeira, Greece, Portugal and France; Table S1, Supporting information). For each individual, gonads, embryos (when present) and a piece of arm were preserved in RNA later and later used for RNA extraction (Table S1, Supporting information); a piece of arm was also preserved in EtOH 95% for DNA extraction using a Chelex protocol (Walsh *et al.* 1991). Species delimitation was performed according to the mitochondrial lineages described by Boissin *et al.* (2011). For each specimen, a COI gene fragment was PCR-amplified (forward primer: 5' CTT GCA GGA GGA GGA GAY CC 3'; reverse primer: 5' AGT ATA AGC GTC TGG GTA GTC 3') and Sanger-sequenced (LGC Genomics, Germany). Individuals from Madeira, Sagres and Marseille belonged to the broadcasting species C3 (mitochondrial lineage L1 in Boissin *et al.* (2011)), whereas individuals from Crete represented species C3 and C5 (mitochondrial lineages L1 and L3, respectively), in line with the species distribution described by Weber *et al.* (2015). To illustrate the presence of two distinct species, phylogenetic trees for COI and for the nuclear marker EF1 $\alpha$  (Elongation factor 1 $\alpha$ ; retrieved from the transcriptomes generated in this study) are available in Fig. S1, Supporting information. RNA extractions were performed using a combination of standard guanidium thiocyanate–phenol–chloroform (GTPC) RNA extraction followed by an extra purification step with a RNeasy kit for RNA extractions (Qiagen). Both methods were used in combination with maximize quantity and purity of RNA, as recommended by Gayral *et al.* (2011). Twelve libraries for RNA sequencing were prepared (Table S1, Supporting information); seven with RNA from unique individuals (two from species C5 and five from species C3) and five with RNA from pooled individuals from four different localities (C5-Crete, C3-Crete, C3-France, C3-Madeira, C3-Portugal). Pools of individuals were performed using the same quantity of RNA per individual.

### *Sequencing and assembly*

Library preparation and sequencing were performed by the genomic platform Genotoul (Toulouse). Libraries

were constructed using TruSeq library preparation kit (Illumina) following manufacturer's instructions. Sequencing was performed with a HiSeq 2000 sequencer (Illumina). The complete bioinformatics pipeline used in this study is described in Gayral *et al.* (2013). For this assembly, only the individual libraries were used, to calculate the per-species nucleotide diversity and the individual heterozygosities. Briefly, raw reads were filtered for length ( $\geq 60$  bp) and quality (Phred score  $\geq 30$ ). De novo assembly was performed using Abyss (Simpson *et al.* 2009) and Cap3 (Huang & Madan 1999), according to the method B described in (Cahais *et al.* 2012). The combination of Abyss and Cap3 was selected based on a combination of reliability and computational efficiency. In addition, this combination was recently shown to perform better than Trinity in the assembly of 26 species of plants (Sarah *et al.* 2016). Reads were mapped to the contigs using BWA (Li & Durbin 2009). Raw contigs were filtered for length ( $\geq 200$  bp) and coverage ( $2.5\times$  per individual). ORFs were predicted from the filtered contigs, and the longest ORF was kept. Genotypes were called for each position of each contig using read2SNPs and applying a filter for hidden paralogy as described in Gayral *et al.* (2013). Genotypes were only called if supported by a posterior probability above 0.95 and at least 10 reads. For each species, the per-site synonymous ( $\pi_S$ ) and nonsynonymous ( $\pi_N$ ) diversities were calculated, as well as the per-individual heterozygosity.

### *Research and annotation of orthologs*

Orthologs between C3 and C5 were predicted using a reciprocal best BLAST hit approach, similarly to Gayral *et al.* (2013). BLASTP searches were performed using the 13 990 C3 ORFs as query and the 20 790 C5 ORFs as target, and vice versa with an e-value cut-off of  $10e^{-5}$ . Results with no hit, with an overlap  $<60\%$  or with an identity percentage  $<90\%$  were discarded. In addition, results with a ratio of the score of the second best hit over the score of the best hit higher than 0.8 were removed, to avoid the inclusion of paralogs. This analysis yielded 4740 putative orthologs, a surprisingly low number for two species so closely related. A potential problem seemed to be the low number of ORFs retrieved for the broadcasting species C3: because we used the same methodology for sample preparation and sequencing, we hypothesized that this problem stemmed from a combination of high polymorphism in C3 and too much stringency in the coverage criterion.

To test this possibility, we attempted to recover C3 contigs using C5 contigs as references for mapping, and vice versa. To maximize the chances of recovering lost contigs, all the reads of each species were used (from

single and pooled individuals) and coverage constraints were relaxed. C3 reads were mapped on the 11 405 C5 contigs displaying less than 80% identity with C3 contigs after the first BLAST search using BOWTIE2 (Langmead & Salzberg 2012), and allowing a maximum of five differences (for 100-bp reads) with the reference contig to account for the fact the reads and the reference belong to two different species. C3 contigs were then reconstructed using MIRA (Chevreux *et al.* 1999). Contigs with gaps in the coverage, or with an average coverage  $<10\times$  or smaller than 250 bp, were discarded. A reciprocal best BLAST hit analysis was then performed to compare the newly reconstructed C3 contigs to the 20 790 C5 contigs and to verify whether these newly reconstructed C3 contigs indeed corresponded to some of the original C5 references. Using this method, 6156 additional C3 contigs were reconstructed, raising the total number of C3 contigs to 20 146. The equivalent analysis for C5 led to the reconstruction of 1333 additional C5 contigs, raising the total number of C5 contigs to 22 123. Comparison of the 20 146 C3 contigs to the 22 123 C5 contigs using a reciprocal best BLAST hit approach isolated a set of 12 229 orthologs. To ensure that the C3–C5 comparison only involved orthologous contigs, we used the ratio between the first and the second best BLAST hit score as a guide, and contigs with a ratio  $>0.8$  were excluded.

Functional annotation was performed via BLASTP against the NR (nonredundant) database of NCBI using an *e*-value cut-off of  $10e^{-5}$ . The top 20 hits were extracted and loaded in BLAST2GO for annotation (Conesa *et al.* 2005). Mapping, annotation and slim ontology were performed with BLAST2GO using default parameters, except for the annotation cut-off parameter that was set to 45. GO categories were described using the level 3 of slim ontology. For the genes that did not display GO term annotations, BLASTP search was performed against the NR (nonredundant) database of NCBI using an *e*-value cut-off of  $10e^{-5}$ .

### *Positively selected genes and gamete recognition genes*

To characterize genes evolving under positive diversifying selection, we performed a first screen using the pipeline PSGfinder (Montoya-Burgos laboratory: <https://genev.unige.ch/research/laboratory/Juan-Montoya>). This pipeline performs standard pairwise dN/dS calculation using a pair of aligned nucleotide sequences of orthologous protein-coding genes (one consensus sequence per species). The dN/dS ratio is calculated for alignment windows which size is determined by the presence of at least three nonsynonymous substitutions; this provides a sufficient dN starting signal that reduces the number of windows to

be tested. The estimation of dN and dS values were obtained using the method 'yn00' (Yang & Nielsen 2000), including its assumptions, as implemented in PAML version 4.8 (Yang 2007). Windows with a dN/dS ratio  $> 1$  were considered as indicative of gene parts displaying a signal of positive diversifying selection. The advantage of PSGfinder is that it uses pairwise comparison of orthologs, so positively selected candidate genes can be found even when only two species are available. Then, we confirmed that the most interesting candidate genes found with PSGfinder displayed signals of positive diversifying selection using a codon substitution model [modified branch-site model A; Bayes Empirical Bayes (BEB) analysis; *O. longicauda* C5 as foreground branch] of codeml implemented in PAML version 4.8 (Yang 2007), using outgroup sequences from public databases (sea urchin *S. purpuratus*) or kindly provided by Timothy O'Hara (brittle stars *Ophiothrix angulata* and *Ophiophragmus wurdemani*). Because of its change in reproductive mode, we expect the genes of the brooding species C5 to have evolved under positive selection. As control, we also performed the same analysis but using *O. longicauda* C3 as foreground branch (where we do not expect positive selection to occur). In addition, we used the recently developed BUSTED method which allows to identify genewide evidence of episodic positive selection (Murrell *et al.* 2015), implemented in the HYPHY package (Pond & Muse 2005). Furthermore, we used the branch-sites random effects likelihood method which allows to detect lineages evolving under positive selection without an a priori hypothesis (Pond *et al.* 2011), also implemented in the HYPHY package (Pond & Muse 2005). Finally, we used ancestral sequence reconstruction methods (marginal reconstruction method; WAG amino acid replacement matrix) implemented in PAML version 4.8 (Yang 2007) to confirm which species was affected by natural selection.

Several proteins involved in reproduction (in sperm chemotaxis or in sperm–egg interaction) and whose evolution in other species involved diversifying selection were specifically isolated in our C3 and C5 contigs using BLASTP searches. These proteins were the bindin and its receptor EBR1, as well as the sperm receptor for egg jelly, suREJ, involved in activation of the acrosome reaction, sperm-activating peptides from *S. purpuratus* (speract) and from *Asterias amurensis* (asterosap) as well as their associated receptors (speract receptor and asterosap receptor), known to induce sperm chemotaxis. For the egg receptor for sperm Obi1, we used the sequence of the sea star *Patiria miniata* as query. We also searched for C3/C5 homologs of the sperm-specific calcium channels (Catsper 1-4) known to evolve under positive selection in rodents (Podlaha *et al.* 2005) and primates (Podlaha & Zhang 2003).

## Results

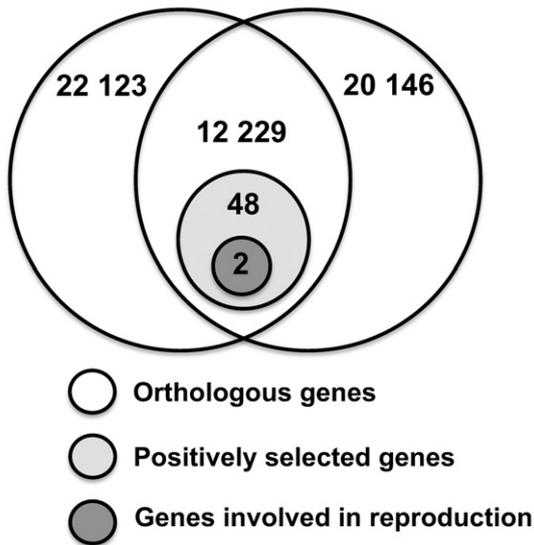
### Assembly statistics and annotation of orthologous genes

Illumina sequencing provided about 250 million raw reads, which were trimmed for length and quality-filtered. Assembly yielded 523 565 and 259 916 raw contigs for C3 and C5, respectively (Table 1). After filtering for length and coverage, 110 225 C3 and 118 043 C5 filtered contigs were obtained. Open reading frame prediction yielded 13 990 and 20 800 ORFs for C3 and C5, respectively. After ORF reconstruction based on 'unique' genes of each species, we obtained 20 146 and 22 123 ORFs for C3 and C5, respectively, of which 12 229 were clearly orthologous genes (Fig. 1). The average nucleotide divergence of the 12 229 orthologs

**Table 1** Transcriptome assembly statistics generated in study

|                             | C3<br>(5 individuals) | C5<br>(2 individuals) |
|-----------------------------|-----------------------|-----------------------|
| Raw reads                   | 164 464 891           | 87 959 550            |
| Raw contigs                 | 523 565               | 259 916               |
| Filtered contigs            | 110 225               | 118 043               |
| ORFs                        | 13 990                | 20 790                |
| Reconstructed ORFs          | 20 146                | 22 123                |
| Orthologous genes           | 12 229                | 12 229                |
| Annotated orthologous genes | 5925                  | 5925                  |

### Brooding species C5 Broadcasting species C3



**Fig. 1** Summary of the gene number, orthologous gene number and positively selected genes between *Ophioderma longicauda* C3 and C5 found in this study.

at the nucleotide level was 0.93%. The higher number of genes in C5 might partly be due to the fact that developing embryos were included in the species C5 transcriptomes, contrary to the species C3 that was not sexually mature at the time of sampling.

Of the 12 229 identified orthologs, 5925 (48%) could be successfully annotated with GO terms. For biological process, primary metabolic process was the most abundant GO term (10.8%), followed by organic substance metabolic process (10.6%) and cellular metabolic process (8.1%) (Fig. S2, Supporting information). For molecular function, genes coding for binding were highly represented (67.8%) as well as transferase (10.5%) and hydrolase (12.9%) activities (Fig. S2, Supporting information).

### Broadcast spawners display higher genetic diversity compared to brooders

To investigate genetic diversity, 1388 and 6700 genes were used for C3 and C5, respectively (Table 2). The smaller number of genes in C3 is most likely due to the stringent quality thresholds applied for genotype calling. Indeed, a position had to be covered with at least 10 reads per individual in order to be called. Given that the C3 individuals were not sampled at the same time of the year nor in the same localities (Table S1, Supporting information), they most likely displayed differences in gene expression levels and therefore differences in gene coverage. Using only C3 individuals from Crete (e.g. same locality as C5), we retrieved more than 5000 genes for the analysis, which gave the same diversity estimates as with the data set of 1388 genes (data available upon request). A total of 7607 and 18 408 SNPs were called for C3 and C5, respectively. The average per-site synonymous diversity ( $\pi_S$ ) was about twice

**Table 2** Comparison of transcriptomic genetic diversity between broadcasting C3 and brooding C5 species

|  | C3                 | C5                 | Diversity ratio<br>C3/C5 |
|--|--------------------|--------------------|--------------------------|
| Reproductive strategy                                  | Broadcasters       | Brooders           |                          |
| N individuals  | 5                  | 2                  |                          |
| N contigs  | 1388               | 6700               |                          |
| N SNPs   | 7607               | 18 408             |                          |
| Average $\pi_S$ [ $\times 10^{-3}$ ]                   | 17                 | 8                  | 2.12                     |
| Average $\pi_N/\pi_S$ ratio                            | 0.181              | 0.269              | 0.67                     |
| Average heterozygosity [ $\times 10^{-3}$ ]( $\pm$ SD) | 6.27 ( $\pm 0.6$ ) | 4.16 ( $\pm 0.2$ ) | 1.51                     |

SNPs, single nucleotide polymorphisms; Sd, standard deviation;  $\pi_S$ , synonymous nucleotide diversity;  $\pi_N$ , nonsynonymous nucleotide diversity.

higher in C3 compared to C5, resulting in a higher  $\pi_N/\pi_S$  ratio in C5 (Table 2). In addition, the average heterozygosity was about 1.5 higher in C3 compared to C5.

### *Sperm-specific ion channels exhibit positive diversifying selection*

Comparison of the 12 229 orthologs between the species C3 and C5 isolated 187 sequence windows displaying evidence of positive diversifying selection (PSGfinder,  $dN_{window}/dS_{gene} > 1$ ,  $P$ -value  $< 0.001$ ). These 187 windows were contained in 48 distinct genes, eleven of which could be successfully annotated with GO terms (Table 3). Another 17 of the 48 genes could be linked to biological process such as immunity, DNA transcription and phosphorylation through BLAST searches (Table S2, Supporting information); 45% (5/11) of the annotated genes encode proteins involved in ion transport, which is a significant enrichment compared to the global transcriptome annotation (3.6%; 215/5925). Interestingly, two genes involved in ion transport are well characterized functionally: the sperm-specific sodium-proton exchanger (NHE) and the tetrameric potassium-selective cyclic nucleotide gated channel (TetraKCNG) (Fig. 1, Table 3).

Positive selection analyses performed with PAML were concordant with the results of PSGfinder, with the majority of the positively selected sites retrieved with PAML were also observed in regions detected with PSGfinder (19/26; Figs 2 and 3). In addition, ancestral sequence reconstructions revealed that positive diversifying selection particularly targeted the *TetraKCNG* and *NHE* genes in the brooding species C5 (Figs 2 and 3). Interestingly, for both genes, >75% (21/27) of the positively selected sites detected by PAML and divergent in the brooding species are conserved in all other species, even in the distantly related sea urchin *Strongylocentrotus purpuratus* (Figs 2 and 3). Finally, the BUSTED method confirmed that both genes are evolving under positive selection (NHE:  $P$ -value = 0.00422; TetraKCNG:  $P$ -value = 0.0235), whereas only NHE was confirmed using the branch-sites random effects likelihood method (NHE:  $P$ -value = 0.016; TetraKCNG:  $P$ -value = 1).

NHE and TetraKCNG are well studied in the sea urchin *S. purpuratus* and are involved in sperm motility after activation of sperm with chemoattractants released by the eggs, such as speract [Fig. 4; see (Darszon *et al.* 2008) for the complete speract signalling model]. If sperm chemoattractants are species specific, they may be different between the brooding and the broadcasting species. To our knowledge, sperm chemoattractants are not described in brittle stars. Therefore, we attempted to find an ortholog of sperm chemoattractant in brittle

stars based on the ones described in the literature, which are speract (in the sea urchin *S. purpuratus*) and asterosap (in the sea star *A. amurensis*). Unfortunately, we could not identify any ortholog, likely because of the small size of the peptides (ten amino acids for speract and 56 amino acids for asterosap; Hirohashi *et al.* 2008; Nakachi *et al.* 2008). As ligand and receptor are likely to co-evolve, we isolated the sequences of the sperm chemoattractant receptor in the two *O. longicauda* species (guanylyl cyclase) but comparison of the two sequences did not reveal any evidence of positive diversifying selection (Table 4). Interestingly, however, of the ten nonsynonymous mutations found in the sperm chemoattractant receptor gene, nine occur in the extracellular (ligand-binding) part, whereas none are detected in the transmembrane part and only one is found in the intracellular part. In addition, a four-codon gap was found in the ligand-binding part of C5, suggesting that C3 and C5 may display different sperm chemoattractants. Therefore, mechanisms of species recognition could occur during sperm chemotaxis in the *Ophioderma longicauda* species complex. Finally, we found orthologs of the proteins involved in fertilization of Bindin, EBR1, suREJ1, Catsper 1-4, ARIS 1-3, Obi1 and Rendezvin. Yet, the evolution of these proteins did not reveal any evidence of positive diversifying selection in *O. longicauda* C3 and C5 (Table 4).

## Discussion

### *Genetic diversity is lower in brooders*

In this study, we used transcriptomes of two sympatric brittle stars belonging to the species complex *Ophioderma longicauda* to compare their level of genetic diversity and investigate how natural selection targeted genes potentially involved in their reproductive isolation. Genetic diversity, a central metric in species response to environmental disturbance (Reusch *et al.* 2005; Hughes *et al.* 2008), is about 1.5–2 times higher in the broadcasting species C3 compared to the brooding species C5, despite low average nucleotide divergence (<1%). These results are in line with a recent study investigating genetic diversity of 76 metazoan species, which showed that the main determinant of genetic diversity is parental investment (Romiguier *et al.* 2014). In particular, species categorized as K-strategists (high longevity, high parental investment, low number of large offspring) displayed lower genetic diversity than r-strategists (low longevity, low parental investment, high number of small offspring). These strategies highlight different responses of species to environmental disturbances (MacArthur & Wilson 1967). In this context, the brooding *O. longicauda* C5 might be more

**Table 3** Positively selected genes between *O. longicauda* C3 and C5 that display GO term annotations. Gene name, matching organism after BLAST search and available GO terms are displayed

| Contig C3       | Contig C5   | Gene name    | Organism                             | Full gene name  | Biological process   | Molecular function                | Cellular component    |
|-----------------|-------------|--------------|--------------------------------------|---|--|-----------------------------------|-----------------------|
| Contig157183    | Contig10800 | NHE          | <i>Strongylocentrotus purpuratus</i> | Sperm-specific sodium-proton exchanger  | Ion transport  | Ion channel activity              | Cell                  |
| Contig5262      | Contig45826 | TetraKCNG    | <i>Strongylocentrotus purpuratus</i> | Tetrameric potassium-selective cyclic nucleotide gated channel  | Ion transport  | Ion channel activity              | Cell                  |
| Contig132964    | Contig75158 | LOC576432    | <i>Strongylocentrotus purpuratus</i> | PREDICTED: excitatory amino acid transporter 3-like   | Ion transport; cellular component organization             | Transporter activity              | Cell; plasma membrane |
| Contig22861     | Contig23039 | LOC584316    | <i>Strongylocentrotus purpuratus</i> | PREDICTED: disrupted in renal carcinoma protein 2 homolog   | Ion transport  | Ion channel activity              | Cell                  |
| Contig141406    | Contig7832  | LOC585510    | <i>Strongylocentrotus purpuratus</i> | PREDICTED: solute carrier family 13 member 5-like   | Ion transport  | Transporter activity              | Plasma membrane       |
| Contig17797     | Contig45476 | /            | <i>Crassostrea gigas</i>             | Thymidine phosphorylase   | Nucleobase containing compound metabolic process           | Transferase activity              | No                    |
| Contig168194    | Contig2036  | LOC100376819 | <i>Saccoglossus kowalevskii</i>      | PREDICTED: arylacetamide deacetylase (esterase)-like  | Metabolic process  | Hydrolase activity                | No                    |
| Contig_C3_26484 | Contig26484 | /            | <i>Crassostrea gigas</i>             | Tripartite motif-containing protein 59  | No   | Binding; peptidase activity       | No                    |
| Contig_C3_21879 | Contig21879 | /            | <i>Homo sapiens</i>                  | AChain A, Structural Studies Of Protein Tyrosine Phosphatase Beta Catalytic Domain In Complex With Inhibitors | Response to external stimulus; signal transduction         | Phosphoprotein phosphate activity | Plasma membrane       |
| Contig_C3_32532 | Contig32532 | LOC100566073 | <i>Anolis carolinensis</i>           | PREDICTED: zinc finger protein 167-like   | Transcription DNA dependant                                | DNA binding                       | Nucleus               |
| Contig_C3_2308  | Contig2308  | LOC581462    | <i>Strongylocentrotus purpuratus</i> | PREDICTED: nephrin-like   | Multicellular organismal development; cell differentiation | Protein binding                   | Plasma membrane       |

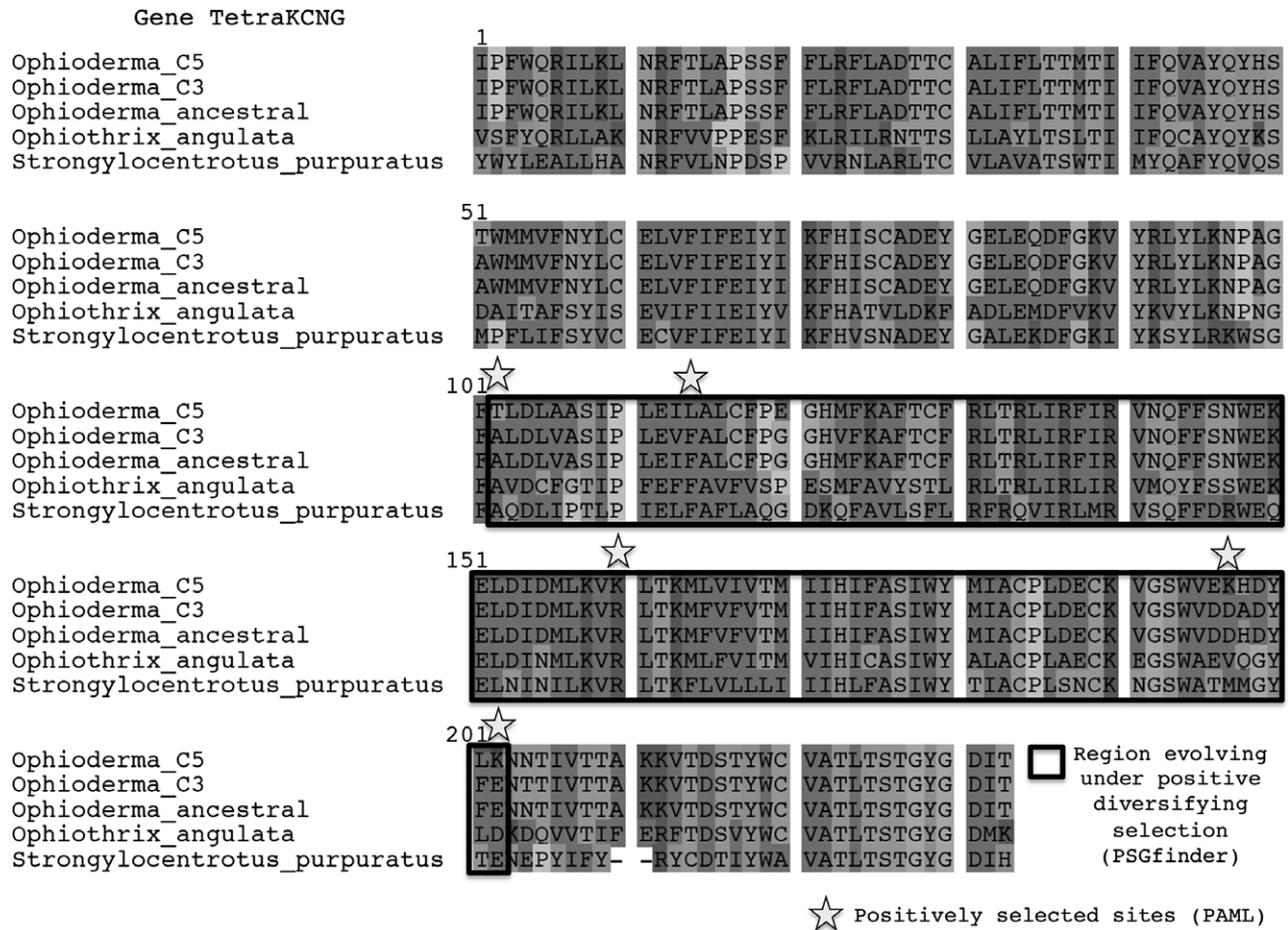


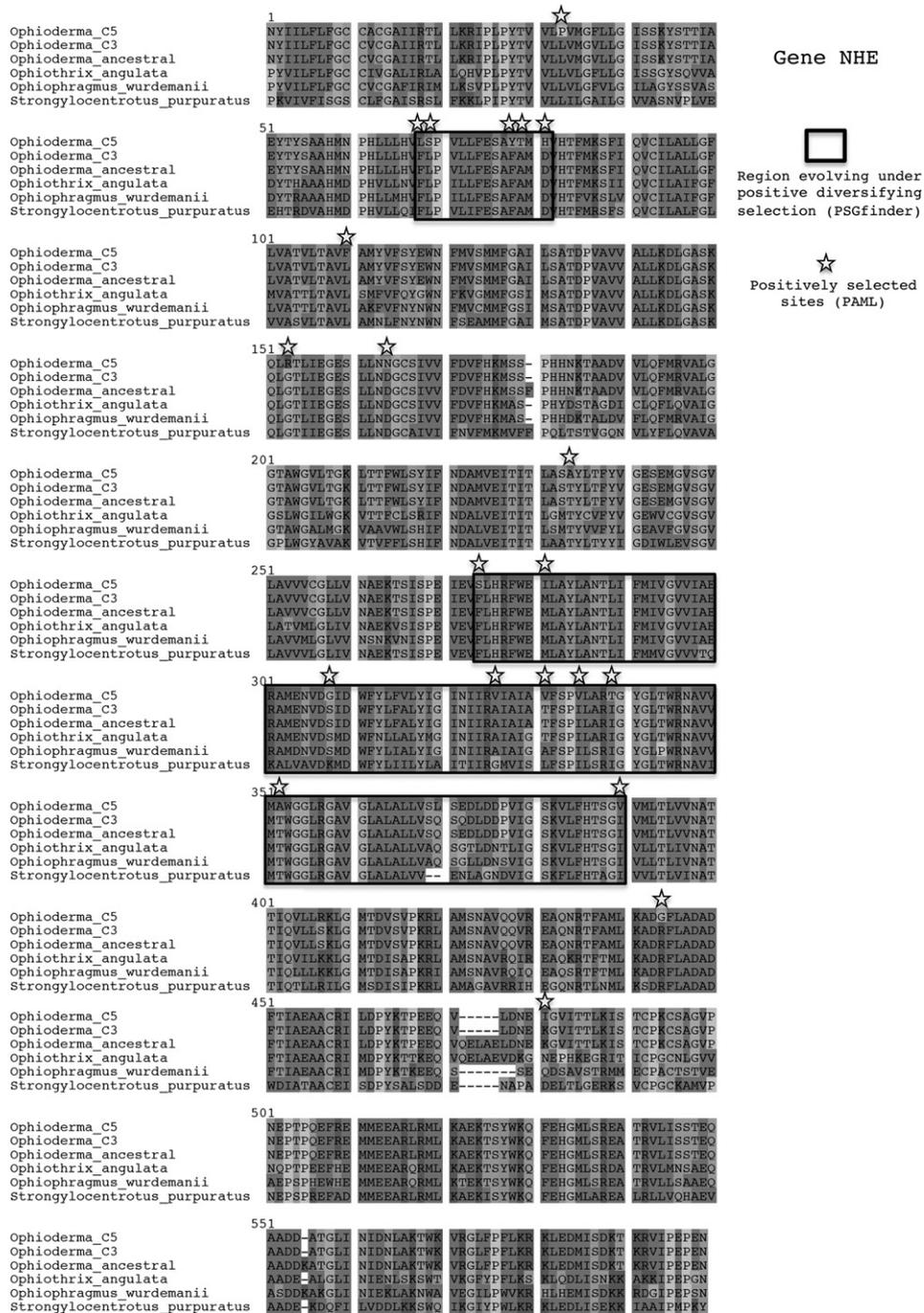
Fig. 2 TetraKCNG protein alignment including *Ophioderma longicauda* C3 and C5, as well as outgroups *Ophiothrix angular* (brittle star) and *Strongylocentrotus purpuratus* (sea urchin). Ancestral sequence reconstruction of *Ophioderma* was performed with PAML version 4.8. The region evolving under positive selection detected by PSGfinder and the positively selected sites detected by PAML are displayed. The alignment highlights that positive diversifying selection occurred in the brooding species *Ophioderma longicauda* C5. Levels of grey correspond to different amino acid families.

vulnerable to rapid environmental changes than the broadcasting *O. longicauda* C3.

### Mechanisms of prezygotic isolation

We focused on genes displaying positive Darwinian selection involved in reproduction, because they may have played a role in reproductive isolation of *O. longicauda* brooding and broadcasting species. There are several steps leading to fertilization in free-spawning invertebrates: (i) sperm chemotaxis, which is the activation and attraction of sperm mediated by chemoattractants released by the egg, (ii) interaction of sperm with the egg jelly, which induces the acrosome reaction, (iii) binding of sperm to egg vitelline envelope, (iv) penetration of sperm through the egg envelope and (v) fusion of gamete membranes (Vacquier 1998). So far, positively selected proteins contributing to prezygotic isolation in

marine invertebrates were involved in sperm-egg interactions, either at the level of egg jelly (step ii) with the sperm receptors suREJ (Mah *et al.* 2005) or at the level of vitelline layer binding and penetration (steps iii-iv) with the sperm protein bindin (Lessios 2011; Vacquier & Swanson 2011; Evans & Sherman 2013), as also shown recently for sea stars (Patiño *et al.* 2016). In *Ophioderma longicauda*, none of these proteins were found to evolve under positive selection. Interestingly, we could sequence and assemble, to our knowledge, the first bindin sequences of brittle stars, which displayed very low divergence between the brooding and the broadcasting species. Using domain prediction analyses, we did not detect any repetitive regions as found in sea stars (Patiño *et al.* 2016). It is therefore likely that the *Ophioderma* bindin sequences are incomplete, due to the difficulty of assembling repetitive regions using short read sequences. Further research on brittle star bindin should be

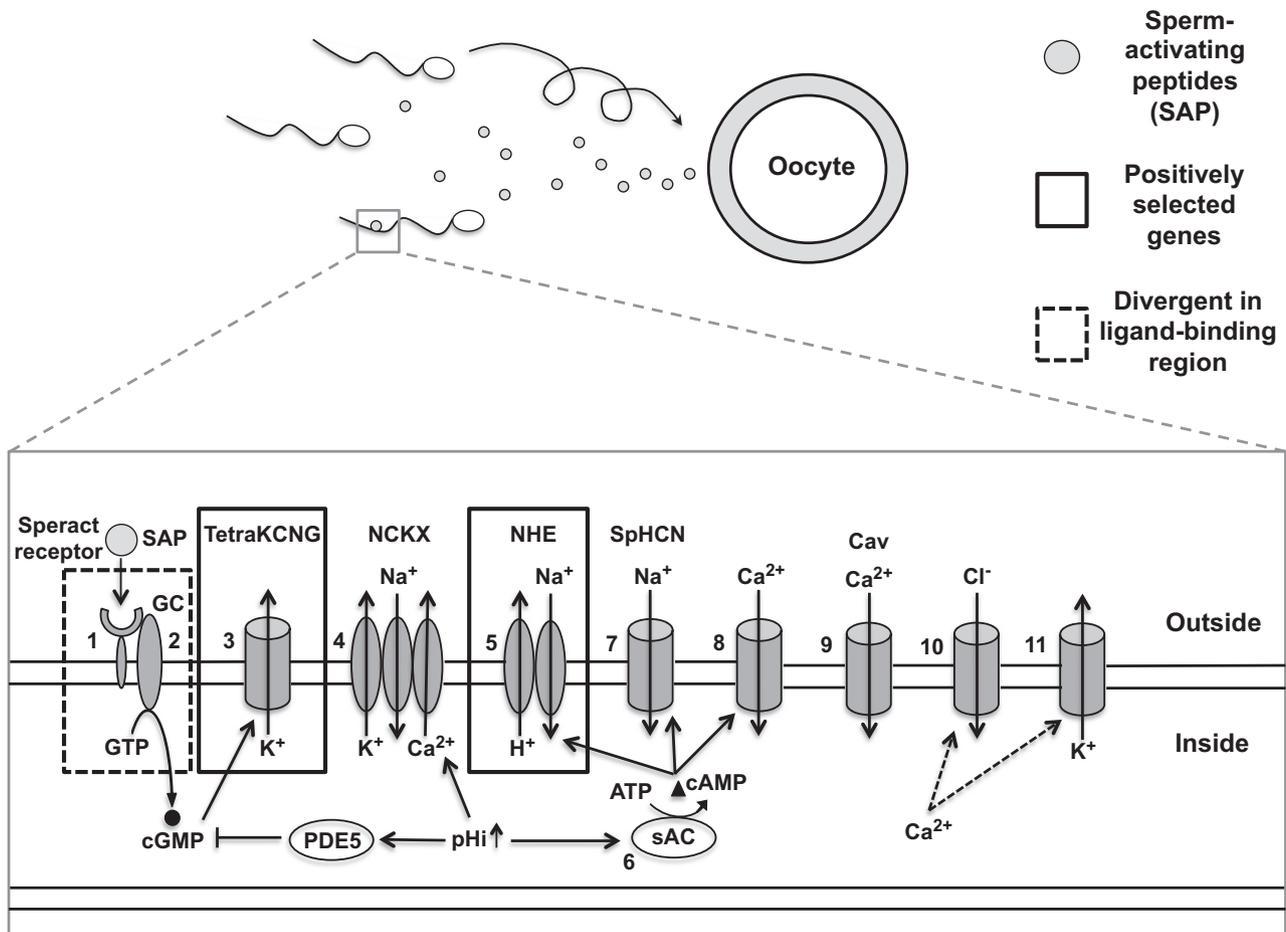


**Fig. 3** NHE protein alignment including *Ophioderma longicauda* C3 and C5, as well as outgroups *Ophiothrix angulata* (brittle star), *Ophiophragmus wurdemanii* (brittle star) and *Stronglyocentrotus purpuratus* (sea urchin). Ancestral sequence reconstruction of *Ophioderma* was performed with PAML version 4.8. The region evolving under positive selection detected by PSGfinder and the positively selected sites detected by PAML are displayed. The alignment highlights that positive diversifying selection occurred in the brooding species *Ophioderma longicauda* C5. Levels of grey correspond to different amino acid families.

performed to conclude on the role of bindin in species recognition and speciation.

In contrast, positive selection was found to act on two ion channels involved in sperm activation and motility. Interestingly, these genes have never been

described as being positively selected between closely related species so far. The two sperm-specific ion channels (TetraKCNG and NHE) and their involvement in sperm activation have been extensively studied in sea urchins (Beltrán *et al.* 2007; Galindo *et al.* 2007; Darszon



**Fig. 4** Speract signalling model (modified from Darszon *et al.* 2008). In the sea urchin, *Strongylocentrotus purpuratus*, the sperm-activating peptides (SAP) speract, released by the eggs, bind to speract receptor (1) and activate a guanylyl cyclase (GC), which activates sperm motility through changes in intracellular cGMP (2), pH (3-5), cAMP (6-7) and calcium concentration (8-11). Sperm is attracted to the eggs along a gradient of chemoattractants (sperm chemotaxis). The positively selected genes (NHE and TetraKCNG) in *O. longicauda* C3 and C5 are highlighted. In the sea star *Asterias amurensis*, the SAP asterosap bind directly to the guanylyl cyclase to activate sperm motility. This is also likely the case in brittle stars, which are more closely related to sea stars compared to sea urchins. In *O. longicauda* C3 and C5, the guanylyl cyclase orthologs are divergent in the ligand-binding region (nine nonsynonymous mutations and one-four-codon gap), suggesting the existence of different chemoattractants in C3 and C5. Reproduced with permission from The International Journal of Developmental Biology (Int. J. Dev. Biol.) (2008) Vol: 52595-606.

*et al.* 2008). In addition, the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) is also involved in the signalling of the acrosome reaction (Beltrán *et al.* 2007). NHE is essential for sperm motility, as shown with NHE-null male mice which displayed severely diminished sperm motility (Wang *et al.* 2003). Yet, the exact mechanism explaining how mutations at the protein level influence the movement of ions remains to be investigated. Interestingly, positive selection was also found to occur in other sperm-specific ion channels, Catsper (1-4), which are involved in sperm motility in primates and rodents (Podlaha & Zhang 2003; Podlaha *et al.* 2005). These four channels, also found in *O. longicauda*, did not display signals of positive selection, contrary to TetraKCNG and NHE.

#### Positive selection on putative sperm motility genes: consequence of brooding evolution?

Although fertilization has not been directly observed in brooding *O. longicauda*, fertilization is very likely to occur internally, within the brooding pouches. Furthermore, all brooding females contained juveniles at a single stage of development (Weber *et al.* 2014), in favour of a single fertilization event. Several hypotheses were investigated to explain positive selection on ion channels TetraKCNG and NHE: (i) brooding conditions per se, (ii) male/female conflict, (iii) sexual selection via sperm competition and (iv) reinforcement, the most convincing hypotheses being (i) and (ii).

**Table 4** List of known genes involved in gamete recognition and fertilization in echinoderms (*Strongylocentrotus purpuratus*, *Patiria miniata* and *Asterias amurensis*) and their occurrence in *Ophioderma longicauda* C3 and C5 transcriptomes. Unless specified, BLAST was performed using sequences from *Strongylocentrotus purpuratus*

| Gene                | Function  | Contig name                           | BLAST result<br>(%ID and<br>%coverage)           | Divergence<br>C3-C5 [nt/<br>protein %] | Positively<br>selected | References                   |
|---------------------|---|---------------------------------------|--|--|------------------------|------------------------------|
| Speract             | Sperm chemoattractant from <i>S. purpuratus</i>   | C3: no hit<br>C5: no hit              | —  | —                                      | —                      | Hansbrough & Garbers (1981)  |
| Asterosap*          | Sperm chemoattractant from <i>A. amurensis</i>  | C3: no hit<br>C5: no hit              | —  | —                                      | —                      | Nakachi <i>et al.</i> (2008) |
| Speract receptor    | Guanylyl cyclase, involved in activation of sperm motility  | C3: Contig16770<br>C5: Contig35656    | 57–93  | [1/1.4]                                | No                     | Dangott & Garbers (1984)     |
| Asterosap receptor* | Guanylyl cyclase, receptor to sperm chemoattractant and activation of sperm motility                | C3: Contig16770<br>C5: Contig35656    | 72–56  | [1/1.4]                                | No                     | Nakachi <i>et al.</i> (2008) |
| Catsper1            | Sperm-specific calcium channel involved in sperm motility   | C3: Contig69549<br>C5: Contig55755    | 56–96  | [0.8/1.1]                              | No                     | Ren <i>et al.</i> (2001)     |
| Catsper2            | Sperm-specific calcium channel involved in sperm motility   | C3: Contig38361<br>C5: Contig49537    | 58–97  | [1.2/0.8]                              | No                     | Ren <i>et al.</i> (2001)     |
| Catsper3            | Sperm-specific calcium channel involved in sperm motility   | C3: Contig50793<br>C5: Contig16275    | 61–96  | [0.1/0.3]                              | No                     | Ren <i>et al.</i> (2001)     |
| Catsper4            | Sperm-specific calcium channel involved in sperm motility   | C3: Contig119271<br>C5: Contig251057  | 41–74<br>(4: against <i>Ciona intestinalis</i> ) | [0.7/1.0]                              | No                     | Ren <i>et al.</i> (2001)     |
| suREJ1              | Sperm receptor for egg jelly, involved in activation of acrosome reaction                           | C3: Contig31292<br>C5: Contig12876    | 45–19  | [1.2/2]                                | No                     | Vacquier & Moy (1997)        |
| Bindin              | Sperm protein involved in attachment with egg vitelline envelope and fusion of sperm-egg membranes  | C3: Contig72545<br>C5: Contig18807    | 62–20  | [0.5/1]                                | No                     | Vacquier & Moy (1997)        |
| EBR1                | Egg bindin receptor   | C3: Contig41363_bb<br>C5: Contig41363 | 45–4   | [1.2/1.3]                              | No                     | Kamei & Glabe (2003)         |
| Obi1 <sup>#</sup>   | Egg receptor for sperm in <i>Patiria miniata</i>  | C3: Contig63504<br>C5: Contig9261     | 39–25  | [0.5/0.3]                              | No                     | Hart & Foster (2013)         |
| ARIS1*              | Gene involved in induction of acrosome reaction in sea stars (Acrosome Reaction Inducing Substance) | C3: Contig79388<br>C5: no hits        | 63–10  | —                                      | —                      | Ikadai & Hoshi (1981)        |
| ARIS2*              | Gene involved in induction of acrosome reaction in sea stars (Acrosome Reaction Inducing Substance) | C3: Contig13709<br>C5: no hits        | 58–25  | —                                      | —                      | Ikadai & Hoshi (1981)        |
| ARIS3*              | Gene involved in induction of acrosome reaction in sea stars (Acrosome Reaction Inducing Substance) | C3: Contig69716<br>C5: no hits        | 54–44  | —                                      | —                      | Ikadai & Hoshi (1981)        |
| Rendezvin           | Gene encoding for egg proteins involved in organization of fertilization envelope                   | C3: Contig123340<br>C5: no hits       | 40–89  | —                                      | —                      | Wong & Wessel (2006)         |

\*Sequences from the sea star *Asterias amurensis* were used as query sequences.

<sup>#</sup>Sequence from the sea star *Patiria miniata* was used as query sequence.

First, positive selection may be a direct consequence of brooding. Indeed, the evolution of brooding causes important changes in the environmental conditions

surrounding the eggs. The eggs are gathered into pouches (the bursae) and are therefore present at higher densities than in the open water, and in an

environment possibly reduced in oxygen (Strathmann & Chaffee 1984). According to Strathmann & Chaffee (1984), oxygen is a limiting factor in many situations of brooding or in marine species providing brood protection, and the smaller size of such species with respect to their close relatives could be explained as an adaptation to oxygen availability (Strathmann *et al.* 1984). As a matter of fact, C3 individuals are on average larger than C5 individuals and reach also larger maximal sizes (Stöhr *et al.* 2009; Weber *et al.* 2014). If oxygen levels are lower (or limiting) in the brooding pouch, sperm with enhanced motility might have been positively selected to reach the eggs despite lower oxygen concentrations.

Second, another, indirect consequence of brooding may be an increase in the sexual conflict between males and females regarding the optimal ratio of egg/sperm encounters, due to the risk of polyspermy and its contrasted cost among sexes. This lethal event, occurring when several spermatozoa fertilize the same oocyte, might occur more often if they are present at high density (Dale & Monroy 1981; Wong & Wessel 2004). Natural selection would thus favour eggs emitting less (or less efficient) chemical attractants, thereby causing an increase in natural selection for sperm motility.

Third, sperm motility is an important component of sperm competition (Birkhead *et al.* 1999; Levitan 2000; Gage *et al.* 2004; Snook 2005). In sea stars, patterns of binding evolution are contrasted between gonochoric external fertilizers, which are subject to sperm competition, and selfing hermaphrodites (brooders or not), which are not (Patiño *et al.* 2016). Selfing hermaphrodites displayed no or low evidence of positive selection, whereas external fertilizers displayed numerous sites under positive selection. In *O. longicauda*, fertilization might occur as a single event within a given brooding chamber (cf above, and Weber *et al.* 2014). If adult behaviour ensures that sperm from a single male enters a brooding pouch, there will not be any sperm competition. However, without parentage analyses, the possibility that sperm from several males reach a brooding pouch cannot be ruled out. Indeed, multiple paternity was reported in several brooding sea urchins (Chenuil *et al.* 2004; Maturana *et al.* 2016). If sperm from several males enter a brooding chamber, competition among males may be increased due to higher gamete concentration, favouring positive selection on sperm motility genes.

Fourth, natural selection may have favoured mutations leading to prezygotic isolation between divergent populations or species to avoid wasting gametes in fertilizations producing unviable or sterile offspring, corresponding to the reinforcement hypothesis (Lessios 2011). According to this hypothesis, when two species

diverge in allopatry, their genomes become incompatible whereas their fertilization systems may remain compatible. If hybridization occurs after secondary contact, hybrid embryos may have reduced fitness or may not develop at all, wasting reproductive effort. Consequently, divergent proteins preventing heterospecific fertilization would be positively selected by natural selection. In the case of *Ophioderma*, hybrids with reduced fitness may have occurred from the two possible types of crosses (C3 sperm with C5 oocytes and C5 sperm with C3 oocytes). Yet, as the cost of gamete loss is presumably higher in the brooding species (due to the higher parental investment observed in this species), selective pressures may have been higher in brooders (particularly in genes expressed in females and eggs, but by co-evolution, also on genes expressed in males and spermatozoa), explaining why positive selection was detected in this lineage. Why positive selection would be detected in sperm motility genes, and not other reproduction genes, has no conspicuous explanation under a strict reinforcement hypothesis. The reality may be a combination of several explanations. It is likely that selection due to the confined fertilizing environment (the first or second explanations) led to rapid evolution of some sperm proteins, which contributed to accelerate prezygotic isolation between brooders and broadcasters.

#### *Sperm chemotaxis: a common mechanism of species recognition in brittle stars?*

Our results suggest for the first time that sperm motility genes could have contributed to prezygotic isolation between closely related echinoderm species. Such an isolation could also have been favoured through chemotaxis differences, as sperm chemotaxis (through the occurrence of different sperm chemoattractants) plays for example an important role in sexual selection in the mussel *Mytilus galloprovincialis* (Evans *et al.* 2012). Consistent with this, the brooding and broadcasting species likely display differences in their chemoattractants and related receptors. Indeed, we found that the sperm chemoattractant receptor of *O. longicauda* C5 displayed nine nonsynonymous mutations and a four-codon gap in its extracellular (ligand-binding) domain. In contrast, the transmembrane and intracellular domains remained conserved between *O. longicauda* C3 and C5.

Previous research showed that sperm chemotaxis is generally not species specific in echinoderms, except for brittle stars. For example, sperm of the sea urchin *Lytechinus pictus* could successfully be activated by speract chemoattractants of the species *Strongylocentrotus purpuratus* (Guerrero *et al.* 2010). In addition, a

recent study on speract and its receptor in five species of the sea urchin genus *Diadema* showed that these molecules evolve under strong purifying selection and not under diversifying selection (Jagadeeshan *et al.* 2015). Furthermore, sperm chemotaxis appeared to be mostly family specific in 19 species of holothurians (Miller 1997), as well as in six sea star species (Nakachi *et al.* 2006). In contrast, sperm chemotaxis was species specific in 10 of 15 species of brittle stars (Miller 1997) suggesting that chemoattractants are mostly species specific in brittle stars. In particular, sperm chemotaxis specificity allowed separating two unidentified species belonging to the *Macrophiothrix* genus. Therefore, mechanisms of species recognition may occur during sperm chemotaxis in brittle stars, contrasting with previous research on marine invertebrates. Research on brittle star sperm-activating peptides, associated receptor and more generally, genes involved in sperm activation should be pursued, as they might be involved in numerous speciation events in this particular class of echinoderms as in other groups of animals.

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A.A.-T.W., L.A.-R. and A.C. designed research; A.A.-T.W. performed sampling and laboratory work; A.A.-T.W., L.A.-R., N.G., A.B. and J.I.M.-B. analysed the data; A.A.-T.W., L. A.-R. and A.C. wrote the manuscript; all authors contributed to the final version of the manuscript.

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### Data accessibility

The raw transcriptome reads were deposited on the Sequence Read Archive (SRA) (Accession no.: SRP096095) under the BioProject PRJNA360063. The

assembled transcriptomes of *Ophioderma longicauda* C3 and C5 and all the alignments listed in Tables 3 and 4 are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.713q3>.

### Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** Details of sampling localities, dates and individuals used in this study.

**Table S2** BLASTP search for 37 positively selected genes between *O. longicauda* C3 and C5 that did not display GO term annotations.

**Fig. S1 A:** Neighbor-Joining tree of the mitochondrial marker COI for the five *O. longicauda* C3 individuals and two *O. longicauda* C5 individuals. **B:** Neighbor-Joining tree of the nuclear marker EF1 for the five *O. longicauda* C3 individuals and two *O. longicauda* C5 individuals.

**Fig. S2** Results of Blast2Go annotation (Level 3) of 5925 orthologs of *Ophioderma longicauda* C3 and C5 species.