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Loss of the six3/6 controlling pathways might have resulted in pinhole-eye evolution in *Nautilus*

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Coleoid cephalopods have an elaborate camera eye whereas nautiloids have primitive pinhole eye without lens and cornea. The *Nautilus* pinhole eye provides a unique example to explore the module of lens formation and its evolutionary mechanism. Here, we conducted an RNA-seq study of developing eyes of *Nautilus* and pygmy squid. First, we found that evolutionary distances from the common ancestor to *Nautilus* or squid are almost the same. Although most upstream eye development controlling genes were expressed in both species, six3/6 that are required for lens formation in vertebrates was not expressed in *Nautilus*. Furthermore, many downstream target genes of six3/6 including crystallin genes and other lens protein related genes were not expressed in *Nautilus*. As six3/6 and its controlling pathways are widely conserved among molluscs other than *Nautilus*, the present data suggest that deregulation of the six3/6 pathway led to the pinhole eye evolution in *Nautilus*.

Coleoid cephalopods, such as squid, octopus and cuttlefish, have the most sophisticated eye among invertebrates; that is, a camera eye, which is almost equivalent to the vertebrate camera eye in terms of function. *Nautilus*, (subclass Nautiloidea) often referred to as a living fossil, diversified from the ancestral cephalopods 415.0 ~ 452.6 million years ago[1–3]. *Nautilus* does not possess a camera eye but rather, a pinhole eye, which does not have lens or cornea. The eye can be subdivided into modules such as photoreceptor cells, retina, lens and cornea. The pinhole eye of *Nautilus* provides a unique example to explore how these modules evolve. Other molluscs or the ancestor of cephalopods did not have an elaborate camera eye, although some molluscs have a primitive camera eye-like structure[4]. Thus, it is of interest to clarify the ancestral structure of cephalopods eyes and the processes involved in the evolution of nautiloids and coleoid cephalopod eyes.

There are three possible scenarios to explain cephalopod eye evolution. Firstly, the cephalopod ancestor once acquired a camera eye, but *Nautilus* lost the lens and cornea during evolution. In this scenario, the loss of lens developmental processes might be the cause of pinhole eye evolution. Animals have commonly lost tissues and organs while adaptating to their environment by the loss of a corresponding gene regulatory network[5]. Secondly, a cephalopod ancestor acquired a pinhole eye, then, coleoid cephalopods added a lens and cornea as an upgrade of their visual system[6]. As paleontological evidence suggests, ancestral cephalopods, such as ammonites, had eyes without a lens[7], thus this argument appears persuasive. Thirdly, an ancestor possessed a more primitive eye than either the pinhole or camera eye. Then, the descendant lineages acquired different eye forms independently, such as the pinhole eye in nautiloids, and the camera eye in coleoid cephalopods. Molluscs other than cephalopods are known to have various types of eyes, such as a cup eye (e.g. some shell fishes), lens eye, (e.g. chitons) mirror eye, (e.g. scallops) and compound eye (e.g. ark clams), but no pinhole eye or camera eye[4]. This has led to the belief that various eye forms can be evolved independently in different species, nevertheless, molecular studies, especially conservation of the pax6 regulatory pathway has also clearly pointed out to a conserved mechanism that underlies eye evolution[8–10].

We addressed these evolutionary issues of the cephalopod eye, by analyzing gene expression during eye development in *Nautilus* and pygmy squid. Even though several transcriptome studies using cephalopods have been reported, only a few papers have used developmental stages of the embryo and none of them have focused on comprehensive transcriptome studies[11–13]. In this study, we compared gene expression in the nautiloid embryonic

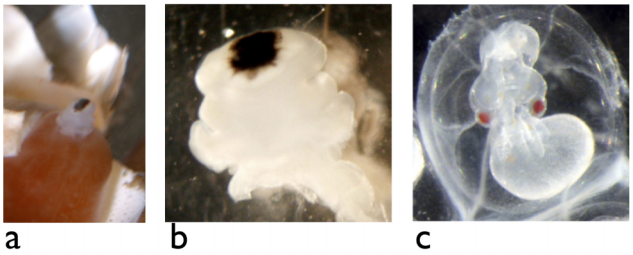

Figure 1 | Embryos of *Nautilus* and baby squid used in this study. (a,b) The *Nautilus* pinhole eye in two different magnifications at 30 days of development, (c) baby squid at stage 24.

eye and that in the coleoid cephalopods embryonic eye, to investigate gene regulatory networks involved in eye formation. For nautiloids, we utilized 30-day-old *Nautilus* embryos from Toba aquarium. For coleoid cephalopods, we utilized stage 24 embryos of Idiosepius paradoxus, the pygmy squid, which is only 1 cm in length, and is easy to maintain and breed in laboratory conditions (fig. 1). It was hoped that transcriptome comparisons of the developing eye in *Nautilus* and squid would pinpoint to genes and regulatory networks that led to their evolution. Comparative analysis of these genes and networks could clarify the evolutionary process underlying their eye development. Our data provide the first molecular framework that could account for the lack of lens and cornea in the *Nautilus* eye.

Results

The transcriptome of *Nautilus* and Squid developing eye. Even though *Nautilus* and squid belong to the same class, Cephalopoda, *Nautilus* display relatively primitive morphological characters in comparison to those of squid, such as a protective shell and pinhole eye. In this study, we explored the transcriptome of the developing embryonic eye of *Nautilus* and squid using an Illumina GAII sequencer, which produces shorter but far more numerous sequence reads. We have sequenced 79 mega (M) and 91 M reads from 75 bps pair end libraries for *Nautilus* and squid (fig. S1). In addition, we have added sequence reads obtained from Roche FLX sequencers, which produce longer but less numerous sequence reads. We obtained 120 K and 50 K reads from single libraries for *Nautilus* and squid (fig. S1). For de-novo assembly, we first removed low-quality data and bacterial contamination. Next, we assembled the Illumina GAII short reads using the Trinity, the RNA-assembler[14]. We then merged the Trinity-assembled GAII data with the Roche FLX data, and further assembled the data using Newbler software provided by Roche. Using this hybrid *de novo* assembly pipeline, we obtained 141,774 and 50,811 contigs for *Nautilus* and squid with gene expression frequencies, respectively (fig. S1)[15].

Expression frequencies and RNA-seq coverage. To assess the variety of genes in *Nautilus* and squid, we counted the number of unique homologs from *Nautilus* and squid contigs found by homology search against Non-Redundant protein database of NCBI (NR). From this analysis, 141,774 *Nautilus* contigs were matched to 13,772 genes in the NR database, whereas 50,811 squid contigs were matched to 5,606 genes. Similarly, when compared to another mollusc genome (that of pearl oyster), we found that there are 14,597 and 4,761 pearl oyster genes that are homologous to *Nautilus* and squid, respectively (table 1). Numbers of homologous genes with human, fly, lancelet, sea anemone and *Ciona* genomes are also presented in the same table (table 1). In table 2, we also show comparisons with several EST databases from human brain, as well as from *Aplysia*, *Lottia* and scallops. Additionally, in table 3, we present comparisons with GO related to eye development, visual perception and lens development.

Evolutionary rate of homologous gene pairs between *Nautilus* and squid. It is known that molecular evolutionary rates differ among species, and might affect species specific traits and phenotype. If the evolutionary rate is not uniform between *Nautilus* and squid, the difference of evolutionary rates might reflect the different evolutionary process of *Nautilus* and squid eyes. To assess this problem, we calculated the distribution of the evolutionary distances of homologous gene pairs as a function of the Neighbour-Joining (NJ) distance (fig. 2). This result suggests that evolutionary distances from the ancestor are almost the same between *Nautilus* and squid.

Genes and gene regulatory networks for eye formation in *Nautilus* and squid. Since the main aim of our study was to gain some insight or knowledge pertaining to the absence of the lens in *Nautilus*, we first examined for the presence of the main structural constituents of the lens, which are crystallins. Genes for crystallins are known to be recruited from enzyme-encoding genes and original genes are different in each species. Cephalopods use glutathione S-transferase (GST) as S-crystallin, and aldehyde dehydrogenase/intermediate filament protein as Omega-crystallin[16,17]. These genes tend to be highly expressed in the eye for rapid metabolism in lens. As a result, we found three copies of glutathione S-transferase highly expressed at the FPKM of 24.4, 21.7, 19.5 in squid, and we found none in *Nautilus* (table 4). There are two GST homologs in *Nautilus*, but they do not possess a conserved consensus S-crystallin domain, indicating they are GST-like proteins but not S-crystallin (fig. 3). These results indicate that the loss of transcriptional factors that regulate crystallin gene expression might be the reason for no crystallin and thus for no lens formation in *Nautilus*. Thus we proceeded to examine expression of "Eye Field Transcription Factors (EFTFs)" in our datasets.

EFTF genes are known to be important for the specification of eye fields and the development of the eye and eye substructures in vertebrates[8,18,19]. In invertebrates, the functions of a few EFTF genes have already been surveyed, but most EFTF genes have not been well studied[20–23]. We found otx2, pax6, and lhx2 in both *Nautilus* and squid. Tbx3 and rx1 are only found in *Nautilus*, whereas Six3/6 is

Table 1 | Homology search against various databases. Homology search against Non-Redundant protein database of NCBI (NR), Human all proteins, pearl oyster all proteins, fruit fly all proteins, lancelet all proteins, sea anemone all proteins and ciona all proteins

Databases		Nautilus		Squid			
DB	# Genes in db	Unique queries	Unique subjects	Unique queries	Unique subjects	Common genes	Squid specific genes
NR	-	17,893	13,772	6,700	5,606	1,611	3,995
Human	37,151	15,682	7,239	5,418	3,518	2,930	588
Pearl Oyster	72,597	14,597	7,954	4,761	3,474	2,623	851
Fly	21,899	12,628	5,493	4,301	2,807	2,484	323
Lancelet	50,817	16,101	8,793	5,439	3,814	2,923	891
Sea anemone	27,273	14,315	6,755	4,706	3,212	2,696	516
Ciona	19,858	12,515	6,224	4,325	2,905	2,309	596

Table 2 | Homology search against various databases. Homology search against expressed genes of scallops, aplysia, lottia, human brain, and human eye

Databases		Nautilus		Squid			
DB	# Genes in db	Unique queries	Unique subjects	Unique queries	Unique subjects	Common genes	Squid specific genes
hotate EST	3,834	336	117	138	76	58	18
Aplysia EST	256,289	10,097	6,600	4,741	3,194	1,656	1,538
Lottia EST	252,091	12,188	8,466	4,222	3,574	1,870	1,704
Human brainEST	511,370	13,057	9,568	4,900	3,974	1,700	2,274
Human NEIB	200,876	10,993	7,122	3,974	3,188	1,744	1,444

Table 3 | Homology search against various databases. Homology search against Fly and Human GO classification related to eye development

Databases		Nautilus		Squid			
DB	# Genes in db	Unique queries	Unique subjects	Unique queries	Unique subjects	Common genes	Squid specific genes
Fly GO eye development	452	2,121	226	715	176	166	10
Human GO eye development	479	1,981	178	506	126	117	9
Fly GO visual perception	41	598	28	132	19	18	1
Human GO visual perception	260	1,688	136	387	74	72	2
Human GO lens development	41	454	39	107	25	23	2
Human brain H-angel	896	3,894	531	1,086	348	329	19

only found in squid (table 4). Among these EFTF genes, lhx2 and Six3/6 are involved in lens formation in animals.

Six3 and six6, which were duplicated from the ancestral form of the six gene at the time of divergence of vertebrates, are also known to be involved in lens development in vertebrates. Liu et al. (2006) confirmed that the loss of Six3 in mice interrupted the lens development process[24]. Also, Six3 has been shown to be a major player in lens induction. Oliver et al. (1996) have shown that ectopic expression of Six3 leads to ectopic lens induction in fish[25] and Grogg et al. (2005) showed that ectopic expression of Six3 induces lens regeneration from the ventral iris in newts[26]. In squid, six3/6, the ancestral gene of Six3 and six6, was expressed at the FPKM of 1.9 ~ 2.1, but there is no expression in *Nautilus*. As other EFTF genes were found in *Nautilus* with the FPKM of 1.3 ~ 23.1, six3/6 is likely not expressed in *Nautilus*. Six3/6 is widely conserved and expressed not only in squid but also among molluscs[27], suggesting lineage-specific loss of six3/6 expression in *Nautilus*. To confirm that *Nautilus* has no six3/6 expression and squid has authentic six3/6 expression, we performed phylogenetic analysis of the squid six3/6 gene and *Nautilus* six3/6 like gene (NP_contig88602; NP represents *Nautilus* contig) that are related to six gene family. NJ tree confirms that the squid six3/6 homolog has been clustered with human six3 and six6, but the *Nautilus* gene (NP_contig88602) is clustered with six5 genes and PS_contig06570 (PS represents squid contig) (fig. 4a). Nautilus has an ancestral Six4/5 gene only, and there is no Six1/2 or Six3/6 orthologs expressed in eye development (fig. 4a). Also we confirmed that the squid six3/6 homolog (PS_contig13865) was expressed at the lentigenic cells where lens protein is expressed (fig. 4b).

Sox, Nkx2, Fox, POU and TGIF transcriptional factors have been shown to work cooperatively with Pax-6 on lens formation of vertebrates[28–30]. Among major soluble signal proteins, BMP2/4 and BMP3

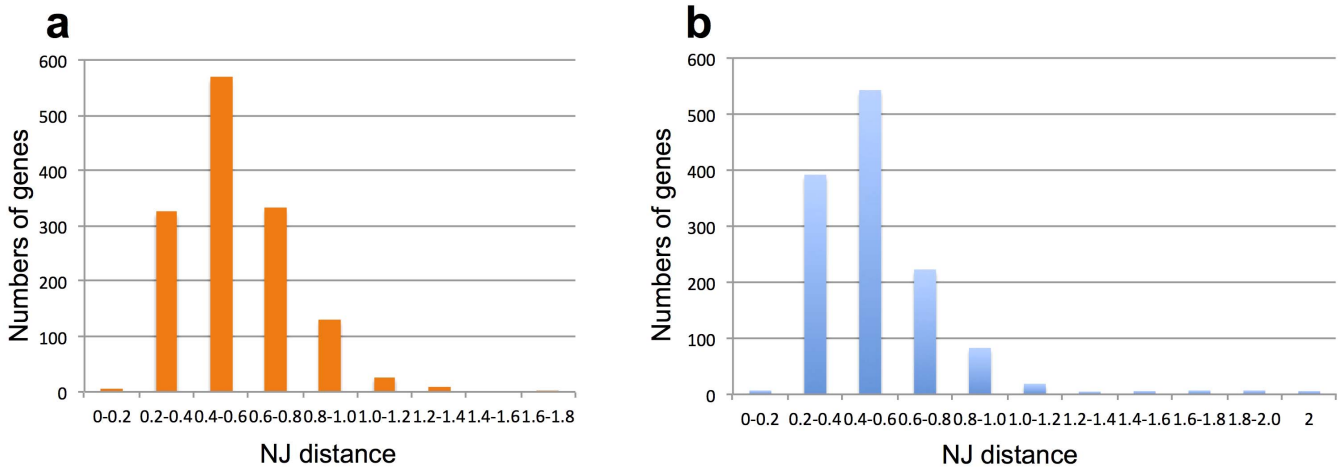

Figure 2 | Genetic distances between homologous genes of squid or *Nautilus*, and *Aplysia*. (a) Histograms of genetic distance of homologs between squid and *Aplysia* and (b) between *Nautilus* and *Aplysia*.

Table 4 | Expression of eye field transcription factor genes and lens protein coding gene. The table shows that homologous genes of the molluscs to eye field transcriptional factors (EFTFs) of the vertebrates and S-crystallin of the cephalopods. NP contig ID and PS contig ID represent isoforms matched to each gene. FPKM represents the fragment per kilobases per million reads that shows relative abundance of gene expression. Left three column represents the presence of homologs in closely related species

Gene Name HIX ID	NP contig ID	NP FPKM	PS contig ID	PS FPKM	Aplysia EST	Limpet EST	Pearl oyster genome
Pax6 HIX0009529.18	comp4923_c0_seq1	15.4	comp7037_c0_seq1	2.6	-	FC563366	pfu_aug1.0_8418.1_67856.t1
	comp4923_c0_seq2	4.9					
	comp4923_c0_seq3	2.6	comp7037_c0_seq2	22.9			
	comp5935_c0_seq1	23.1	comp7037_c0_seq3	2.7			
	comp21022_c1_seq1	2.3	comp7580_c0_seq1	29.0			
	comp21022_c1_seq2	2.8	comp100382_c0_seq1	1.5			
rx1/Rax1 HIX0202674.1	comp265385_c0_seq1	1.8	-	-	EB227521	-	-
Tbx3/ET HIX0011028.16	comp182732_c0_seq1	2.6	-	-	EB253303	FC583587	pfu_aug1.0_294.1_36469.t1
	comp256561_c0_seq1	2.4					
tailless/Nr2e1 HIX0006116.14	comp109275_c0_seq1	2.2	comp205035_c0_seq1	2.8	AY327135	FC592666	pfu_aug1.0_4198.1_37830.t1
			comp248187_c0_seq1	2.6			
			comp274669_c0_seq1	2.5			
	comp118662_c0_seq1	2.8	comp300144_c0_seq1	1.5			
	comp154975_c0_seq1	2.4	comp300144_c0_seq2	1.4			
	comp224412_c0_seq1	2.2	comp303977_c0_seq1	1.3			
	comp342153_c0_seq1	1.8	comp303977_c0_seq2	1.2			
Six3/Six6 HIX0029857.12/ HIX0037682.12	-	-	comp182859_c0_seq1	2.1	EB275185	FC753769	pfu_aug1.0_806.1_22307.t1
			comp182859_c0_seq2	1.9			
Lhx2/9 HIX0008367.14/ HIX0001444.16	comp60271_c0_seq1	2.8	comp451347_c0_seq1	0.7	EB350439	FC764248	pfu_aug1.0_8005.1_53075.t1
	comp96799_c0_seq1	2.6					
	comp96799_c0_seq2	1.3					
	comp96799_c0_seq3	1.6					
	comp264946_c0_seq1	2.4					
	comp343477_c0_seq1	2.0					
Otx1/2 HIX0011692.15/ HIX0002090.13	comp180575_c0_seq1	2.7	comp128859_c0_seq1	2.0	EB253122	FC696152	pfu_aug1.0_595.1_65534.t1
			comp128859_c0_seq2	4.6			
	comp433132_c0_seq1	1.3	comp150711_c0_seq1	3.4			
			comp150711_c0_seq2	3.8			
S-crystallin/GST AAA91343	-	-	comp14159_c0_seq1	24.4	-	-	-
			comp137735_c0_seq1	3.8			
			comp122201_c0_seq1	0.9			
			comp26952_c0_seq1	21.7			
			comp12240_c0_seq2	21.0			
			comp12240_c0_seq3	19.5			
			comp122201_c0_seq2	1.4			
			comp154828_c0_seq1	2.5			
			comp128004_c0_seq1	3.4			

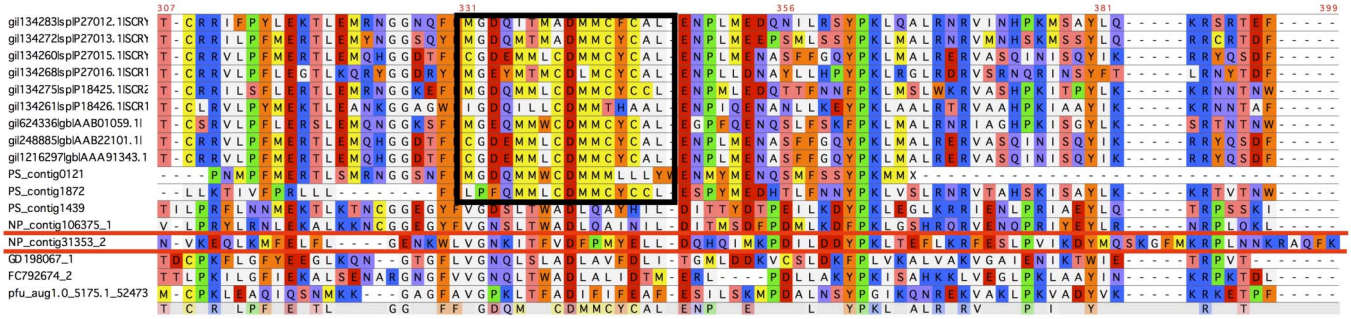

Figure 3 | Sequence alignment of S-crystallin (GST) genes. The alignment indicates that coleoid cephalopods possess the characteristic consensus sequences domain of S-crystallin indicated by the box. Red underlines represent GST homologs in *Nautilus*.

showed expressions in the embryonic squid eye but not in *Nautilus* pinhole eyes (table 5). Prompted by the interesting result that six3/6 was not expressed in pinhole eye, we then checked for expression of genes downstream of six3/6, which are known to be related to lens formation, such as: SoxB2, Nkx2, FoxB, FoxN1/4, PouIII, TGIF, BMP2/4, BMP3. All of these genes were expressed in squid at FPKM of 1.1 ~ 6.7, but not expressed in *Nautilus* at all (table 5). These genes are functional when they interact with the Pax6/Six3 complex for the formation and maintenance of lens vesicles[31]. Among them, SOXB2, Nkx2, FoxB, FoxN1, POU3, and TGIF transcription factors work cooperatively with Pax6 during lens formation in vertebrates. We have not found differences in expression of FGFs and Wnts, which are involved in lens formation but also expressed in other processes, such as retina differentiation. Our results, thus, pinpoint the six3/6 regulatory network as the prime culprit for no lens and cornea formation in *Nautilus*, which resulted in pinhole eye evolution (fig. 5). Such results could provide the impetus for further studies in the evolution of pinhole eyes in other animals and to understand the role of Six3/6 in eye development across species.

Discussion

The number of contigs and unique genes is larger (almost triple) in *Nautilus* than in squid. One possibility for the higher number of contigs in *Nautilus* is that the coverage of RNA-seq reads is too small to construct full-length genes for *Nautilus*, and resulted in gapped fragments from the same gene. As the total reads of *Nautilus* and squid RNA-seq are almost equal, difference of numbers of contigs could come from the insufficient coverage of RNA-seq. To assess this problem, we utilized the two following approaches. First, if a small number of genes are highly expressed and occupy sequence reads, low-expression genes tend to be missed in RNA-seq data. We, therefore, checked the distribution of gene expression frequencies for each species. We counted "Fragments Per Kilobase of transcript per Million mapped reads (FPKM)"[15], for each gene and drew a distribution graph (fig. S2). The average FPKM for *Nautilus* is higher than that for squid, and the mean FPKM for *Nautilus* is smaller than that of squid. This result indicates that the proportion of highly expressed genes is larger in *Nautilus*, and RNA-seq coverage is slightly better in squid. Second, to assess the influence of RNA-seq coverage to find lowly expressed but important genes, such as transcription factors, we performed the following tests. We searched for EFTFs that are essential for eye development in vertebrates. We searched for otx2, tbx3, pax6, and lhx2 in our RNA-seq data, as these genes are already known to be involved in eye development in molluscs. As a result, all genes were found to be expressed with a FPKM of 1.7 ~ 2.5. These FPKMs do not differ between *Nautilus* and squid. Then, we assembled contigs using 1/4, 1/2, 3/4 random data sets of sequence reads, and counted the number of unique homologous genes in the human eye EST database. As shown in fig. S3, the number of unique homologs obtained at 3/4 data is equivalent to that at the complete data set, indicating that the variation in genes is almost saturated at the 3/4 dataset level. In conclusion, despite differences in RNA-seq

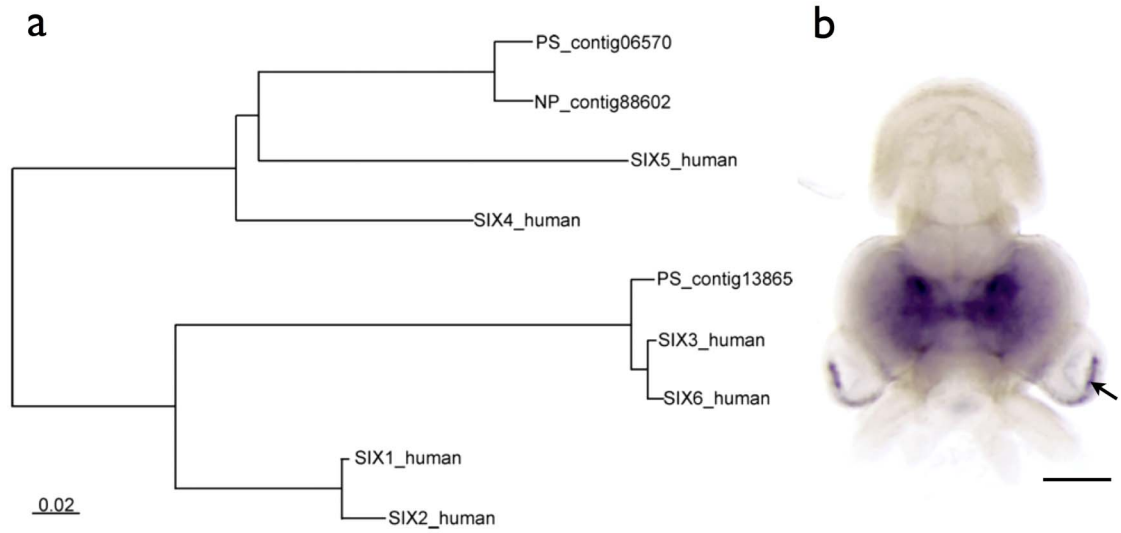

Figure 4 | Phylogenetic analysis and gene expression analysis of six3/6-like genes. (a) Phylogenetic tree shows that the *Nautilus* six3/6-like gene (NP_contig88602) is clustered with human six5 and squid PS_contig06570, while the squid PS_contig13865 is clustered with human six3/6 genes. (b) squid six3/6-like gene was expressed at lentigenic cells where lens protein is expressed (arrowhead). Bar = 100 μm.

Table 5 | Expression of six3/6 and downstream network. Genes downstream of six3/6 pathways were searched in *Nautilus* and squid

Gene Name	Human	Nautilus	FPKM	Squid	FPKM
SIX3/6	SIX3/HIX0029857.12	-	-	comp182859_c0_seq1	2.1
				comp182859_c0_seq2	1.9
	SIX6/HIX0037682.12	-	-	-	-
SoxB2	Sox14/HIX0030875.8	-	-	comp229006_c0_seq1	1.4
				comp243466_c0_seq1	1.3
Nkx2	Nkx2.1/HIX0011603.17	-	-	comp114549_c0_seq1	6.7
				comp140806_c0_seq1	4.1
				comp173024_c0_seq1	3.9
				comp226298_c0_seq1	2.5
FoxB	Fox B1/HIX0038075.12	-	-	comp369806_c0_seq1	1.6
				comp433872_c0_seq1	1.2
FoxN1/4	Fox N1/HIX0039248.12	-	-	comp45891_c0_seq1	1.1
PouIII	POU3F4/HIX0028415.13	-	-	comp235306_c0_seq1	2.7
TGIF	Tgif/HIX0014308.15	-	-	comp142128_c0_seq1	4.7
				comp142634_c0_seq1	3.6
BMP2/4	HIX0011668.15	-	-	comp211559_c0_seq1	2.4
BMP3	HIX0031518.12	-	-	comp95586_c0_seq1	5.9
				comp124917_c0_seq1	4.6

coverage, our data can be used to detect lowly expressed genes in both species.

From our results, downstream genes and networks of the Pax6/Six3 complex appear to have been lost in *Nautilus* due to the loss of six3, resulting in the inactivation of the lens formation process during *Nautilus* evolution. Thus our data support the first scenario presented in the introduction; that most likely the *Nautilus* lineage lost its lens and cornea and that its pinhole eye might have evolved from a camera-type eye by deregulation of a particular regulatory network, in this case the six3/6 one, which is well conserved from the common ancestor of cephalopods and vertebrates. Furthermore our approach and results strongly argue that whole transcriptome studies are quite useful to delineate the mechanisms or evolution of the eye. Also, our results indicate that despite a common master gene for eye evolution, perturbance of downstream networks and factors might account for the diversity of eye types during evolution.

Methods

Sample collection, mRNA extraction, and sequencing. Specimens of the pygmy squid, *Idiosepius paradoxus* were collected from Chita Peninsula, Nagoya, Japan. Eggs of *Nautilus pompilius* were obtained from Toba aquarium, Mie, Japan. All experiments were performed in accordance with relevant guidelines and regulations in Ochanomizu University. Total RNA was extracted from RNAlater-fixed tissue using the Molluscan RNA kit (Omega biotech) with on-column DNase digestion according to the protocol described in our previous paper[32]. For long-read sequencing, cDNAs were fragmented into 500–800 bp using a GS FLX Titanium Rapid Library Preparation Kit (Roche) according to the manufacturer's protocol. These libraries were sequenced by staff at the Functional Genomics Center Zurich and the Institute of Plant Biology, University of Zurich. The fragments were then amplified on beads by emulsion polymerase chain reaction, and the amplified fragments in each cDNA library were pyrosequenced on a 1/2 section of pico-titerplate (one plate in total) using the 454 GS FLX Titanium system and reagents (Roche). Primer sequences of the 454 reads were trimmed from all reads prior to the subsequent analysis. The sequence cleanup program Lucy was used to trim low-quality sequences from the raw data[33]. mRNA from *Nautilus* and squid embryos was also used for short-read sequencing by Illumina GAII. cDNAs were first amplified, and then fragmented to 150 bps and used for construction of pair-end libraries. Sequencing of the *Nautilus* and squid libraries were performed by Cofactor Genomics inc. Files containing the sequences and quality scores have been deposited at the DDBJ Sequence Read Archive (ID:DRA000453).

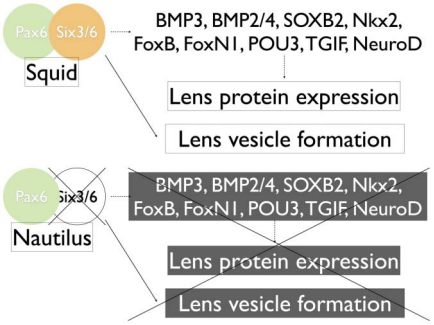

Figure 5 | Model for lens loss in *Nautilus*. Loss of six3/6 cause no lens and cornea formation in *Nautilus*.

RNA-seq assembly. For the hybrid assembly of Illumina data and GS FLX data, we first assembled short reads from Illumina GAII using Trinity software. As Newbler 2.6 does not accept reads with more than 2000 bps, we then assembled Illumina GAII contigs (<2000 bps) and GS FLX reads by Newbler 2.6 software. Larger contigs produced by Trinity were then merged with the contigs by Newbler.

Homology search. Homology searches were performed against several protein databases by using blastx (implemented in Blast + ver. 2.25). Nr database (as of September 9, 2011) was downloaded from the NCBI. We used all H-Invitational transcripts (HITs) as human all protein data set. Since the data set includes alternative splicing products, unique genes were counted based on H-Inv cluster ID (HIX). We obtained all protein data set of lancelet (*Branchiostoma floridae* v1.0), sea anemone (*Nematostella vectensis* v1.0) and *ciona* (*Ciona intestinalis* v2.0) from the JGI. All protein data set of fly (BDGP5.25.64) was obtained from Ensembl. Predicted Proteins of the pearl oyster were obtained from Marine Genomics Unit, OIST, Japan (Pinctada fucata Genome Ver 1.00). The scallop ESTs were obtained as previously described in Yoshida et al.[13]. *Aplysia* and *Lottia* EST sequences were obtained from the NCBI dbEST. For comparison with the vertebrate eye and brain EST data, we used data from the NCBI, H-invDB and NEI Bank. Human brain EST data was obtained from the NCBI dbEST. We obtained human-eye ESTs of cornea (NbLib0003, NbLib0077) fovea (NbLib0004), ciliary body (NbLib0006), corneal_stroma (NbLib0008), trabecular_meshwork (NbLib0009), fetal_cochlea (NbLib0010), cochlea (NbLib0011), retina (NbLib0013, NbLib0129), lacrimal_gland (NbLib0054, NbLib0076), fetal_eye (NbLib0065, NbLib0124), lens (NbLib0068), optic_nerve (NbLib0069 NbLib0119), RPE_choroid (NbLib0072), whole_eye (NbLib0079, NbLib0132), pterygium (NbLib0106), conjunctiva_epithelium (NbLib0108) from the NEIbank. We also used human brain ESTs obtained from the H-angel. Eye development related genes were downloaded from the UniprotKB. For discoveries of evolutionary conserved genes of cephalopod eyes we chose genes related to GO: eye development (GO:0001654), GO: visual perception (GO:0007601) and GO: lens development (GO:0002088). GO: lens development is a part of GO: eye development.

Homologous gene pair estimation and genetic distance. Homologous gene pairs were selected using TBLASTX searches against the Aplysia EST dataset obtained from the NCBI dbEST (Taxonomy ID: 6500) with default settings, an E-value cutoff of 1e-10, and an amino acid length longer than 30 aa. NJ genetic distances were calculated using the clustalw with Kimura 2-parameter setting as this setting was widely used for NJ distance calculation. The average distances of two species were not statistically significant (average 0.556 ± 0.200 versus 0.537 ± 0.259, Student t-test, $P > 0.05$).

In situ hybridization. To generate six3/6 DIG-labeled RNA targeted probes, we performed RT-PCR based on six3/6 homologous contig in the pygmy squid. RT-PCR fragments obtained from previous primers were sub-cloned into T-vector (Promega)

and used as templates for in vitro transcription. Whole-mount in situ hybridization was performed according to the previously published protocol[34].

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Author contributions

A.O. and P.A.T. designed research; T.M. and M.Y. performed experiments for sample collection and library construction; K.S., J.S., and K.S. contributed to sequencing; A.O., Y.O., and M.Y. performed research; and A.O., P.A.T wrote the paper.

Additional information

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