

The *Dugesia ryukyuensis* Database as a Molecular Resource for Studying Switching of the Reproductive System

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The planarian *Dugesia ryukyuensis* reproduces both asexually and sexually, and can switch from one mode of reproduction to the other. We recently developed a method for experimentally switching reproduction of the planarian from the asexual to the sexual mode. We constructed a cDNA library from sexualized *D. ryukyuensis* and sequenced and analyzed 8,988 expressed sequence tags (ESTs). The ESTs were analyzed and grouped into 3,077 non-redundant sequences, leaving 1,929 singletons that formed the basis of unigene sets. Fifty-six percent of the cDNAs analyzed shared similarity (E -value $< 1 E^{-20}$) with sequences deposited in NCBI. Highly redundant sequences encoded granulin and actin, which are expressed in the whole body, and other redundant sequences encoded a Vasa-like protein, which is known to be a component of germ-line cells and is expressed in the ovary, and Y-protein, which is expressed in the testis. The sexualized planarian expressed sequence tag database (<http://planaria.bio.keio.ac.jp/planaria/>) is an open-access, online resource providing access to sequence, classification, clustering, and annotation data. This database should constitute a powerful tool for analyzing sexualization in planarians.

Key words: planarian, EST analysis, sexualization, asexual-sexual switch, gene ontology

INTRODUCTION

Living organisms have established unique reproductive systems to multiply and sustain their species. Two types of reproduction evolved, namely, asexual and sexual. Asexual reproduction is much simpler than sexual reproduction, and it produces offspring that are genetically identical to the parent and in which no genotypic variation occurs. In contrast, sexual reproduction reshuffles genes and results in offspring genetically different from parents and siblings. Hence, con-

siderable genotypic variation occurs in the case of sexual reproduction (Bell, 1982).

It is well known that planarians possess remarkable regenerative abilities requiring the activity of neoblasts, which are the only known proliferating cells (Agata and Watanabe, 1999; Orii *et al.*, 2005; Agata *et al.*, 2006). It is also known that some planarians switch between asexual and sexual reproduction in response to environmental signals (Curtis, 1902; Hyman, 1939). To understand the mechanism of switching reproductive systems in planarians, we established an experimental bioassay system for sexual switching. *Dugesia ryukyuensis* of the OH strain, which have been maintained under laboratory conditions for more than 15 years through asexual reproduction, were induced to reproduce sexually by feeding them sexually mature individuals of *Bdel-*

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locephala brunnea, an exclusively oviparous species (Kobayashi *et al.*, 1999). Following induction, the *D. ryukyuensis* OH strain gradually developed sexual organs over time, and after a given point (the point-of-no-return), could not return to an asexual state (Kobayashi and Hoshi, 2002).

To understand the selection of reproductive systems and the differentiation of sexual organs, we isolated the key genes that function in sexual induction and that are expressed specifically during the sexualizing process. We detected the gene *Dryg*, which is expressed specifically in

yolk glands of worms after the point-of-no-return (Hase *et al.*, 2002). *Dryg* expression during the sexualizing process is limited to a single cell type possessing characteristics of neoblasts, which are totipotent somatic cells. We also detected other genes expressed in sexual organs (unpublished observations). However, the eventual goal is to identify all genes involved in sexual induction.

To obtain a comprehensive catalog of genes responsible for sexual induction, we are currently conducting a large-scale EST analysis of cDNA obtained from *D. ryukyuensis*.

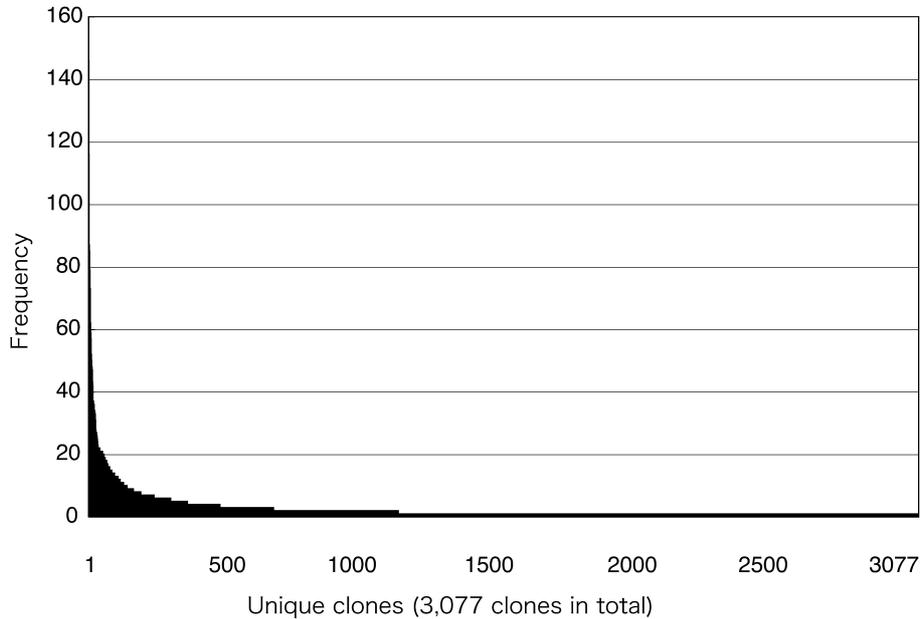


Fig. 1. Gene-expression profile in sexualized planarians (*D. ryukyuensis*). Frequency of occurrence is plotted for each non-redundant clone. Most (63%) of the clones were expressed as singletons.

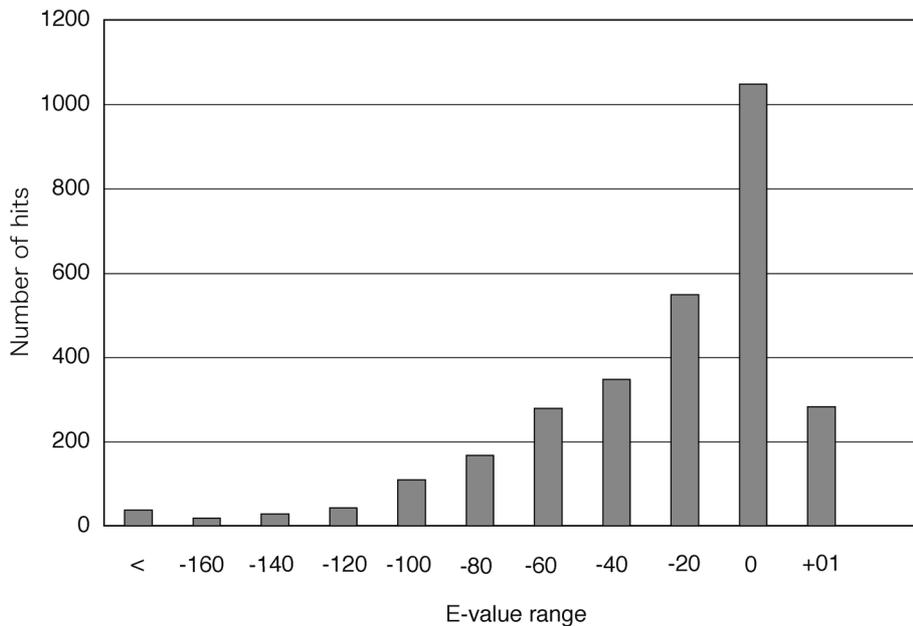


Fig. 2. BLASTX analysis of genes from sexualized planarians (*D. ryukyuensis*). The distribution of the BLASTX matches is according to *E* value. The number of ESTs for the *E*-value ranges is indicated above each bar.

Here, we describe this database and provide an overview of the results obtained thus far. Updated results are available on the website (<http://planaria.bio.keio.ac.jp/planaria/>).

lished by Dr. S. Ishida (Hirosaki University, Aomori, Japan). This strain was named the OH strain. The animals were maintained at 20°C

MATERIALS AND METHODS

Animals

A clonal line of the asexual strain of *D. ryukyuensis* was estab-

Construction of a cDNA library

The OH strain was fully sexualized by using the feeding procedure of Kobayashi *et al.* (1999). Total RNA was extracted from more than 30 fully sexualized planarians. Poly (A) RNA was purified using

Table 1. High-ranking redundant clusters of EST sequences.

cluster_id	length	num	representation	id	description	E-value
DrC_00023	665	146	Dr_sW_001_I17	gb AAP44511.1	progranulin-b [<i>Danio rerio</i>]	6.00E-26
DrC_00499	1433	116	Dr_sW_013_D21	dbj BAC44866.1	actin [<i>Galaxea fascicularis</i>]	<1.0E-170
DrC_00802	759	87	Dr_sW_020_K06	embl CAD21525.1	hypothetical protein [<i>Taenia solium</i>]	1.50E-02
DrC_00591	1065	85	Dr_sW_015_H08	ref NP_571273.1	hatching gland gene 1 [<i>Danio rerio</i>]	3.00E-91
DrC_00208	1418	79	Dr_sW_006_C12	gb AAA29882.1	fimbrin	2.00E-43
DrC_00505	2152	73	Dr_sW_013_F10	dbj BAD12572.1	heat shock protein [<i>Numida meleagris</i>]	<1.0E-170
DrC_00493	607	66	Dr_sW_013_C02	embl CAB62280.1	hydroxyproline-rich glycoprotein DZ-HRGP [<i>Volvox carteri f. nagariensis</i>]	3.30E-01
DrC_00181	853	62	Dr_sW_005_J06	dbj BAA25398.1	CsCA1 [<i>Ciona savignyi</i>]	3.00E-27
DrC_00521	1174	59	Dr_sW_013_K05	gb AAC23561.1	ADP/ATP carrier [<i>Trypanosoma brucei brucei</i>]	7.00E-91

Table 2. Reproduction and regeneration-related genes in sexualized planarian EST sequences.

cluster_id	length	num	representation	id	description	E-value
DrC_00023	665	146	Dr_sW_001_I17	gb AAP44511.1	progranulin-b [<i>Danio rerio</i>]	2.00E-25
DrC_00812	1085	22	Dr_sW_020_N13	embl CAA68079.1	Y-box protein [<i>Dugesia japonica</i>]DjY1	1.00E-138
DrC_00495	513	8	Dr_sW_013_C11	pir S20471	class V zygote-specific protein - <i>Chlamydomonas reinhardtii</i>	4.00E-24
DrC_00081	637	5	Dr_sW_003_B12	dbj BAB12217.1	vasa homolog [<i>Ciona savignyi</i>]	2.00E-62
DrC_00456	1591	5	Dr_sW_L10	embl AJ439094.1	Y-box protein [<i>Dugesia etrusca</i>]DeY1	1.00E-159

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DrC-00456  MADIQTSPTEERSVHATEKKILAYDYLATKINGKVKWFNVKSGYGFINRNDNQEDIFVHQT  60
DeY1      *****
DjY1      **NN*EKTD*YEGN*ADSS-KQKQL*H*G*****R***VC*****I**S

DrC-00456  AILKNNPRKWQRSVGDGEEVEFDVVEGEKGLEAANVTGPDGIPVQGSKYAADKRHYRSRS 120
DeY1      *****
DjY1      **V*S**DHPRK**E***IL**I*K*A**N*****SAI**KC*K**E**LRYPRG*G*G

DrC-00456  FGRAVGRGFGRVRSRLRNGPRGRSLSREMDSTNEDAGPEGDAVFGTERRRRLPSRRRSY 180
DeY1      *****K***K*****T*****I*****
DjY1      R*VFR**RSLVVSEEGDMHTQEFARGRGRPFRRGGRPV*QYRKPNYNDMPQQYF*DY

DrC-00456  PRPMPPPPQGGYYMNRRLPSRDDYREPDEYENRTMIMGYRGGVPRRRFIGGFGRGHA  240
DeY1      *****S*****
DjY1      RG*PRQGEDFVDEVLGGVSP*EVPKYEQFD*EMFDRRPF*R**G*GR*RSRGR*RG

DrC-00456  NGSGANTYIRGYSPPPNARRYMDSRPGPVMNFSDSGP  280
DeY1      *****I*QIQDQ
DjY1      R*SPRDNPKANENEEQHQSQQPLQD

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Fig. 3. Comparison of the deduced amino acid sequence of DrC-00456 with those of other Y-box proteins in planarians: *DeY1* in *D. etrusca* (accession no. AJ512636) and *DjY1* in *D. japonica* (accession no. AJ512635). Asterisks indicate identical residues. The multiple alignment was obtained by CLUSTAL W (1.83) multiple-sequence alignment.

an Oligotex-dT30<Super> mRNA Purification Kit (Takara, Shiga, Japan) and was converted to oligo(dT)-primed cDNAs with a cDNA Synthesis Kit with oligo(dT)18 anchor primer (Stratagene). Both ends of the cDNAs were linked to *EcoRI* adaptors. The cDNAs were digested with *EcoRI* and ligated into the Bluescript II SK(+) plasmid digested with *EcoRI* and *XhoI*. Clones containing the inserts were then selected for nucleotide sequencing.

EST sequences

The cDNAs were introduced into *E. coli* DH10B competent cells (Invitrogen) by using a Gene Pulser electroporation system (Bio-Rad Laboratories) and plated onto LB-ampicillin plates (25 cm \times 25 cm). Purified plasmid DNA was sequenced with a 3700 ABI sequencer and a Big-Dye Terminator Sequencing Kit according to the manufacturer's instructions (Applied Biosystems). The 5' end one-pass sequences were read, and a total of 10,745 ESTs were obtained. Prior to the computational analysis of ESTs, contaminating subsequences such as repeat and vector sequences were identified by a BLASTN similarity search against the UniVec vector database (<ftp://ftp.ncbi.nih.gov/pub/univec>) and were eliminated.

Classification, clustering and annotation

To control EST quality, the sequence data were processed automatically to remove the polyA tail and vector sequences. For classification, sequences obtained underwent automatic BLASTX searches against NCBI's GenBank. The redundancy of sequences was checked with the Contig Assembling Program (CAP3) (Huang and Madan, 1999). Sequences were annotated with gene ontology (GO) terms by searching against the Gene Ontology Database (Harris *et al.*, 2004).

In situ hybridization and histology

Whole-mount *in situ* hybridization was performed using the protocols described by Umesono *et al.* (1997). Sense and anti-sense riboprobes were prepared by *in vitro* transcription using RNA polymerase with digoxigenin-UTP (Roche).

RESULTS AND DISCUSSION

An overview of ESTs derived from the sexualized planarians

The asexual *Dugesia ryukyuensis* OH strain was fully sexualized by the method of Kobayashi *et al.* (1999), and a cDNA library was generated. From this cDNA library, we obtained from the fully sexualized planarians 8,988 5' EST cDNA sequences after the removal of clones with no inserts. The average length of sequences was 600 bp. Sequences longer than 100 bp were registered with GenBank (accession numbers BW632996–BW643983).

Annotation and clustering of the sexualized planarian EST sequences

To classify the sequences obtained, ESTs were subjected to a BLASTX homology search and annotated against the Uniprot / Swissprot database maintained by the European Bioinformatics Institute (EBI). Of the 8,988 sequences obtained, 4,902 exhibited high similarity (E -Value $<1E-20$) to proteins in other organisms. To eliminate sequence redundancy, EST clustering was performed using CAP3. A group of sequences was considered clustered when sequence similarity was greater than 90% (-p option) and an overlap of more than 49 bp existed (-o option). The 8,988 ESTs were assembled into 3,077 clusters consisting of 1,147 contigs and 1,930 singletons. Fig. 1 shows the frequency distribution of clusters based on the number of clusters and the

frequency of each cluster obtained. A unique identifier starting from DrC-00001 and continuing to DrC-03077 was assigned to each assembled sequence to provide annotations in a manner similar to EST. Annotations of consensus sequences are also available in the database.

These planarian EST sequences were subjected to BLASTX analysis for annotation. Of the 3,077 clusters, 1,646 (53%) were highly similar (E -value $<1E-20$) to functionally characterized genes in other organisms (Fig. 2). A total of 1,158 clusters showed low similarity ($1E-20$ $<E$ -value $<1E-$

Table3. The distribution of GO slim in the Biological Process category associated with sexualized *D. ryukyuensis* ESTs.

Cellular component		
GO slim ID	description	Number of cluster
GO:0005622	intracellular	610
GO:0005623	cell	195
GO:0005575	cellular component	46
GO:0005576	extracellular	40
GO:0005941	unlocalized protein complex	10
GO:0008372	unknown	0
total		901
Molecular function		
GO slim ID	description	Number of cluster
GO:0003824	catalytic activity	557
GO:0005488	binding	547
GO:0005198	structural molecule activity	128
GO:0003676	nucleic acid binding	127
GO:0005215	transporter activity	72
GO:0003774	motor activity	37
GO:0004871	signal transducer activity	33
GO:0030234	enzyme regulator activity	28
GO:0005554	molecular function unknown	9
GO:0030528	transcription regulator activity	4
GO:0016209	antioxidant activity	2
total		1534
Biological process		
GO slim ID	description	Number of cluster
GO:0007582	physiological process	570
GO:0006810	transport	208
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	131
GO:0007154	cell communication	68
GO:0006118	electron transport	46
GO:0006519	amino acid and derivative metabolism	35
GO:0007275	development	34
GO:0050896	response to stimulus	20
GO:0006928	cell motility	3
GO:0009405	pathogenesis	2
GO:0000003	reproduction	1
GO:0050789	regulation of biological process	0
GO:0000004	biological process unknown	0
total		1118

1), and no similarity was observed for 283 clusters.

High-ranking redundant clusters of EST sequences

It has previously been shown that highly redundant clusters usually correspond to highly expressed genes. Highly redundant clusters of ESTs for *D. ryukyuensis* are shown in Table 1. The most redundant cluster, DrC-00023, is homologous to granulin (*grn*, E -value $<2E-25$), a cytotrophic factor modulating the growth of various cells *in vitro* (Bateman and Bennett, 1998). DrC-00023 was present in 146 copies in the EST-DB, suggesting that it is very abundantly expressed. This indicates that DrC-00023 could act as a growth factor for promoting strong regeneration in planarians. Interestingly, it has been reported that *grn* precursor gene expression increases in the neonatal rat hypothalamus in response to estrogen treatment during the perinatal period (Suzuki *et al.*, 2001) and that the *grn* product functions as a hormone in the brain during sex differentiation in mammals. Other highly redundant clusters included housekeeping gene families such as actin and heat-shock proteins.

A list of highly redundant gene clusters involved in reproduction and regeneration is shown in Table 2. Cluster DrC-00812 exhibited high similarity to the planarian Y-box gene *DjY1* (E -value $<1E-138$), which is specifically expressed in regeneration blastemas in *D. japonica* (Salveti *et al.*, 1998). Cluster DrC-00456 was very similar to another planarian Y-box gene, *DeY1* (E -value $<1E-159$; Fig. 3). This gene is expressed in testicular germ cells (spermatogonia, spermatocytes, and spermatids) of the sexual planarian *D. etrusca*, whereas no expression is detected in blastemas

(Salveti *et al.*, 2002). *DjY1* is not highly similar to DrC-00456. DrC-00495 is similar to a class V zygote-specific gene (E -value $4E-13$) that functions in gametogenesis (Matters and Goodenough, 1992). DrC-00081 is similar to *Vasa* (E -value $1E-40$), which is a member of the ATP dependent helicase family expressed specifically in stem cells in many organisms and which is regarded as a stem-cell marker (Hay *et al.*, 1998; Shibata *et al.*, 1999).

Classification of genes according to their functions

Genes were classified according to gene ontology (GO). If the E -value of a sequence annotated by the BLASTX search against the Uniprot protein database was lower than $1E-15$, the sequence was annotated with corresponding GO terms by referencing the Gene Ontology Annotation (GOA) database. Additionally, GO slims, which are slimmed-down versions of GO terms, were used for summarizing the GO term distribution of GO-annotated sequences. Table 3 shows the distribution of GO slim categories of consensus sequences. The major transcripts in the EST-DB compiled here encode intracellular proteins playing catalytic roles during physiological processes. The updated results are available on the website (<http://planaria.bio.keio.ac.jp/planaria/>).

Expression patterns of high-ranking redundant clusters related to development

To identify highly redundant gene clusters specifically expressed during the sexualization process, whole-mount *in situ* hybridization was performed for the highly redundant gene clusters DrC-00023 (a granulin homolog) and DrC-

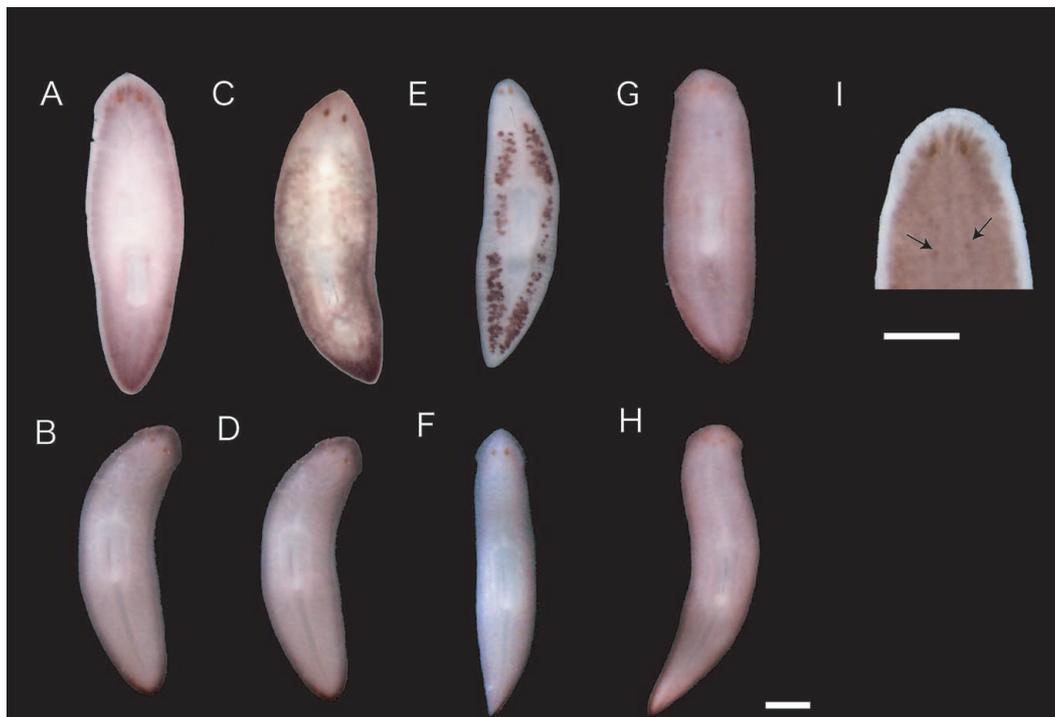


Fig. 4. Whole-mount *in situ* hybridization of highly redundant gene clusters in sexualized (A, C, E, and G) and asexual (B, D, and F) planarians (*D. ryukyuensis*). (A–D) Selected ESTs that comprise highly redundant gene clusters: (A, B) DrC-00023, a granulin-like gene; (C, D) DrC-00499, an actin-like gene. (E–I) Selected ESTs that comprise highly redundant gene clusters involved in reproduction: (E, F) DrC-00456, a Y-box protein-like gene; (G, H) DrC-00081, a vasa-like gene. (I) High-magnification view of (H). Arrows indicate the germinal-disc region of the ovary. Scale bar is 1 mm.

00499 (an actin homolog). Expression patterns of these genes are shown in Fig. 4A–D. DrC-00023 and DrC-00499 are expressed in the whole body of sexual and asexual worms, not specifically in the brain, suggesting that DrC-00023 may not function as a brain hormone during sexual differentiation. As granulin is known as a pleiotropically expressed growth factor that mediates cell-cycle progression and cell motility in mammals (Bateman and Bennett, 1998; He and Bateman, 2003), DrC-00023 may function as a growth factor in planarians.

Since DrC-00456 (*DeY1* homolog) and DrC-00081 (*Vasa* homolog) have high copy numbers in the EST-DB and high *E*-values, whole-mount *in situ* hybridization was also performed for these highly redundant gene clusters. DrC-00456 was specifically expressed in the testes of sexual worms, but not expressed in asexual worms (Fig. 4E, F). Interestingly, *DeY1* from *D. etrusca* (Salveti *et al.*, 2002) is likely a homolog of DrC-00456.

Asexual worms do not maintain genital organs such as the testis, yolk gland, etc., but exhibit small ovarian primordia with a few oogonia. DrC-00081 was expressed specifically in the ovary of sexual worms and in these ovarian primordia in asexual worms (Kobayashi *et al.*, 1999). Planarian *Vasa*-like genes, *vlg-A* and *vlg-B*, are transcribed in the ovary and testis (Shibata *et al.*, 1999). Since DrC-00081 mRNA was detected only in the ovary in sexual worms or the ovarian primordia in asexual worms, but not in the testis (Fig. 4G–I), DrC-00081 could be a novel *Vasa*-like gene.

Future directions

Several planarian databases have recently been established. An EST-DB generated from the head portion of *D. japonica* should facilitate understanding of the evolutionary divergence of the planarian central nervous system (Mineta *et al.*, 2003). EST-DBs generated from asexual and sexual stages of the hermaphroditic strain of *Schmidtea mediterranea* (Zayas *et al.*, 2005) enable a comparison of the asexual and sexual stages of planarians and the analysis of germ-cell specification. However, they cannot trace the sexualization process and are not appropriate for studying processes such as oogenesis or spermatogenesis.

In contrast, our EST-DB facilitates studies related to these processes by identifying genes activated in the switch from asexual to sexual reproductive mode. For example, microarray probes of sexualized planarians could be prepared from our EST-DB and used to analyze gene expression at each stage of sexualization. We can also classify genes according to their functions, such as sexual hormones, sexual hormone receptors, or hormone-synthesizing enzymes. This sexualized planarian EST-DB is therefore an excellent tool for studying the process of the sexualization in planarians.

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