Isolation of mixis-related genes from the rotifer *Brachionus plicatilis* using subtractive hybridization

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This paper has not been submitted elsewhere in identical or similar form, nor will it be during the first three months after its submission to *Hydrobiologia*.

Abstract

The monogonont rotifer *Brachionus plicatilis* produces resting eggs through sexual reproduction (mixis), which is affected by external and internal factors. We collected resting eggs from rotifers cultured at 15 and 25ºC and hatched them with and without 14-day dormancy in the dark. Stem females hatched from both conditions were further cultured at 15, 20 and 25ºC. We obtained two phenotypes, one with high mixis (more than 50%), which was hatched from resting eggs formed at 15ºC without dormancy, and another in which sexual reproduction occurred at 25ºC and resting eggs were formed at 15ºC with a 14-day dormancy. In the latter phenotype, mictic females appeared at 15ºC, but not at 25ºC. Using subtractive hybridization, we isolated one gene from the latter phenotype of females that had no significant similarity to known genes in BLAST searches. We propose that this gene is unique to rotifer mictic reproduction. Ongoing characterization of this gene attempts understand its role in mixis.

Introduction

The life cycle of monogonont rotifers includes both sexual and asexual reproduction, and is regulated by external and internal factors. Many studies have been conducted with the goal of inducing rotifer sexual reproduction, termed mixis, and summaries of these works have been published (Gilbert, 1974; Pourriot & Snell, 1983; Hagiwara et al., 1994; Denekamp et al., 2009). It is well documented that sexual reproduction resulting in formation of ephippia in cladoceran species is induced when the environment turns worse (D'Abramo, 1980). Cysts of *Artemia* also appear under poor environmental conditions (D'Agostino & Provasoli, 1968; Versichele & Sorgeloos, 1980). In contrast, sexual reproduction of monogonont rotifers occurs under moderate conditions (Hino & Hirano, 1984; Lubzens et al., 1985; Snell, 1986; Hagiwara et al., 1988). The optimal condition for inducing mixis coincides with that for asexual reproduction. New insight was brought by our recent finding through the treatment of stem females hatched from resting eggs. When stem females experienced starvation after hatching, this induced a higher percentage of mixis in their offspring (Hagiwara et al., 2005). An important next step is to understand the genetic basis of resting egg formation. Various studies on single gene function have been published in recent years (Wheelock et al., 1999; Kaneko et al., 2002, 2005), and increasing molecular information has been available on bdelloid rotifers (Mark Welch & Meselson, 2001; Mark Welch & Mark Welch 2005; Pouchkina-Stantcheva et al., 2007) and monogonont brachionid rotifers (Suga et al., 2007a, 2007b, 2008); however, there is almost no information on gene regulation in the brachionid rotifer life cycle.

In order to isolate mixis-related genes from brachionid rotifers, our strategy was to establish a phenotypically altered rotifer clone whose mixis induction could be fully manipulated by regulation of culture condition. For this purpose, we applied several former findings regarding rotifers, indicating that some sexual reproductive features are inherited by offspring through the maternal cytoplasm. Hino & Hirano (1985, 1988) reported that temperature and salinity during resting egg formation affect mixis induction in the derived clone hatched from the resting eggs. Hagiwara & Hino (1989) found that rotifer clones with active mixis appear when they are hatched from resting eggs without dormancy. By obtaining a phenotypically altered rotifer clone in which mixis is induced only under restricted conditions, we conducted cloning of sex-related genes using the subtraction hybridization method.

Materials and methods

Selection of phenotypically altered rotifer clone for sex-related gene study

The euryhaline rotifer *Brachionus plicatilis* Clone NH1L (Hagiwara et al., 1989, 1994, 2005) was used for the present study. In order to obtain a rotifer clone with higher mixis induction, we regulated the life history of ancestral individuals. First, the phenotypically altered NH1L strain was established from stem females starved for 12 hours after hatching and fed thereafter, based on the finding that a strain from starved stem females showed higher mixis induction (Hagiwara et al., 2005). The rotifer clone was kept cultured at 25ºC, 11 ppt salinity, 0L:24D and by feeding with *Nannochloropsis oculata*. Rotifers were then cultured at two different temperatures (15 and 25ºC) and the obtained resting eggs were incubated under two different light conditions for each temperature: Condition 1, irradiation with 20W fluorescent bulbs after preservation in darkness for 14 days; Condition 2, irradiation immediately after formation. These treatments were based on the findings of Hagiwara & Hino (1989), who reported that higher mixis induction is observed with rotifer clones from resting eggs that were not put in a dormant stage. Hatched clones were cultured at three temperature levels (15, 20 and 25ºC) for 14 days, and the mixis rate of each clone was calculated: (No. of unfertilized and fertilized mictic females) / (total egg carrying females).

Clones in which mixis was not induced during the above treatment were cultured at 15, 20, 25 and 30ºC to monitor the population growth and mixis induction.

Isolation of total RNA

Brachionus plicatilis clone, which was phenotypically altered clone derived from clone NH1L, was cultured at 25ºC in 11 ppt, and fed *Nannochloropsis oculata*. The rotifer culture was divided into 2 cultures when the rotifer density reached 12.5 individual per ml. One rotifer was cultured at the same condition for 6 days; another was cultured at 15ºC for 7 days. Each rotifer was harvested by filtration onto a plankton net (45 micron mesh) that was washed with 11 ppt seawater. Each rotifer was resuspended in 700 ml 11 ppt seawater that was exchanged every 3 h to allow the rotifers to consume any remaining *Nannochloropsis* and excrete their gut contents. The washed and starved rotifers were collected by plankton net and suspended in Isogen (Nippon Gene, Japan). The rotifers were homogenized with 0.8 mm glass beads using a vortex mixer, and total RNA was isolated from the homogenate according to the manufacturer's instructions of Isogen.

Construction of subtractive cDNA library

Approximately 1.0 μg of total RNA from each cultured clone (NH17L at 15 and 25ºC) was used to construct a subtractive cDNA library using a PCR-select cDNA subtraction kit (Clontech, USA) according to the manufacturer's instructions. PCR amplification was conducted using a GeneAmp PCR System 9700 (Applied Biosystems, USA). The optimal number of cycles for the 1st and 2nd PCR was 28 and 13, respectively. An aliquot of the subtractive cDNAs was ligated into pGEM-T Easy vector (Promega, USA) and then transformed into *Escherichia coli* strain DH5α. The transformed DH5α strains were infected on the LB agar plates with ampicillin (final concentration was 100 μg/ml), and then cultured at 37ºC. Randomly picked up about 800 colonies were cultured in LB broth media containing ampicillin (final consecration was 100 μg/ml) at 37ºC. The plasmid DNAs were isolated using R.E.A.L. Prep 96 Plasmid Kit (Qiagen, Germany). To detect specific expressed cDNA clones, dot blot hybridization analysis using Hybond-N^+ nylon membranes (GE Healthcare, UK) was performed with the DIG-labeled subtractive cDNAs as the probe, according to the manufacturer's instructions of the PCR-select cDNA subtraction kit and the DIG DNA Labeling and Detection Kit (Roche, Switzerland).

Sequence analysis and BLAST search

The isolated plasmid DNAs were sequenced with ABI BigDye v1.1 chemistry using M13 forward and reverse primers. Fragments were electrophoresed on an ABI PRISM 310 Genetic Analyzers (Applied Biosystems). These sequence data were compared to the EMBL/GenBank database using BLSTIN and BLSTX (Altschul et al., 1997).

Statistical Analysis

Chi-square contingency test was conducted to observe the different hatching rates of resting eggs. The average mixis rates of the hatched clones cultured at different temperatures on the same culture day were compared by the Tukey-Kramer post-hoc test followed by one-way ANOVA ($p<0.05$).

Results

Selection of phenotypically altered rotifer clone

The percent hatching of resting eggs was the highest (63.8%) when resting eggs were produced at a higher temperature (25ºC) and incubated after dormancy (Table 1).

When rotifers were cultured at three temperature levels (15, 20 and 25ºC) for 14 days, the highest mixis was observed at 20ºC (Fig. 1). Two out of 6 rotifer clones at 20ºC showed the highest mixis, at more than 50%; these originated from resting eggs at 15ºC with immediate irradiation. Among them, one clone showed the highest mixis, at 65 %, 3 times higher than the parental clone NH1L. Clones from resting eggs formed at 15ºC and irradiated after preservation at darkness for 14 days and cultured afterwards at 25ºC did not engage in sexual reproduction at 25ºC. There were 28 clones in total in which mixis was not induced at 25ºC.

Among the 28 clones in which mixis was not induced at 25ºC, we selected one clone (designated as NH17L) that originated from a resting egg produced at 15ºC and hatched without a dormant stage. This clone grew well at 20–30ºC (Fig.2). However, mixis was not induced at higher temperature (25 and 30ºC) and the highest mixis rate was observed at 15ºC (Tukey-Kramer post-hoc test, $p<0.05$, one-way ANOVA, df=3, F=5.023-136.962, $p<0.04$; Fig .3). The phenotype, controlled mixis induction against culture temperature, was constantly maintained for 2 years (data not shown).

Subtractive cDNA clones

From the subtractive cDNA library 768 clones were randomly selected using dot blot hybridization analysis, and 6 colonies (designated Subt2C4, Subt2F5, Subt2F7, Subt3C3, Subt3G, and Subt3H1) were picked up as the specifically expressed cDNAs in NH17L cultured at 15ºC. These clones were sequenced and submitted to the NCBI database using BLSTIN and BLSTX (Table 2). The Subt3H1 clone had a sequence homologous to that of retinoblastoma-binding protein 4, whereas the other 5 clones had no significant similarity to genes in the GenBank database.

Discussion

From our past findings in *B. plicatilis*, some sexual reproductive features appear to be inherited by offspring through the maternal cytoplasm. (Hino & Hirano, 1985, 1988; Hagiwara & Hino 1989; Hagiwara et al., 2005). When we encountered a *B. plicatilis* Yashima clone in which mixis was induced only at 10ºC when the temperature was changed between 10 and 30ºC, we hypothesized that culture history during resting egg production and diapause may have effects (Hagiwara et al., 1989). Based on these previous findings, we systematically varied the conditions for resting egg formation, diapause and hatching in this study, and obtained variable mixis induction in F1 progeny (Fig. 1). One clone appeared with a very high mixis rate (65%), 3 times higher than that of the parental NH1L clone, and may be applicable for large-scale resting egg production for long-term preservation. From the report of Hino & Hirano (1985) and Hagiwara et al. (1989), we anticipated that rotifer clones that did not show mixis at 25ºC may undergo mixis at higher temperatures when these clones were hatched from resting eggs without dormancy and produced under low temperature (15ºC). Indeed, the clone in which mixis was not induced at temperatures greater than 20ºC showed mixis only at 15ºC.

To isolate mixis-related genes, we used NH17L derived from NH1L resting eggs, the conditions of which were regulated for formation, diapause and hatching. We compared the expressed genes of the clone NH17L cultured at 15 and 25ºC using subtractive hybridization. It has been shown that the Y-box protein (YP) family is characterized by an evolutionarily highly conserved nucleic acid-binding domain, called a cold-shock domain, from bacteria to humans (Zend-Ajusch et al., 2002). In this study, we did not isolate the YP gene, although subtractive cDNAs were constructed from rotifers cultured at 10ºC. It was suggested that the culture temperature at 15ºC was not cold for this clone or that the rotifer acclimated to this temperature over 7 days.

Among the 6 isolated clones, Subt3H1 showed high similarity to Retinoblastoma-binding protein (RBBP) from *Tribolium castaneum*. RBBP P55 in *Drosophila* is homologous to the WD40 repeat protein Multicopy Suppressor of IRA1 (MSI1) from *Arabidopsis* (Guitton & Berger, 2005). Guitton & Berger (2005) reported that single mutations in the gene *MSI1* were able to initiate parthenogenetic development of the embryo in *A. thaliana* from eggs cells produced by meiosis. In several animal species, female gametes are able to initiate embryogenesis in the absence of fertilization, a process referred to as parthenogenesis (Mittwoch, 1978). In sexual reproduction of

Brachionus, mictic females produce haploid oocytes through meiosis, which develop either into haploid males or, if fertilized, into resting eggs. Several authors reported that mictic females did not produce resting eggs unless they were mated with haploid male until 9 h after birth (Snell & Childress, 1987; Hagiwara et al., 1988; Gomez & Serra, 1996). These findings suggested that the embryogenesis into a haploid male from a haploid oocyte in a young mictic female was inhibited during the possible mating period. It could be suggested that Subt3H1 inhibits the development of haploid oocytes into haploid males in young mictic females during the possible mating period, so that Subt3H1 was estimated to have the same function as the *MSI1* gene.

The other 5 clones have no significant similarity to GenBank database entries by BLAST search. Because there is little genomic data for Gnathifera, the transcripts that we found with no known function may represent genes that are species-, class- or phylum-specific. Further study of these clones will no doubt greatly expand our understanding of the mixis and biology of rotifers.

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Labels of Figures:

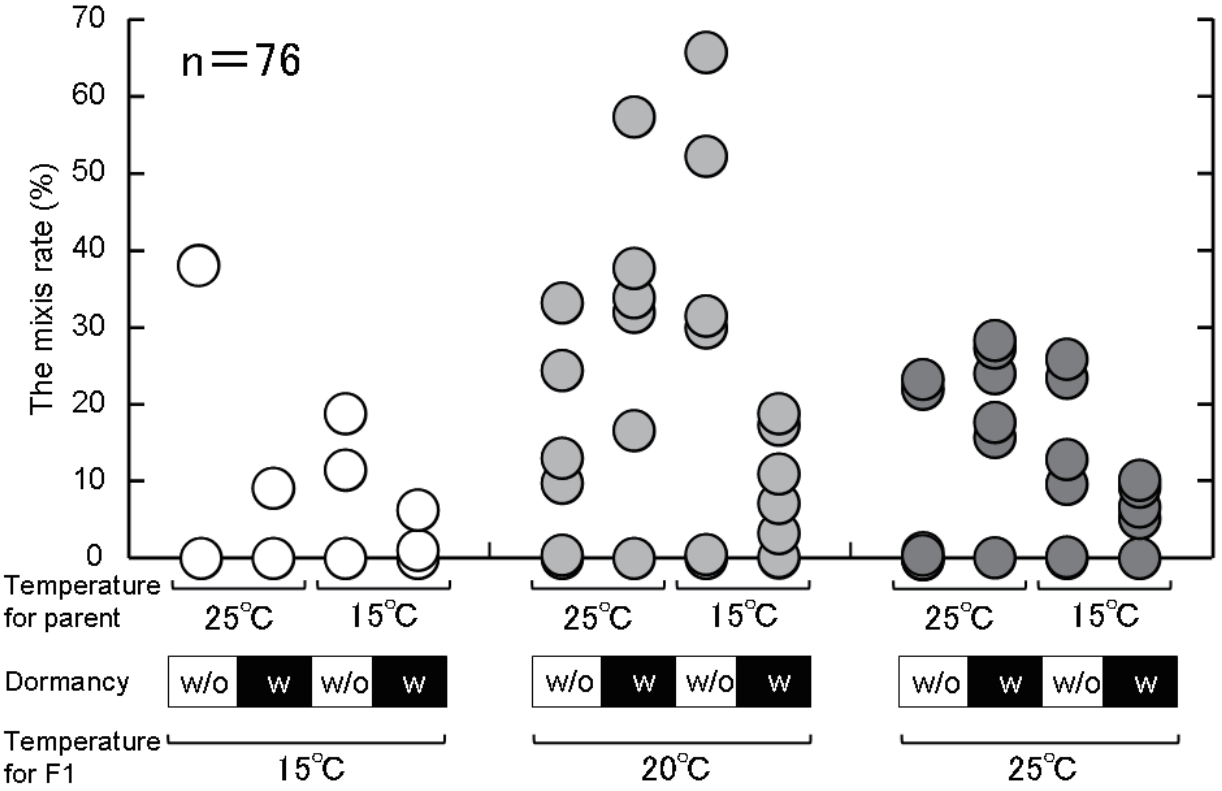

Fig. 1. Mixis induction of F1 progenies, obtained by different treatments of culture conditions.

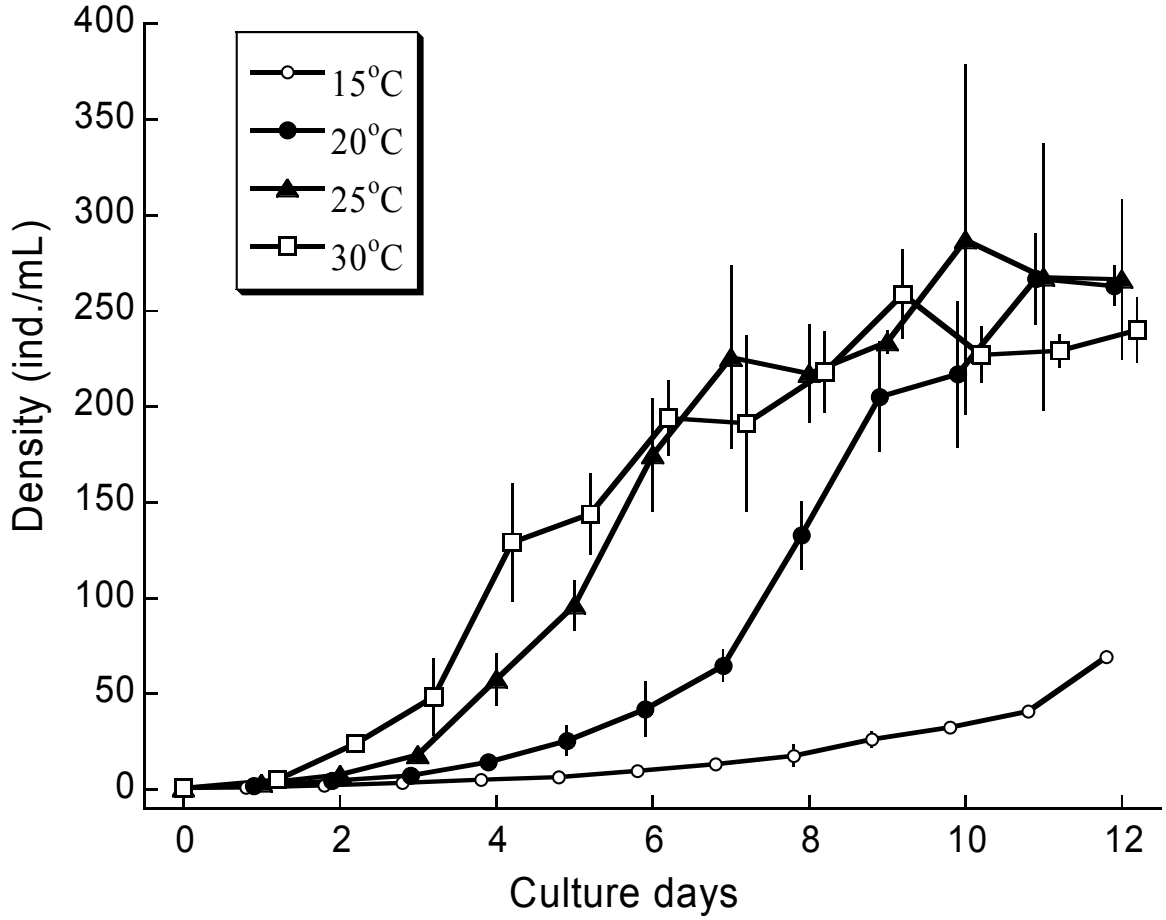

Fig. 2. Population growth of a phenotypically altered clone at 4 culture temperatures. Each plot and bar indicates average and standard deviations of 3 replicates, respectively.

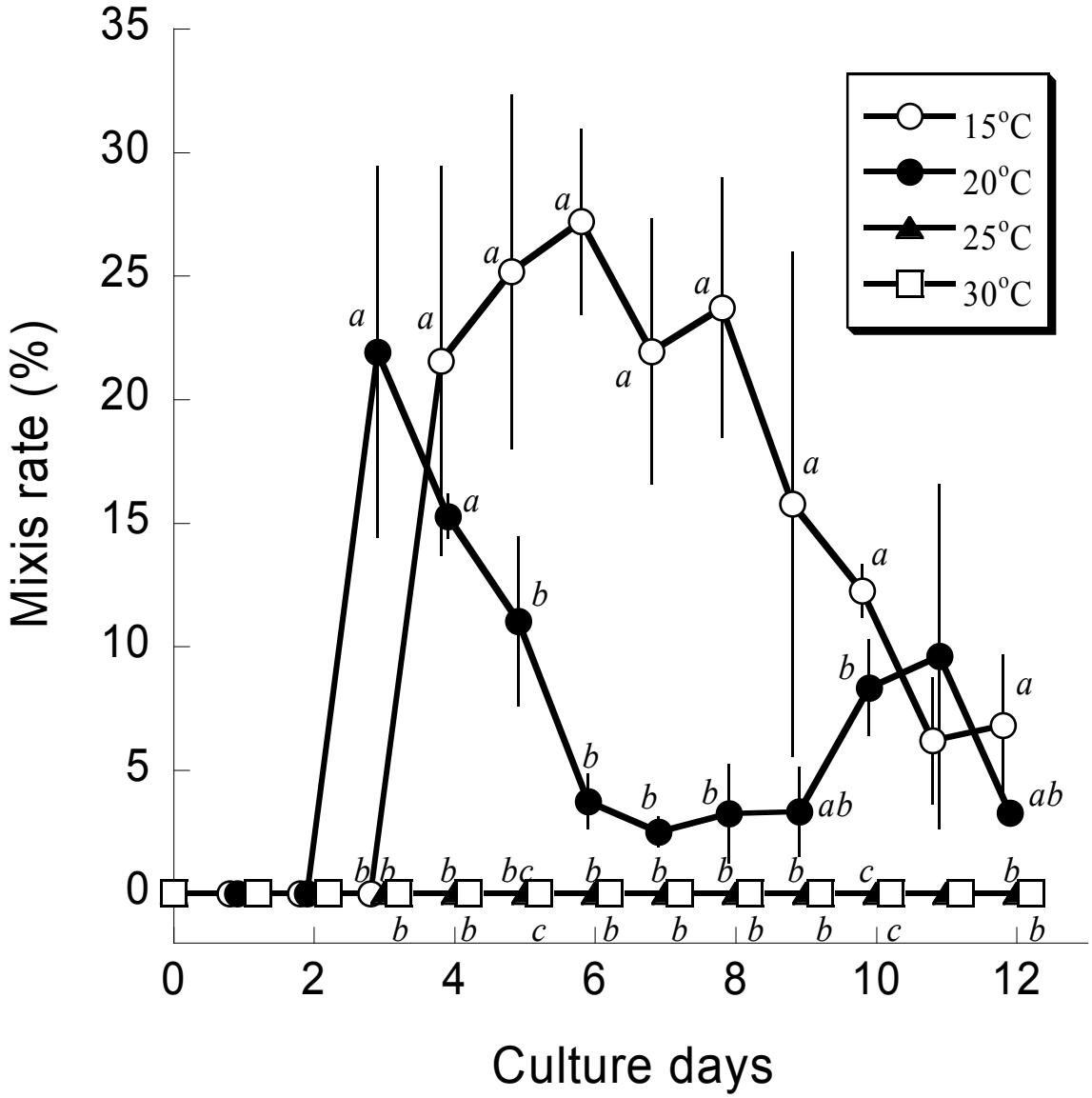

Fig. 3. Daily change of mixis rate of a phenotypically altered clone at 4 culture temperatures. Each plot and bar indicates the average and standard deviations of 3 replicates, respectively. Plots with different alphabets indicate significant differences among the same culture day (a>b>c, Tukey-Kramer post-hoc test, $p<0.05$).

Table 1 Cumulative hatching rate of resting eggs prepared at 4 conditions.

Water temperature of resting eggs formation (ºC)	Timing of irradiation	n	Number of hatched neonates	Hatching rate （%）
25	14th day after formation	600	383	63.8[a]
25	Immediate after formation	298	127	43.3[b]
15	14th day after formation	360	114	31.7[c]
15	Immediate after formation	1200	142	11.8[d]

a>b>c>d, χ^2 test, $p<0.05$

Table 2 Highly expressed genes in *Brachionus plicatilis* NH17L strain cultured at 15ºC and their putative function.

Clone name	Accession number	Clone size (bp)	Gene product name or probable function	Score	E-value
Subt2C4	AB521114	574	Transcription factor BTF3	37.4	0.79
Subt2F5	AB521118	950	Conserved Plasmodium protein (unknown function)	42.4	0.069
Subt2F7	AB521115	683	Nitrilase 1	59.7	2e-07
Subt3C3	AB521116	388	beta-glucosidase	55.5	2e-06
Subt3G3	AB521117	661	Response regulator receiver domain protein	36.6	1.9
Subt3H1	AB521113	1875	Retinoblastoma-binding protein 4	725	0.0