

Expression pattern of *nanos*, *piwil*, *dnd*, *vasa* and *pum* genes during ontogenic development in Nile tilapia *Oreochromis niloticus*

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Abstract

Primordial germ cells (PGCs) are specified by maternally provided determinants in fish. PGCs migrate then into prospective gonadal sites during early development and give rise to germ cell lineage. PGC disrupted animals do not sexually mature which has a range of commercial as well as environmental benefits. To find potential target genes for sterilisation of Nile tilapia, relative mRNA abundance patterns and tissue distribution of four *nanos*, two *piwil*, *dnd1*, *vasa* and three *pum* genes were investigated during ontogenic development from unfertilised eggs to newly hatched larvae and in adult tissues, respectively. The ontogenic pattern of RNA abundance revealed that all the investigated gene transcripts are maternally deposited to varying degrees, except for *nanos2* which is not expressed in eggs. The ontogenic patterns of relative RNA abundance could be grouped into three categories. The first one, including *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa*, showed abundant transcript levels during early developmental stages which are then degraded during the period of maternal to zygotic transition between blastula and gastrula stages with a reduction in expression of four to five orders of magnitude by hatching stage. Another, including *pum2* and *pum3*, showed similar patterns to the first group, but the transcript levels are reduced by only two orders of magnitude. The third group, including *nanos1a*, *nanos1b* and *pum1*, was characterised by a zygotic increase. *nanos2* had no detectable transcripts until hatching stage. The tissue screening of *nanos1a*, *nanos1b*, *pum1*, *pum2* and *pum3* showed that they are expressed in various tissues, implying their potential pleiotropic effects in these tissues apart from gonads. In contrast, *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa* appeared to be exclusively expressed in gonads (both ovary and testis), and *nanos2* showed testis-specific expression. Based on these results *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa* were prioritised amongst the 11 selected genes as potential target genes for sterilisation in Nile tilapia as they have no significant zygotic expression during embryogenesis, they are expressed exclusively in gonads and maternally deposited. These features suggest a potential role of these genes in the specification and maintenance of PGCs during the ontogenic development of Nile tilapia.

Keywords: Primordial germ cells, maternal transcripts, maternal to zygotic transition, early development.

1. Introduction

Primordial germ cells (PGCs) are set aside from somatic cells, migrate into prospective gonadal sites during early development and give rise to germ cell lineage (Cinalli et al., 2008). There is a growing interest within the aquaculture sector to explore new means to induce sterility through disruption of the normal PGC development (Wong and Zohar, 2015; Zhang et al., 2015; Wargelius et al., 2016). If successful, PGC disrupted animals would not sexually mature which has a range of economic as well as environmental benefits. However, before such a technology can be developed and applied in a given species, it is important to understand how PGCs are specified and maintain their germ cell fate.

PGCs are specified by either maternally derived germ plasm (GP) as shown in fish, frogs, fruit flies and nematode worms or inductive signalling from surrounding tissues such as bone morphogenetic protein (BMP) signalling during early embryogenesis as shown in most mammals, axolotls and turtles (Hogan, 1996; Raz, 2003; Extavour & Akam, 2003). The maternally deposited GP consists of maternal mRNAs and proteins of germ cell-specific genes, and they are found in PGCs and shown to have essential roles in PGC specification, germ cell development and/or maintenance (Knaut et al., 2000; Houwing et al., 2007; Kosaka et al., 2007), but their functions are unclear in many teleost species. The application of PGC ablation methods in Nile tilapia (*Oreochromis niloticus*) requires first the identification of germ cell-specific genes that are maternally deposited and prioritising the target genes.

Many GP components are RNA-binding proteins or RNAs, which are required to maintain germ cell fate and totipotency, otherwise they become somatic cells (Cinalli et al., 2008; Tada et al., 2012; Lai & King, 2013). *nanos*, *pumilio* (*pum*), *vasa*, *dead-end* (*dnd*) and *piwil-like* (*piwil*) genes are known to be involved in translational repression in germ cells, which is thought to be key to the maintenance of the germline across animal phyla including zebrafish (Mickoleit et al., 2011), *Xenopus* (Lai & King, 2013), mouse (Carmell et al., 2007) and *Drosophila* (Asaoka-Taguchi et al., 1999; Cox et al., 2000).

In Nile tilapia, to date there has been identified four *nanos* genes (Bellaiche et al., 2014; Škugor et al., 2014a), two *piwil* genes (Xiao et al., 2013) and three *vasa* (Conte et al., 2017; Fujimura et al., 2011). Further *in silico* analysis of public databases revealed predicted sequences for one *dnd1* as well as three *pum* genes. The predicted Nile tilapia Nanos proteins contain two CCHC zinc-finger domains which bind to RNA with low sequence specificity (Bellaiche et al., 2014). In Nile tilapia, Kobayashi

(2010) reported that *nanos1* showed a sexual dimorphic expression in sex differentiation period, expressing in oogenic meiotic cells. Following the publication of this work, however, the target studied (accession no. AB453384.1) has retrospectively been reclassified as *nanos3* in accordance with (Bellaiche et al., 2014; Škugor et al., 2014a). Knock-out (KO) of *nanos2* and *nanos3* genes resulted in complete loss of spermatogonia or germ cells both in mice (Tsuda et al., 2003) and Nile tilapia (Li et al., 2014), suggesting a conserved role in maintaining germ cells across phyla. However, there are four *nanos* genes in Nile tilapia, and their possible roles in PGCs are unknown.

Piwi (P-element induced wimpy testis) family members are components of gene regulatory network of germline cells, together with Tudor domain protein and DEAD box helicase, which maintain germ cell fate and pluripotency of germline stem cells (Ewen-Campen et al., 2010). Piwi proteins are germline-specific Argonautes, which combine with small RNA partners termed Piwi-interacting RNAs (piRNAs) and form RNA-induced silencing complexes (RISC) (Aravin et al., 2007; Tolia and Joshua-Tor, 2007). In *Drosophila*, Piwi proteins appear to regulate the number and division of germline stem cells (GSCs) (Cox et al., 2000). In mouse, *Miwi*, *Mili* and *Miwi2* (murine homologs of *piwi-like 1*, *2* and *4*, respectively) are essential for spermatogenesis (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). Unlike mammals where piRNAs are testis-specific, in zebrafish, the piRNAs exist in both testis and ovary, and many of them are derived from transposons, implicating a role in transposon silencing (Houwing et al., 2007). In zebrafish, loss of *ziwi*, the homolog of *piwi-like 1*, showed consecutive loss of germ cells due to apoptosis during early development, suggesting a role in the maintenance of germ cells (Houwing et al., 2007). In addition, zebrafish *piwi-like 2* homolog, *zili*, is known to be essential for germ cell differentiation and meiosis in this species (Houwing et al., 2008). In Nile tilapia, there are also two *piwi-like* genes (*piwil1* and *piwil2*); both proteins are present in spermatocytes but not in mature sperm (Xiao et al., 2013). Despite the apparent importance of Piwi family members in germline cells in diverse species, little is known about their molecular characteristics in fish.

Dead-end (Dnd) is RNA-binding protein, which protects germ cell-specific RNAs from microRNA mediated degradation by binding to the 3'-untranslated regions (3'UTRs) of germ cell-specific genes in human cell and zebrafish PGCs (Kedde et al., 2007). Vasa belongs to an ATP-

dependent RNA helicase of the DEAD box (Asp-Glu-Ala-Asp) family, and it is one of the well-known components of GP (Hay et al., 1988; Yoon et al., 1997).

The Puf (Pumilio and FBF, fem-3 mRNA-binding factor) domain of the RNA binding protein Pumilio provides the specificity of Nanos by binding specific target sequences through interacting with Nanos proteins, and the Nanos/Pumilio complex represses translation of target RNAs by deadenylation (Ewen-Campen et al., 2010). In *Drosophila*, maternally derived Nos/Pum complex which binds to Pumilio binding element in the 3'UTRs of target mRNAs is essential to maintain germline cells (Asaoka-Taguchi et al., 1999). In zebrafish, PGCs-specific knockdown of a novel *puf-A* gene, clustered separately from *pum1* and *pum2*, caused the reduction of PGCs number or the unsuccessful migration of PGCs (Kuo et al., 2009). In Nile tilapia, there are three predicted *pum* genes, but their molecular functions have not been studied. Overall, while evidence in vertebrates indicates pivotal roles of *nanos*, *piwil*, *dnd*, *vasa* and *pum* genes on PGCs and germline development, these genes have not been characterised in early ontogenic development of Nile tilapia.

The aim of this study was to profile expression patterns of eleven candidate genes reported to play a central role in PGC specification and development during early development of Nile tilapia. In order to optimise a sterilisation method and minimise pleiotropy and potential side effects associated with PGC ablation, target genes should be exclusively expressed in gonads and have no apparent roles during embryonic development apart from PGC maintenance and survival. The refined target list would enable subsequent research into gene editing techniques that have the potential to disrupt PGC development in this species.

2. Materials and methods

2.1. Embryo development of *O. niloticus*

A population of all male (XY) Nile tilapia embryos were produced at the Tropical Aquarium Facility at the Institute of Aquaculture, University of Stirling by crossing YY supermale (Scott et al., 1989) with XX female broodstock. Eggs were stripped from a single mature female Nile tilapia into a Petri dish. Before fertilisation, some unfertilised eggs were collected and preserved (as described below) for future analysis, the remainder of the eggs were fertilised with sperm from a single YY supermale. The

fertilised eggs were cultured in a tumbling egg system (<500 embryos in a 700 mL culture chamber) at $27 \pm 1^\circ\text{C}$ and 12L:12D photoperiod. At routine intervals, developmental progression was checked using a dissection microscope, and samples of embryos at key developmental stages (Table 1) were collected and stored in RNAlater at 4°C until RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA from embryo samples, either as pools or as individual samples (Table 1), was extracted using the routine TRI reagent (Sigma-Aldrich) extraction method, following mechanical disruption (Mini-BeadBeater, BioSpec Products) of the samples with 3 mm glass beads (EMD Millipore). RNA integrity and yield were confirmed by gel electrophoresis and spectrophotometry, respectively, and then all RNA samples were treated with DNase I (DNA-free DNA Removal kit, Ambion) to remove residual genomic DNA contamination. cDNA was reverse transcribed from 400 ng of DNase I treated RNA (High capacity cDNA reverse transcription kit, Applied BiosystemsTM) using a blend of random hexamer & anchored oligo dT primer at a 3:1 ratio. Once produced, cDNA samples were stored at -20°C prior to analysis.

2.3. Quantitative PCR analysis

The Nile tilapia sequences of *nanos1a*, *nanos1b*, *nanos2*, *nanos3*, *piwil1*, *piwil2*, *dnd1*, *vasa*, *pum1*, *pum2*, *pum3*, *elf1a*, *gapdh* and *β -actin*, were identified in NCBI and primers (Table 2) were designed using Primer-BLAST in NCBI. In the case of *vasa*, there are three *vasa* homologs (accession No. AB649031-AB649033) (Fujimura et al., 2011; Conte et al., 2017), however the primer pair designed by Pfennig et al. (2012) which was used in the current study amplifies all of three copies. In the case of genes which have multiple potential transcript variants (*piwil1*, *dnd1*, *pum1* and *pum2*) the primer pair was designed on the common sequence and thus would measure all transcript variants expressed. For each target, absolute quantification PCR assays were designed and validated. A standard curve was generated for each target from a serial dilution of a linearised plasmid which had previously had the target-specific partial cDNA fragment cloned within it using pGEM[®]-T Easy Vector Systems (Promega). The cloned partial sequence within each plasmid was confirmed to have 100 % sequence

identity prior to use within the assay by Sanger sequencing (GATC Biotech). Each qPCR reaction was of a total volume of 10 μ L containing 2.5 μ L of cDNA (1/10 diluted), 5 μ L of SYBR green mix (Luminaris Color HiGreen qPCR master Mix, Thermo Scientific), 0.3 μ M of each forward and reverse primer and MilliQ (Millipore) water up to 10 μ L. The qPCR thermal cycling protocol was: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, annealing for 30 sec at temperatures optimised for specific primers (Table 2) and 72°C for 30 sec, and completed with melt curve analysis. All samples were analysed in duplicate together with non-template controls and standard samples. The qPCR efficiency of all targets ranged from 0.91 to 1, and the linearity (r^2) of all standard curves ranged from 0.998 to 1. The standard curves of *nanos1a*, *nanos3*, *piwil1* and *piwil2* had an absolute range from 10^2 to 10^7 copies per reaction, and the rest of targets had an absolute range from 10^1 to 10^6 copies per reaction. In all assays, melt curve analysis confirmed a single product was generated and no primer dimer artefacts were evident. The absolute copy numbers of every target gene were calculated based on its standard curve. This was thereafter normalised using the geometric mean of *elf1a*, *gapdh* and *β -actin*, as this was determined to be the most stable normalisation approach following ranking of expression stability of all possible single & combinations of the three reference genes using Normfinder (<http://moma.dk/normfinder-software>) (Andersen et al., 2004). To aid interpretation of the results, the pattern of the relative RNA abundance of each target gene during larval ontogeny has been corrected with respect to abundance in the unfertilised eggs, where the abundance at this stage has been given the nominal value of 1.

2.4 Tissue distribution

For tissue screening, various tissues (brain, pituitary, eye, heart, intestine, spleen, kidney, liver & gonads) were removed from a single adult tilapia following euthanasia using an approved schedule 1 method and the samples were stored in RNAlater at 4°C prior to RNA extraction (the tissue samples all derived from a single male except for ovarian sample which came from a single female tilapia from the same stock). Total RNA was extracted and cDNA synthesised using the same method described previously. The tissue-specific abundance of the chosen targets was assessed by routine PCR where each reaction consisted a total volume of 5 μ L containing 2.5 μ L MyTaq mix (Bioline), 0.5 μ L of cDNA (1/10 diluted,

2.5 ng), 0.1 μ M of each forward and reverse primer and MilliQ (Millipore) water up to 5 μ L. The PCR thermal cycling protocol was as follows: 95°C for 1 min, followed by 32 cycles (except for *β -actin* of 28 cycles) of 95°C for 15 sec, annealing for 20 sec at temperatures optimised for specific primers (Table 2) and 72°C for 30 sec and final elongation at 72°C for 2 min. Presence of each gene target was assessed by gel electrophoresis.

2.5 Statistics

Statistical analysis was performed using Minitab 17. The relative expression values were tested for normality and homogeneity of variance and \log_{10} -transformed except for *pum1*, which met the assumptions of normally distributed data without correction. Then, significant differences between different developmental stages were tested by one-way ANOVA, followed by Tukey's HSD test ($p < 0.05$). Data are presented as mean \pm standard deviation (SD).

3. Results

3.1. Absolute transcripts number in unfertilised eggs

In unfertilised eggs, the maternally transferred RNAs were detectable in all but one target (Table 3). *piwil1* had the greatest abundance being recorded at 53524 ± 3090 copies per ng total RNA. The abundance of *nanos3*, *piwil2*, *vasa* and *pum2* was an order of magnitude lower, ranging between 1119 ± 257 and 9293 ± 374 copies per ng total RNA. The abundance of *nanos1a*, *dnd1* and *pum3* was a further order of magnitude lower, registering between 181 ± 17 and 605 ± 227 copies per ng total RNA, while the abundance of *nanos1b* and *pum1* was an order of magnitude lower still and therefore approaching the detection limits of the technique with *nanos2* being undetectable in unfertilised eggs.

3.2 Quantitative relative transcript levels during ontogenic development

All targets were detectable throughout larval ontogeny with the exception of *nanos2* which was undetectable until hatching at which point abundance was at the detection limits of the methodology (data not shown). There were three apparent patterns of target abundance observed within the dataset.

The largest group consisting of *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa* could be characterised as maintaining abundance until blastula stage, after which the target abundance significantly reduced from gastrula to hatching stage with the abundance steadily decreasing by four or five (*vasa* only) orders of magnitude over this developmental window (Fig. 1C, D, E, F&G).

The second group consisted of *pum2* and *pum3* and showed similarities to the first group in that they could be characterised as having relatively stable abundance until blastula stage, thereafter a significant reduction (with respect to unfertilised eggs) in abundance was recorded. This reduction in transcript abundance continued until hatching stage; however, unlike the first group, this reduction was in the region of two orders of magnitude in comparison to levels recorded in unfertilised eggs (Fig. 1I&J).

In the final group, which includes *nanos1a*, *nanos1b* and *pum1*, the abundance remained relatively constant over the duration of the study, recording no absolute changes more than an order of magnitude different from unfertilised eggs (Fig. 1A, B&H). Instead of target degradation in the later developmental stages, all targets showed evidence of active expression with the abundance in *nanos1a* being significantly elevated from neurula stage onwards with respect to the gastrula stage. Equally, for *nanos1b*, the expression levels increased significantly from pharyngula to peak at hatching, while in *pum1* there was a significant elevation in the expression at gastrula and neurula stages before the expression levels returned to being comparable to the abundance in unfertilised eggs. (Fig. 1A, B&H).

3. Tissue distribution

In adult tilapia *nanos1a*, *nanos1b*, *pum1*, *pum2* and *pum3* were expressed in a wide range of tissues including brain, pituitary, testis and ovary (Fig. 2). *nanos2* was exclusively expressed in testis, and *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa* were gonad-specific, being expressed in both testis and ovary (Fig. 2).

4. Discussion

In this study, 11 candidate genes were identified as putative targets for gene knockout to induce sterility in Nile tilapia, based on their published regulatory role in germ cells to repress somatic cell fate in a

range of vertebrate species. The optimal candidate gene target should be clearly gonad-specific and also not associated with the normal developmental process during embryogenesis. The targeted removal/silencing of such a candidate gene should therefore specifically remove germ cells without having effects on the early developmental process. Maternal deposition of the transcripts would also be desirable as this would imply that the target gene plays a potential role in PGC specification and/or maintenance. With this in mind, an ontogenic expression study with tissue screening was undertaken to rationalise the target gene list further in Nile tilapia.

Through the observation of the ontogenic pattern of the relative RNA abundance of each candidate gene, inappropriate targets could be excluded based on the presence or absence of apparent zygotic expression. Active zygotic expression would imply a role played by the gene in normal development rather than specification and maintenance of PGCs. This was shown in Atlantic salmon (*Salmon salar*) and medaka (*Oryzias latipes*) where maternally provided germ cell-specific genes such as *dnd*, *Tudor Domain Containing 7 (tdrd7)* paralogue (*tdrd7-2*), *Deleted In Azoospermia Like (dazl)* paralogue (*dazl-2*), *piwill1*, *tdrd1* showed a rapid decrease during the maternal to zygotic transition (MZT) without significant increase in mRNA levels (Xu et al., 2007; Liu et al., 2009; Zhao et al., 2012; Kleppe et al., 2015). The MZT has been reported to occur from fertilisation when maternal transcripts begin to be degraded, and generally a bulk zygotic transcription initiates at midblastula transition (MBT) when cell cycles become longer as described in a range of both vertebrate and invertebrate species (reviewed in Langley et al., 2014). In the current study, the degradation of the majority of the maternal transcripts of *nanos3*, *piwill1*, *piwil2*, *dnd1* and *vasa* occurred between the blastula and gastrula stages during MZT and there was no apparent zygotic transcription to recover the abundance levels of these targets up to the point of hatch when the study ended. The functional mechanism of this degradation has not been studied as yet in tilapia and requires further research. Evidence from other species including zebrafish, *Xenopus* and *Drosophila* suggested that this degradation is controlled by a family of microRNAs including zebrafish miR-430, *Xenopus* miR-427 and *Drosophila* miR-309, known to promote deadenylation and decay of maternal mRNAs during the MZT (Giraldez et al., 2006; Bushati et al., 2008; Lund et al., 2009; Giraldez, 2010). In contrast to this group of genes, *nanos1a*, *nanos1b*, *pum1* and *pum2* all showed a significant increase at different developmental stages, while *pum3* did not

show a sudden decrease in its transcripts level, which all imply an active zygotic expression. The ontogenic expression patterns of these genes suggest they play an active role in normal embryonic development apart from PGCs in Nile tilapia and thus should be excluded from the potential target list for sterilisation through gene editing techniques (Kleppe et al., 2015; Liu et al., 2009).

Gonad-specific expression is critically important to ensure that gene editing has no off-target effects. In the current study, both *nanos1a*, *nanos1b* as well as *pum1*, *pum2* and *pum3* were all ubiquitously expressed in a range of tissues. Such a wide-ranging expression of *nanos1a* and *nanos1b* has been reported in Chinese sturgeon (*Acipenser sinensis*) (Ye et al., 2012). However, contrasting findings have also been reported for tissue-specific expression of *nanos* genes with differential specificity in tissue expression in medaka (Aoki et al. 2009) and lack of gonadal expression in orange-spotted grouper *Epinephelus coioides* (Sun et al. 2017). A wide tissue distribution was also reported for *pum1*, *pum2* and *pum3* in zebrafish, while expression of another *pumillio* gene, *puf-A*, was found in various tissues and the knockdown of this gene resulted in eye defects, reduction of PGCs number and mis-migration of PGCs, indicating its important role in the development of eyes and PGCs survival and migration (Kuo et al., 2009). Therefore, it can be concluded that all of these targets are likely to be involved in various biological roles during the embryonic development of Nile tilapia and not only in gonadal development. In contrast, *nanos2*, *nanos3*, *piwil1* *piwil2*, *dnd1* and *vasa* all appeared to be gonad-specific in Nile Tilapia. *nanos2* was only expressed in testis in this study as shown in mice (Tsuda et al., 2003), however, in many teleosts species including zebrafish, medaka and rainbow trout (*Oncorhynchus mykiss*), the expression of *nanos2* was detected in both oogonia and spermatogonia in adult gonads (Aoki et al., 2009; Beer and Draper, 2013; Bellaiche et al., 2014). While *nanos3* was observed in both testis and ovary in the current study examined by PCR, previous research reported *nanos3* to be specific to ovarian tissue in tilapia using *in situ* hybridisation (Li et al., 2014). These contrasting results may be due to differences in sensitivity of the methodological approaches used between the two studies and further research is needed. In other teleosts, while *nanos3* remains a gonad-specific gene, the gender specificity is not consistent with it being reported in both the testis and ovaries of rainbow trout (Bellaiche et al., 2014), in contrast to being ovary-specific in zebrafish (Köprunner et al., 2001; Beer and Draper, 2013), medaka (Herpin et al., 2007) and grouper (Sun et al., 2017). Taking

an advantage of ovarian germ cell specificity of *nanos3* in zebrafish, Zhou et al. (2018) produced transgenic fish which transgene expression is driven by *nanos3* promoter. In the current study, both the *piwil* homologs were present in both gonadal tissues, this is in agreement with the published studies in zebrafish, medaka and Atlantic salmon, *Salmo salar* for *piwil1* (Houwing et al., 2007; Zhao et al., 2012; Kleppe et al., 2015) and Atlantic salmon and rainbow trout for *piwil2* (Yano et al., 2008; Kleppe et al., 2015). However, tissue-specificity is also not consistent across teleosts as expression of *piwil2* in medaka was reported in a wide range of tissues (e.g. brain, gill, heart, liver, kidney, spleen, intestine, ovary and testes) (Zhao et al., 2012). The gonad specificity of *vasa* observed in the current study is in agreement with the previous work in zebrafish and Atlantic cod (*Gadus morhua*) (Yoon et al., 1997; Presslauer et al., 2012), while the observed gonad specificity of *dnd1* aligns with the work in a wider range of teleosts species including zebrafish, medaka, Atlantic salmon, and turbot (*Scophthalmus maximus*) (Weidinger et al., 2003; Liu et al., 2009; Lin et al., 2013; Kleppe et al., 2015). When considered together these results demonstrate the need to confirm tissue-specificity in target species. Evidently, the diversity in reproductive strategy and development, as well as sex determination and differentiation processes in teleosts, makes it inappropriate to assume the comparable functional roles for these genes (Li et al., 2011; Kobayashi et al., 2013). Therefore, in terms of prioritising targets to be considered for gene editing to induce sterility in Nile tilapia, *nanos1a*, *nanos1b*, *pum1*, *pum2* and *pum3* should clearly be excluded on the grounds of lack of gonad specificity. Arguably, *nanos2* should also be omitted until its functional role in PGC specification and/or early gonadal development, prior to differentiation, is confirmed.

GP components are essential to maintaining germ cell fate, otherwise they become somatic cells (Cinalli et al., 2008; Tada et al., 2012; Lai & King, 2013). *nanos*, *pum*, *vasa*, *dnd* and *piwil* genes are known to be involved in translational repression in germ cells, which is thought to be a key to the maintenance of the germline (Asaoka-Taguchi et al., 1999; Cox et al., 2000; Carmell et al., 2007; Mickoleit et al., 2011; Lai & King, 2013). In Nile tilapia, RNA transcripts of all the investigated genes were present in the unfertilised eggs demonstrating maternal transfer, with the exception of *nanos2*. *nanos2* transcript level was below the detection limits until hatching stage which suggests that it is unlikely to be involved in the specification of PGCs. Among the maternally deposited RNAs in this

study, *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa* did not show evidence of significant zygotic expression, as discussed previously. Such a degradation in abundance is a common feature of many maternal transcripts which are components of GP such as *nanos3*, *vasa*, *dazl*, *dnd* and *tdrd7* in teleosts (Knaut et al., 2000; Köprunner et al., 2001; Kosaka et al., 2007; Strasser et al., 2008; Weidinger et al., 2003; Xu et al., 2005). It should, however, be acknowledged that PGC-specific expression of *nanos3* in embryos was reported in various teleosts species including zebrafish (Köprunner et al., 2001; Beer and Draper, 2013), medaka (Herpin et al., 2007), Atlantic cod (Presslauer et al., 2012) and Atlantic salmon (Škugor et al., 2014a). This suggests that discrete PGC expression may have not been captured by the analysis of whole embryo expression which recorded a decrease by four or five orders of magnitude of these genes. Therefore, we can postulate that in Nile tilapia *nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa* are maternally deposited GP components based on their ontogenic pattern of RNA abundance.

When considered together, the current results suggest that of the eleven candidate genes proposed, five (*nanos3*, *piwil1*, *piwil2*, *dnd1* and *vasa*) would be viable targets for gene editing to induce sterility in tilapia. To date, only *nanos* genes have been targeted for this purpose in Nile tilapia. Using CRISPR/Cas9 gene knock out in XX tilapia, Li et al. (2014) reported the loss of PGCs at hatching stage and germ cell-deficient gonads at 60 dph, suggesting that maternal transcripts of *nanos3* is not enough to maintain germ cells at hatching stage, and the zygotic transcripts during ontogenic development are vital for the survival of PGCs. Furthermore, knockdown of *nanos3* using morpholinos revealed its essential role in PGC specification in zebrafish (Köprunner et al., 2001) and a similar key role in PGCs and germ cell development has been demonstrated in *Drosophila* (Forbes & Lehmann, 1998). Interestingly, Li et al. (2014) also targeted *nanos2* by CRISPR/Cas9 in Nile tilapia, which resulted in the loss of PGCs at hatching stage and germ cell-deficient gonads at 90 dph. This is in agreement with mammalian research where KO mice had no germ cells (Tsuda et al., 2003). From the current study, *nanos2* has been excluded from the candidate list based on the screening results and primarily the apparent testis-specific expression. The successful disruption of PGC development via *nanos2* KO suggests that specificity of expression in differentiated gonads is not necessarily an accurate reflection of the role of the gene in early PGC development which warrants further investigation. Of the remaining candidates (*piwil1*, *piwil2*, *dnd1* and *vasa*) there is no published research exploring their functional role

in PGC and ultimately gonadal development in tilapia. Evidence from other teleosts is indicative that these warrant further research. For example, in zebrafish *ziwi* and *zili* mutants (the zebrafish *piwil1* and *piwil2* homologs), the germ cells are significantly reduced or not present (Houwing et al. 2007 & 2008), while morpholino knockdown of *piwil1* resulted in a reduction in the number of PGCs and defects in PGC migration in medaka (Li et al., 2012). With respect to *dnd1*, Gross-Thebing et al. (2017) reported that in *dnd*-deficient zebrafish the PGCs transdifferentiate into somatic cells, equally *dnd* knockout using CRISPR/Cas9 in Atlantic salmon resulted in germ cell loss (Wargelius et al., 2016). In contrast, Škugor et al. (2014b) reported that the knockdown of *dnd* in Atlantic cod caused a decrease of expression levels of *vasa*, *nanos3* and *tdrd7*, but also showed evident pleiotropic effects suggesting, in Atlantic cod at least, that *dnd* is involved in various developmental processes including suppression of reproduction. These results again clearly demonstrate the need for species-specific research to avoid deleterious effects on the development and health of individuals as part of gene editing research. Finally, in relation to *vasa*, it has been widely reported that the mutation of *vasa* leads to the loss of PGCs, defective germ cell proliferation and differentiation in the gonad across animal phyla, including fruit flies, nematode worms, frogs and mice (Hay et al., 1988; Gruidl et al., 1996; Ikenishi & Tanaka, 1997; Shinomiya et al., 2000). In zebrafish, however, *vasa* knockdown does not appear to affect the specification of PGCs, suggesting the maternal and zygotic *vasa* transcripts during ontogenic development are not essential (Braat et al., 2001), but *vasa* KO zebrafish revealed that the zygotic transcript of *vasa* is required for germ cell differentiation and survival in the later stage (Hartung et al., 2014). In medaka, knockdown of *vasa* led to a number of PGCs not entering the gonad and the ectopic PGCs that lack *vasa* survived and even increased their number, suggesting that *vasa* may play an important role in apoptosis of ectopic PGCs as well as the successful migration of PGCs (Li et al., 2009).

This research has helped rationalise the candidate genes to be taken forward into functional disruption studies with the aim to develop interventions that will induce sterility in Nile tilapia by disrupting normal PGC development. There are nearly 4 million tonnes of Nile tilapia being farmed annually across the global (FAO, 2017), however, production productivity is reduced due to losses associated with maturation in culture. While the industry addresses this challenge currently through the farming of single sex (all male) stocks (Phelps and Popma, 2000), there is a demand for validation of

alternative sterilisation methods. To this end further research will be conducted using genome-editing techniques to analyse the functions of *nanos3*, *piwill*, *piwil2*, *dnd1* or *vasa* in PGCs, which were prioritised by this study, with an aim to induce sterility in Nile tilapia.

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Table 1. Sampled developmental stages of *O. niloticus* from unfertilised eggs to hatching stage in chronological order. Scale bar = 0.5 mm. hpf: hours post fertilisation









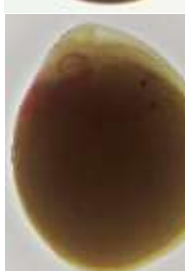

Developmental Stage	hpf	Description of development	Figure	Pooled No.	<i>n</i>
Egg	0	Unfertilised eggs		5	5
Zygote	0-1.5	1-cell		10	6
Cleavage	1.5-2	2-cell		10	6
	2	4-cell		10	4
	3	8-cell		10	6
Blastula	4-12	Early blastula		6	6
Gastrula	22-26	Gastrula, epiboly=30-50%		6	6
Segmentation	26-30	Neurula		6	6
Pharyngula	60-72	Onset of blood circulation		2	6
Hatching	90-110	Jaw extension		1	6

Table 2. Primers used for qPCR and tissue screening PCR.

Gene	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp (°C)	Analysis
<i>nanos1a</i>	XM_003447766.4	TCTCAGGCCATACGAACACCTCG	CTCTGAGCCTGTTTGCGTCTTCG	62	qPCR
		TAAACTGTGCGGCTCTGACAGGA	CTCTGAGCCTGTTTGCGTCTTCG	62	Tissue screen
<i>nanos1b</i>	XM_005467222.3	GAGCCCCTTCCAAAATCAGCTCG	CATGCCGAACAGATCGAACCCAG	62	qPCR
		GAGCCCCTTCCAAAATCAGCTCG	GCTGGGATGGCTGGTCTTTTGAC	62	Tissue screen
<i>nanos2</i>	XM_005448855.3	CGGGAAAGTTTCTGCCCCATCC	AGAACTTGGCCCCGTCTCCATC	62	qPCR
		GTTTCGAGAGACTGTGCAGCAACC	AGAACTTGGCCCCGTCTCCATC	62	Tissue screen
<i>nanos3</i>	XM_005460553.3	GGAGTGTGACATGAGCCGAGCTA	AACTCGTTAGTGCACATTCGCGG	62	qPCR
		GGAGTGTGACATGAGCCGAGCTA	CATACTGCCGCAGAAAGGGACAC	62	Tissue screen
<i>piwil1</i>	XM_003445546.3	ATGATCGTGGGCATCGACTGCTA	ACCACCTGCTCATGCTTTGGTTG	62	qPCR
		ATGATCGTGGGCATCGACTGCTA	CGGGTGACCTCTGAGTCAACGAT	62	Tissue screen
<i>piwil2</i>	XM_003445662.3	TGCCATCAAGAAGCTGTGCTGTG	CTGGGAAATTGTGCGGACGTTGA	62	qPCR
		CAAGAAGCCACCGACCTTTCCAC	CTGGGAAATTGTGCGGACGTTGA	62	Tissue screen
<i>dnd1</i>	XM_003454288.4	CACGGGACACGTATGAGGACATC	ATATTTGGCATAACGAAAGCCGC	60	qPCR
<i>vasa*</i>	AB032467.1	CGATGAGATCTTGGTGGATG	CATGAGATCCCTGCCAGCAGA	58	qPCR
<i>pum1</i>	XM_013270654.2	GCTAACTGGTAAGAAGTTCTGGGAA	CGGGACACCATGATTGGCTG	60	qPCR
<i>pum2</i>	XM_003446310.4	GCGACATTGTCCGTGAGCTTGAT	CAAAAACCTGTCCCTGGAAGGCA	60	qPCR
<i>pum3</i>	XM_005470475.3	TCACAGATGAGCTGTACGGCAAC	CATGGGTGTGAGAACCTGCTTCA	60	qPCR
<i>elf1a</i>	NM_001279647.1	CTGGACAAACTGAAGGCTGAGCG	AAGTCTCTGTGTCCAGGGGCATC	62	qPCR
<i>gapdh</i>	NM_001279552.1	TGGATCTGACATGCCGTCTCTCC	AATCAGAGGACACCACCTGGTCC	62	qPCR
		ATCTACGAGGGTTATGCCCTGCC	CTGTGGTGGTGAAGGAGTAGCCA	62	qPCR
<i>β-actin</i>	KJ126772.1	ATCTACGAGGGTTATGCCCTGCC	CGATGCCAGGGTACATGGTGGTA	60	Tissue screen
		ATCTACGAGGGTTATGCCCTGCC	CGATGCCAGGGTACATGGTGGTA	60	Tissue screen

* based on Pfennig et al. (2012).

Table 3. Absolute copy numbers of maternal transcripts in unfertilised eggs ($n = 5$). Data are represented as mean \pm SD.

	Absolute copy number per ng total RNA
<i>nanos1a</i>	181 \pm 17
<i>nanos1b</i>	5 \pm 1
<i>nanos2</i>	below detection range
<i>nanos3</i>	9293 \pm 374
<i>piwil1</i>	53524 \pm 3090
<i>piwil2</i>	4614 \pm 202
<i>dnd1</i>	605 \pm 227
<i>vasa</i>	1851 \pm 93
<i>pum1</i>	15 \pm 5
<i>pum2</i>	1119 \pm 257
<i>pum3</i>	290 \pm 59

Figure legends

Figure 1. Normalised relative mRNA transcript level of *nanos1a* (A), *nanos1b* (B), *nanos3* (C), *piwil2* (D), *piwil2* (E), *dnd1* (F), *vasa* (G), *pum1* (H), *pum2* (I) and *pum3* (J). Data presented as mean \pm SD ($n = 5 - 6$). Y-axis is shown in logarithmic scale. Superscripts denote statistically significant difference between developmental stages. unf_egg: unfertilised eggs

Figure 2. Tissue distribution of *nanos*, *piwil*, *dnd1*, *vasa* and *pum* genes in Nile tilapia. Br, brain; Pit, pituitary; E, eye; H, heart; In, intestine; Sp, spleen; K, Kidney; L, liver; Tes, testis; Ov, ovary. Marker (M) is 100 bp ladder except for *piwil2* which is 1 kb. Male, 24.1 cm body length, 269.1g body weight, 0.22 % GSI; female, 19.5 cm BL, 124.4 g BW, 0.4 % GSI.

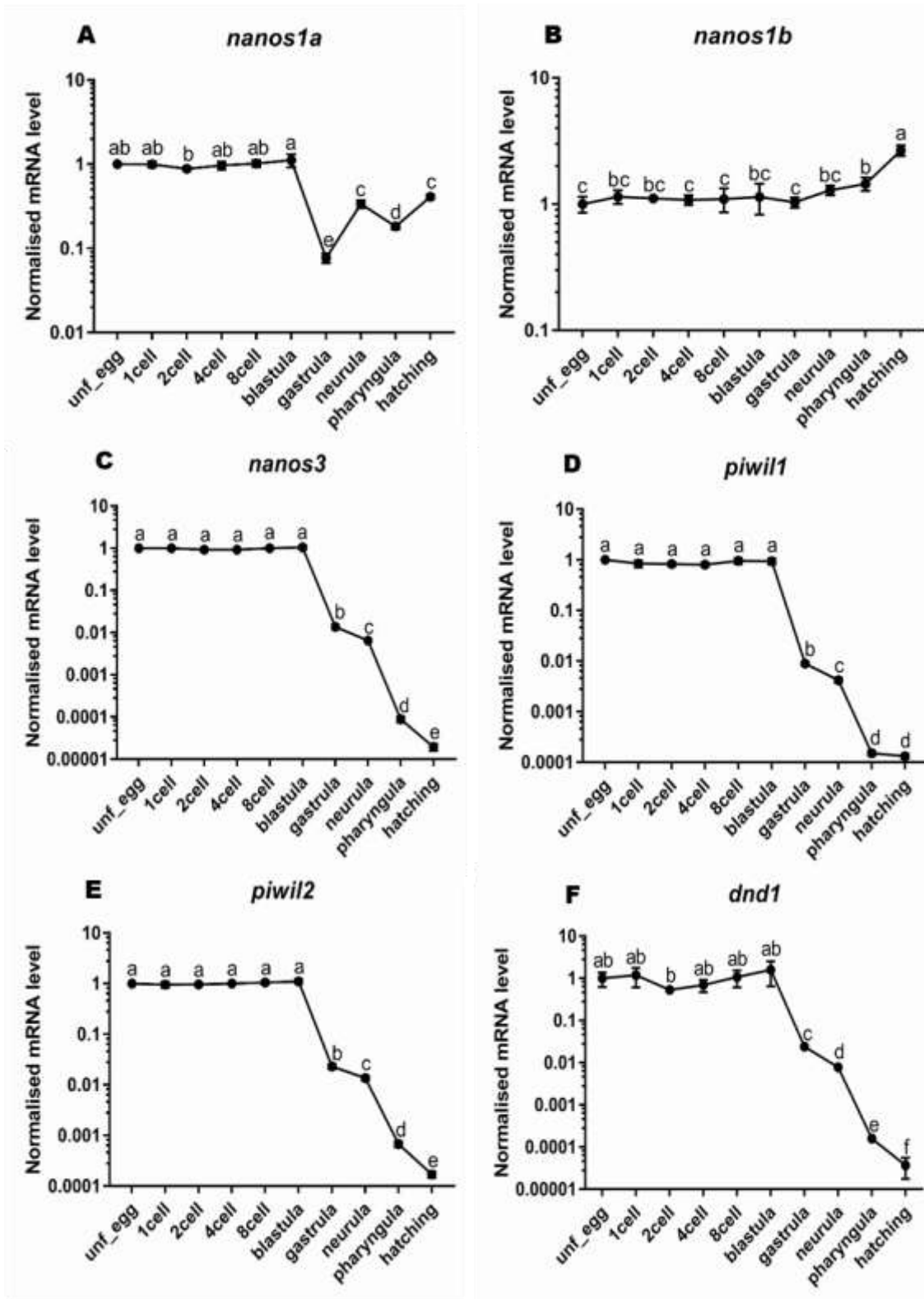


Fig. 1.

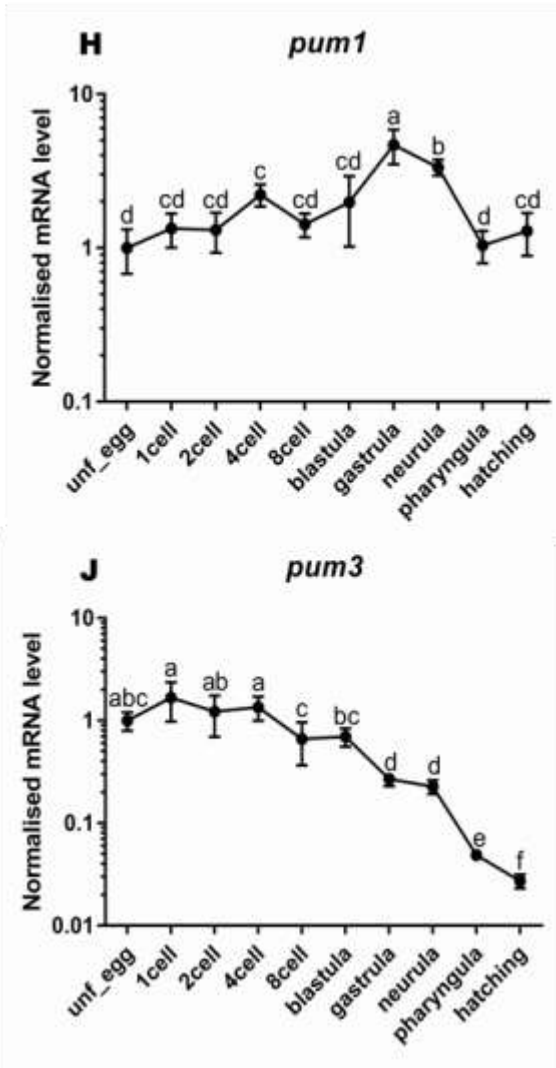
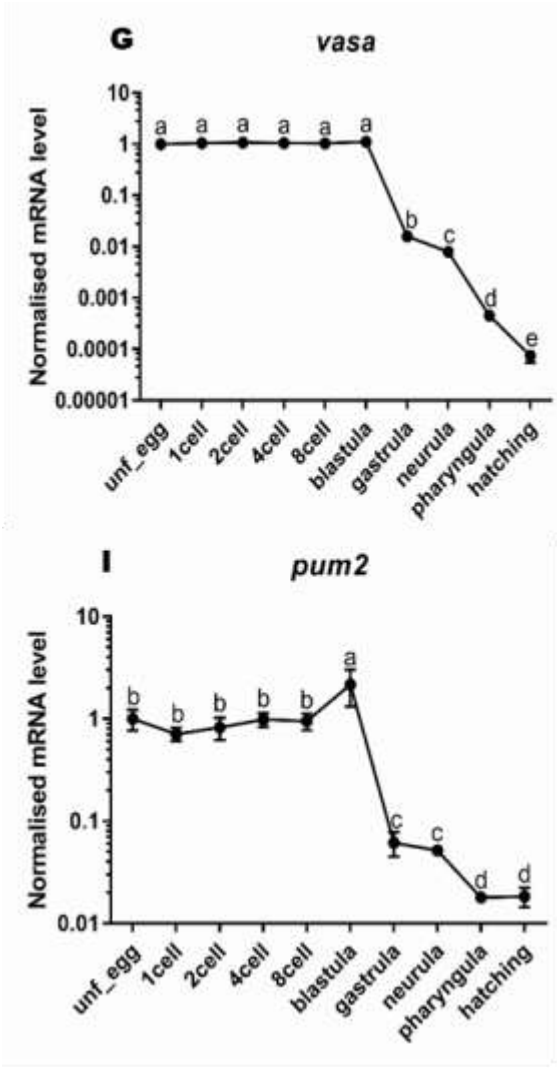


Fig. 1 (continued).

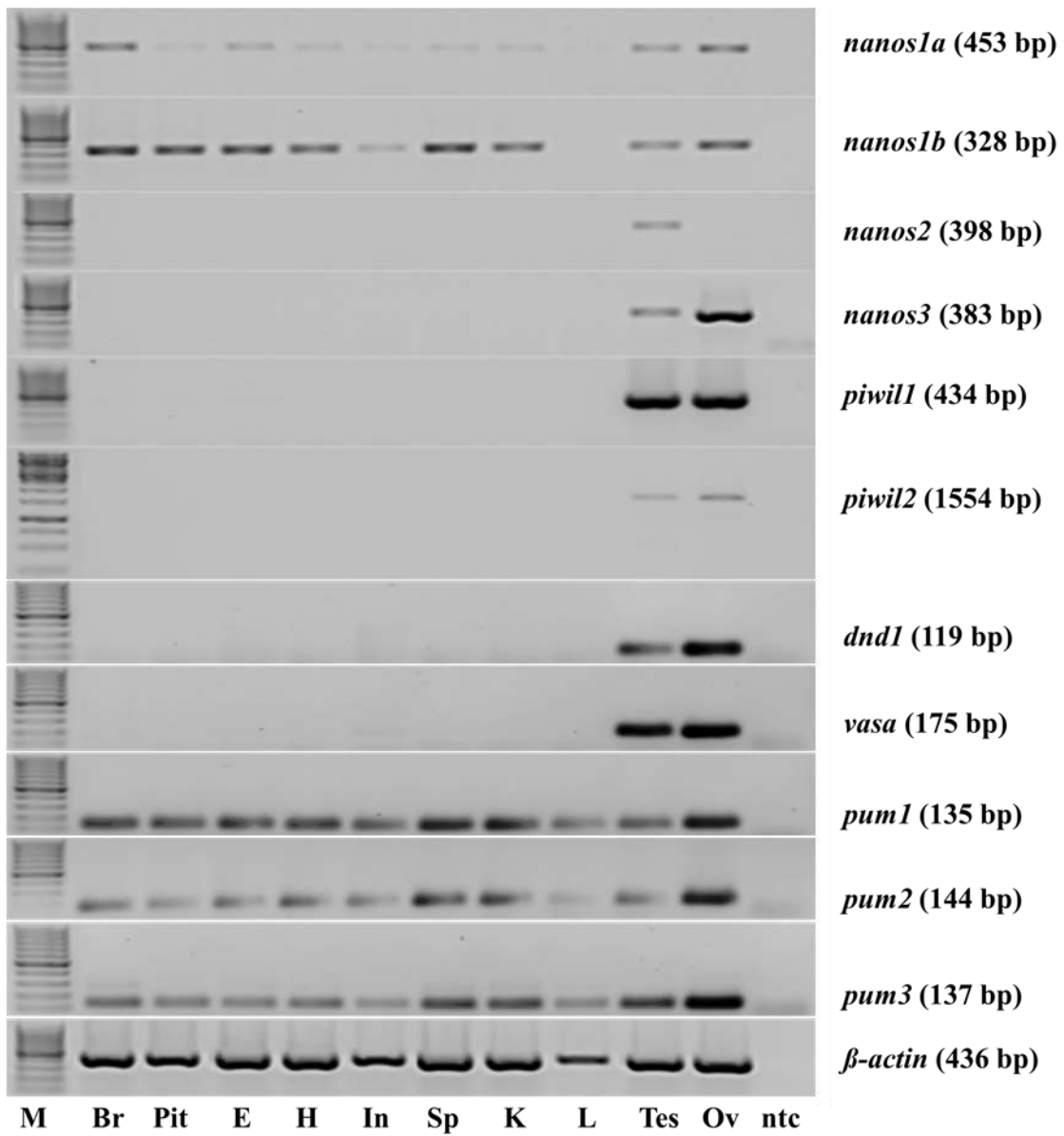


Fig. 2.