2008

Defensins and the convergent evolution of platypus and reptile venom genes

Camilla M. Whittington  
*University of Sydney*

Anthony T. Papenfuss  
*The Walter and Eliza Hall Institute of Medical Research*

Paramjit Bansal  
*University of Sydney*

Allan M. Torres  
*University of Western Sydney*

Emily S.W. Wong  
*University of Sydney*

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Recommended Citation

Whittington, Camilla M.; Papenfuss, Anthony T.; Bansal, Paramjit; Torres, Allan M.; Wong, Emily S.W.; Deakin, Janine E.; Graves, Tina; Alsop, Amber; Schatzkamer, Kyriena; Kremitzki, Colin; Ponting, Chris P.; Temple-Smith, Peter; Warren, Wesley C.; Kuchel, Philip W.; and Belov, Katherine, ’Defensins and the convergent evolution of platypus and reptile venom genes.’ Genome Research. 18, 986-994. (2008).  
[https://digitalcommons.wustl.edu/open_access_pubs/1981](https://digitalcommons.wustl.edu/open_access_pubs/1981)
Defensins and the convergent evolution of platypus and reptile venom genes


*Genome Res.* 2008 18: 986-994 originally published online May 7, 2008
Access the most recent version at doi:10.1101/gr.7149808

Supplemental Material

http://genome.cshlp.org/content/suppl/2008/05/08/gr.7149808.DC1.html

Related Content

Retroposed SNOfallA mammalian-wide comparison of platypus snoRNAs

Jürgen Schmitz, Anja Zemann, Gennady Churakov, et al.
*Genome Res.* June , 2008 18: 1005-1010

Conservation of small RNA pathways in platypus

*Genome Res.* June , 2008 18: 995-1004

Descent in the platypus

B. Tim Park, Jenia Sergeyev, Chia Lin Chang, et al.
*Genome Res.* June , 2008 18: 974-985

Origin of mammal sex chromosomes

Frederic Veyrunes, Paul D. Waters, Pat Miethke, et al.
*Genome Res.* June , 2008 18: 965-973

References

This article cites 47 articles, 13 of which can be accessed free at:
http://genome.cshlp.org/content/18/6/986.full.html#ref-list-1

Articles cited in:

http://genome.cshlp.org/content/18/6/986.full.html#related-urls

Open Access

Freely available online through the *Genome Research* Open Access option.

Creative Commons License

This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see http://genome.cshlp.org/site/misc/terms.xhtml). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported License), as described at http://creativecommons.org/licenses/by-nc/3.0/.

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.

To subscribe to *Genome Research* go to:
http://genome.cshlp.org/subscriptions

Copyright © 2008, Cold Spring Harbor Laboratory Press
Defensins and the convergent evolution of platypus and reptile venom genes

Camilla M. Whittington,1 Anthony T. Papenfuss,2 Paramjit Bansal,3 Allan M. Torres,4 Emily S.W. Wong,1 Janine E. Deakin,5 Tina Graves,6 Amber Alsop,5 Kyriena Schatzkamer,6 Colin Kremitzki,6 Chris P. Ponting,7 Peter Temple-Smith,8,9 Wesley C. Warren,6 Philip W. Kuchel,3 and Katherine Belov1,10

1Faculty of Veterinary Science, University of Sydney, Sydney, New South Wales 2006, Australia; 2Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia; 3School of Molecular and Microbial Biosciences, University of Sydney, Sydney, New South Wales 2006, Australia; 4Nanoscale Organisation and Dynamics Group, College of Health and Science, University of Western Sydney, Sydney, New South Wales 1797, Australia; 5Australian Research Council (ARC) Centre for Kangaroo Genomics, Research School of Biological Sciences, The Australian National University, Canberra, Australian Capital Territory 0200 Australia; 6Genome Sequencing Center, Washington University School of Medicine, St. Louis, Missouri 63108, USA; 7Department of Human Physiology, Anatomy and Genetics, Medical Research Council (MRC) Functional Genetics Unit, University of Oxford, Oxford OX1 3QX, United Kingdom; 8Centre for Reproduction and Development, Monash Institute of Medical Research, Clayton, Victoria 3168 Australia; 9Department of Zoology, University of Melbourne, Parkville, Victoria 3010 Australia

When the platypus (Ornithorhynchus anatinus) was first discovered, it was thought to be a taxidermist’s hoax, as it has a blend of mammalian and reptilian features. It is a most remarkable mammal, not only because it lays eggs but also because it is venomous. Rather than delivering venom through a bite, as do snakes and shrews, male platypuses have venomous spurs on each hind leg. The platypus genome sequence provides a unique opportunity to unravel the evolutionary history of many of these interesting features. While searching the platypus genome for the sequences of antimicrobial defensin genes, we identified three Ornithorhynchus venom defensin-like peptide (OvDLP) genes, which produce the major components of platypus venom. We show that gene duplication and subsequent functional diversification of beta-defensins gave rise to these platypus OvDLPs. The OvDLP genes are located adjacent to the beta-defensins and share similar gene organization and peptide structures. Intriguingly, some species of snakes and lizards also produce venoms containing similar molecules called crotamides and crotamine-like peptides. This led us to trace the evolutionary origins of other components of platypus and reptile venom. Here we show that several venom components have evolved separately in the platypus and reptiles. Convergent evolution has repeatedly selected genes coding for proteins containing specific structural motifs as templates for venom molecules.

[Supplemental material is available online at www.genome.org.]

The monotremes have features that place them definitively within the class Mammalia but are unique in laying eggs rather than giving birth to live young. The gestation period of the platypus probably lasts between 15 and 21 d (Holland and Jackson 2002). The incubation period for eggs is thought to be only 6–10 d (Grant 1995), so at hatching, the nestlings are only about 15 mm long (Grant 1995), during which time they suckle from maternal “milk patches” (Grant 1995). Milk-derived antimicrobial peptides play an important role in immune protection of marsupial young (B.G. Cocks, pers. comm.) and could also protect platypus hatchlings.

To gain more insight into the basic biology of the platypus immune system, we searched the platypus genome for defensin genes that may play a role in protecting altricial platypus young. Defensins are cysteine-rich peptides with antimicrobial properties that have wide distribution, having been identified in plants, insects, and vertebrates (Patil et al. 2005). Animal defensins possess a distinctive pattern of six cysteine residues, and are categorized into alpha-, beta-, and theta-defensins according to the particular arrangement of disulfide bonds between cysteine residues (Lehrer and Ganz 2002). This same pattern of cysteine interconnections is observed in some components of platypus venom called the OvDLPs (Ornithorhynchus venom defensin-like peptides). Male platypuses possess a hollow spur on each hind leg, which is connected via a duct to a venom gland (Grant 1995). Collectively, this is known as the crural system. The platypus venom gland is seasonally active and secretes venom only during the short annual breeding season (Temple-Smith 1973). The venom has mild proteolytic activity and, in laboratory experiments, causes cutaneous anaphylaxis in rabbits and rapid death in mice after intravenous doses of 75–90 mg/kg (Temple-Smith 1973). In the nonbreeding season the platypus crural system is not functional: The venom gland regresses, its secretory epithe-

10Corresponding author.
E-mail kbelov@vetsci.usyd.edu.au; fax 61-2-9351-3957.

Article published online before print. Article and publication date are at http://www.genome.org/cgi/doi/10.1101/gr.7149808. Freely available online through the Genome Research Open Access option.
Defensins and the evolution of platypus venom

By searching the platypus genome, we identified six beta- and four alpha-defensin genes. One of these (DEFA4) resembles both an alpha-defensin, in the arrangement of its first pair of cysteines, and a beta-defensin, in its arrangement of cysteine residues four and five (Fig. 1), supporting the suggestion of Liu et al. (1997) that alpha-defensins have evolved via gene duplication from beta-defensins and subsequently diversified. The identification of platypus alpha-defensins, previously identified only in therians (Belov et al. 2007), pushes back their emergence to prior to the divergence of the three extant mammalian lineages 210 million years ago (Woodburne et al. 2003).

Synteny analysis

Beta-defensin genes are arranged in discrete clusters on vertebrate chromosomes. Chickens have a single cluster of 13 beta-defensin genes (chromosome 3q) (Xiao et al. 2004), opossums possess 37 genes in three clusters (Belov et al. 2007), and dog, human, mouse, and rat have 43, 39, 52, and 43 beta-defensin genes, respectively, in four to five clusters (Patil et al. 2005). There is a clear evolutionary relationship between these conserved syntenic groups (Patil et al. 2005), as the genes within syntenic clusters form separate clades in phylogenetic trees (Patil et al. 2005; Belov et al. 2007). Because of the fragmented nature of the assembly of the platypus genome (Warren et al. 2008), we used a combination of phylogenetic analysis and FISH mapping to deduce which syntenic groups the platypus genes belonged to and to investigate the evolution of defensins.

Neighbor-joining and Bayesian phylogenetic analyses (Fig. 2; Supplemental Figs. 7, 8) suggest that all but one of the platypus beta-defensin genes belong to a single conserved synteny group (group A) (Fig. 3). In the platypus, these genes map to a single chromosome (X2), which supports the topology obtained in our phylogenetic tree (Figs. 2, 3; Supplemental material 5). The single chicken beta-defensin cluster (Xiao et al. 2004) also shares homology with members of synteny group A, making this conserved beta-defensin cluster the most ancient synteny group. Synteny group A also contains the alpha-defensins, which are believed to have evolved from beta-defensins (Liu et al. 1997; Patil et al. 2004). The platypus data suggest that this synteny group gave rise to additional beta-defensin gene clusters through
gene duplication and translocation during mammalian evolution. The first beta-defensin cluster (containing \textit{DEFB6}) to form outside the ancestral cluster appears to be synteny group D, which we have mapped to platypus chromosome X1 (Fig. 3). This group arose after the divergence of the birds and mammals but prior to the divergence of the three mammalian lineages. The fact that no members of synteny groups B and C were found in the platypus genome suggests that these groups probably arose less than 180 million years ago, after the divergence of the marsupials from the eutherians (Woodburne et al. 2003).

**Identification of platypus venom genes**

Searches of the platypus genome using hidden Markov model (HMM) profiles for defensins also identified four other genes encoding putative antimicrobial peptides, with disulfide bonding patterns between cysteine residues that were slightly different from the defensins. Three of these unidentified genes were found to encode defensin-like peptides (OvDLPs), the main peptide components of platypus venom, as they matched partial peptide sequences obtained experimentally (Torres et al. 1999), and the remaining gene appeared to be an intermediate between beta-defensins and OvDLPs. It has also previously been suggested that some components of reptile venom may be homologous to beta-defensins (Zhao et al. 2001). Using this information, we investigated the genetic organization and evolutionary history of venom genes.

**Platypus venom OvDLPs**

The platypus OvDLPs are a family of 5-kD peptides (Torres et al. 1999). The similarity in sequence and structure between beta-defensins and OvDLPs suggests that they are evolutionarily related. Significantly, one gene we identified displays characteristics that are a mixture of both peptide families, perhaps representing an evolutionary intermediate between the beta-defensins.

---

**Figure 2.** Neighbor joining tree of therian and platypus beta-defensins (\textit{DEFB1–6}), in red, with chicken beta-defensins (Chick AvBD1–13) and platypus and reptile venom peptides. Chicken and therian sequences are shaded based on whether they belong to synteny groups A (yellow), B (blue), C (green), or D (pink). These synteny groups were determined by Patil et al. (2004) and Belov et al. (2007) based on genomic localization and phylogeny. Platypus \textit{DEFB1–5}, which we have mapped to chromosome X\textsubscript{P}, belong to synteny group A, while platypus \textit{DEFB6}, mapped to chromosome X\textsubscript{P}, belongs to synteny group D. Similar topologies were recreated using Baysian phylogenetics approaches.
Defensins and the evolution of platypus venom

Figure 3. (Legend on next page)
and OvDLPs. We thus designated this gene DEFB-VL (venom-like beta-defensin). The relatedness of beta-defensins, DEFB-VL, and OvDLPs was confirmed using peptide modeling and gene structure and phylogenetic analysis. There are clear similarities in the structural folds of these peptides (Fig. 4) because of their size and disulfide bond interconnections of the six cysteine residues, and their 1-D NMR spectra also display similarities. DEFB-VL, like the beta-defensins but unlike the OvDLPs (Torres et al. 1999), appears to be antimicrobial (Supplemental material 7). This is due to its N-terminal polyarginine domain, which may allow its interaction with negatively charged phosphate moieties in the cell membranes, and is suggestive of a cell-penetrating domain (Jones et al. 2005). Our fluorescent in situ hybridization (FISH) mapping also supports the hypothesis that OvDLPs are related to beta-defensins, as these were all mapped to the same chromosome (Xp) (Fig. 3; Supplemental material 5).

Features of the defensin and OvDLP genes
Examination of the intron/exon structure of the beta-defensins, DEFB-VL, and OvDLPs showed that they have identical gene structures: two exons, the first encoding a signal peptide and the second encoding the mature peptide containing all six cysteines (Supplemental Fig. 1). Promoter analysis of the defensins and OvDLPs showed that the OvDLP promoters were strikingly similar to one another (89%–94% identity). Moreover, searching with known motifs from the TRANSFAC database, CDPCR1 binding sites were predicted in all OvDLPs and three of the defensin promoters. This supports the recent expansion of the OvDLP gene subfamily. These promoters are also present in three of the defensins. All beta-defensins have predicted HNF1A binding sites, and beta-defensins 1–5 have predicted GATA4 binding sites, which suggest tissue specificity (Brahmachary et al. 2006). DEFB-VL and beta-defensin 5 have predicted NF-kB binding sites, but only DEFB-VL possesses a glucocorticoid receptor (GR) transcription factor binding site (TFBS). Promoter analysis also revealed putative HNF1A and NF-kB TFBS in DEFB-VL. GR and NF-kB binding sites have previously been found in the promoters of defensins and suggest antimicrobial activity (Brahmachary et al. 2006). Despite the similarity between DEFB-VL and the beta-defensins, the spacing of cysteines within the DEFB-VL peptide differs slightly from the beta-defensins, and its amino acid sequence is more similar to that of the OvDLPs than it is to that of the beta-defensins (Supplemental Table S1).

Dating of OvDLP gene duplication
The OvDLPs and DEFB-VL fall within a pseudoautosomal region on the sex chromosome chain that shares conserved synteny with human chromosome 8p and chicken chromosome 3q (Fig. 3). We propose that the gene DEFB-VL, which lies within 5 kb of the venom OvDLP genes, gave rise to these genes through gene duplication. We date this duplication to ~192 million years ago. Subsequent duplications of OvDLPs occurred around 98 and 47 million years ago. These venom peptides have thus evolved prior to the divergence of the platypus and the echidna 17–34 million years ago (Gemmell and Westerman 1994; Janke et al. 2002; Belov and Hellman 2003). This suggests that the last common ancestor of echidnas and platypuses may have been venomous. However, although extant male echidnas have a venom delivery system similar to that of the platypus, the crucial gland does not migrate to the same position, the spur and its associated os calcis are less than half the size of the platypus spur–os calcis complex (Temple-Smith 1973; Temple-Smith and Grant 2001), and the complex cannot be locked in position for spurring (Temple-Smith 1973). This suggests that the echidna crural system may be nonfunctional. There are no published records of echidnas using their spurs, and given that echidnas possess strong spines that provide ample defense against predators, we propose that the crural system, and perhaps its venom peptides, may have been lost during the evolution of the echidna lineage but retained in the platypus lineage for use in defense and conspecific aggression.

Convergent evolution of venom molecules
The venom of some sauropsid reptiles contains crotamine and crotamine-like peptides (vCLPs) (Torres and Kuchel 2004; Fry et al. 2006), which have a similar disulfide bonding pattern and...
structure to the beta-defensins (Zhao et al. 2001). Our phylogenetic analyses show that these genes appear to have evolved independently from genes belonging to the same ancestral beta-defensin synteny group as the platypus OvDLPs (Fig. 5): Lizard vCLPs and chicken beta-defensins 1, 2, and 11 share a common ancestor, and snake venom crotamines and mouse beta-defensin 51 share a common ancestor. The platypus OvDLPs share a common ancestor with mouse beta-defensin 33.

The independent evolution of both reptile venom crotamines and vCLPs and platypus OvDLPs are not the only examples of venom evolving from antimicrobial peptides. Other groups of antimicrobial proteins, including gamma-thionins and whey acidic protein (WAP) proteins, appear to be similar to animal peptide toxins (Kaplan et al. 2007). Similar selective pressures on antimicrobial peptides (which evolve to target and kill a wide range of rapidly evolving microbes) and venom peptides (which have evolved to specifically cover a wide range of prey) suggest an evolutionary connection between antimicrobial and venom peptides (Kaplan et al. 2007).

In the platypus, antimicrobial peptides are not the only molecules that have given rise to venom peptides. We have also characterized the genes of the only other two sequenced venom components, OvCNP and OvNGF, which have homologs in other species that function in nontoxin roles (Supplemental material 3). Homologous proteins too can be found in reptile venom, and phylogenetic analysis suggests that these venom components may also have originated independently in reptiles and platypuses (Supplemental material 3). Clearly, recruitment of genes involved in nonvenom cellular pathways is a common feature in the evolution of venom, when it is considered that at least 18 molecules in reptile venom have evolved in this way, including acetylcholinesterase, CRISP (cysteine-rich secretory protein), CVF (cobra venom factor), and Factor X (Fry 2005).

Conclusions

We have identified genes in the platypus for beta-defensins and the earliest known alpha-defensins. We have shown that venom molecules have evolved independently in the platypus and sauropsid reptiles, and we propose that there are specific protein motifs that are preferentially selected for evolution to toxin molecules (Fry 2005). Independent origin of venom molecules appears to coincide with independent origin of venom glands: Snake venom glands evolved from modified salivary glands, whereas platypus venom glands evolved from modified sweat glands (Temple-Smith 1973).

Based on the existence of several species of venomous shrews, it has been proposed that the ancestral mammal may have been venomous (Dufton 1992). Our findings do not support this and nor do those of others who have discovered the fossil of an extinct venomous insectivore that has evidently evolved venom independently from the extant insectivores (Fox and Scott 2005). Instead, we hypothesize that a phylogenetic analysis of shrew venom molecules, impossible at the moment because of the lack of a venomous shrew genome and difficulties in obtaining venom tissues (Dufton 1992), would show an independent origin of these venom molecules from those in platypus and snake venom. However, as our results show, there appears to be a preponderance of certain types of molecules that evolve to have venom function; we speculate that some shrew venom components will be similar to those in reptile and platypus venom.

The availability of the platypus genome has provided an intriguing glimpse into the evolutionary past of mammals and unique insight into the convergent evolution of venom systems by showing that duplication of immune genes has independently given rise to venom molecules several times in the vertebrate lineage. The platypus has much more to offer the field of comparative biology, and given the vast number of useful compounds that have been developed from reptilian venom (Dufton 1992; Fry et al. 2006), platypus venom has considerable potential for the discovery and development of novel therapeutic drugs.

Methods

Platypus genome searching

Alpha- and beta-defensins and OvDLPs

Platypus alpha- and beta-defensins were identified by searching the six-frame translation of the platypus genome using a custom-
built alpha-defensin profile HMM and the Pfam beta-defensin profile HMM (PF00711) with HMMPer (http://hmmer.janelia.org). Genomic sequences that aligned to the HMM were extracted. Peptides that contained stops were discarded.

Next, 267 known mammalian beta-defensins (Patil et al. 2005) were aligned to the platypus genome using tblastn (Altschul et al. 1997). The sequences of all HSPs with E-values < 1 were extracted and translated. Once again, peptides that contained stops were discarded. The presence of the defensin motif was checked by searching with the alpha- or beta-defensin HMMs and manual inspection. Peptides that did not match the profile HMM or did not contain the six-cysteine motif were discarded.

The HMMPer and filtered BLAST data sets were then merged using overlap on the genome. Gene prediction using GenScan (Burge and Karlin 1997) and GenomeScan (Yeh et al. 2001) was performed around each of the features identified in this way. If a defensin motif was found on a contig, gene prediction was performed on the entire contig. For ultracontigs and chromosomes, sequence from the neighborhood was extracted. Predicted proteins were recursively checked using the signal peptide using SignalP (Bendtsen et al. 2004) and a defensin propeptide using a Pfam model (PF00879). Next, these sequences were checked for the presence of a signal peptide using SignalP (Bendtsen et al. 2004) and a defensin propeptide using a Pfam model (PF00879).
Defensins and the evolution of platypus venom

NMR on DEFB-VL
All NMR experiments were performed at 25°C on a Bruker AVANCE-600 DRX spectrometer using a 5-mm 1H inverse probe. The 1D NMR spectra were acquired in phase-sensitive mode using time-proportional phase detection.

Acknowledgments
We thank the platypus genome consortium for inspiring conversations and the opportunity to work on this project. This work was supported by a grant from the University of Sydney Research and Development Scheme to K.B. and the Australian Research Council to P.K. C.W. is supported by an Australian Postgraduate Award.

References


Holland, N. and Jackson, S. 2002. Reproductive behaviour and food consumption associated with the captive breeding of platypus.

Modeling and synthesis of DEFB-VL

DEFB-VL structural prediction
The DEFB-VL peptide three-dimensional model structure was created from OdVLP-B peptide NMR structure (1zu1) using the MOdELLer program (Sali and Blundell 1993). The “very fast modelling protocol” in MOdELLer (version 6) was implemented in which DEFB-VL fragments were manipulated to adopt similar structures (secondary and tertiary) to those in the analogous fragments in OdVLP-B peptide NMR structure. Sequence similarities between DEFB-VL and OdVLP-B structures were determined using the LALIGN program (Huang and Miller 1991). A similar modeling scheme was implemented in creating a platypus defensin model from human beta-defensin 1 crystal structure (1iju).

Synthesis of DEFB-VL and OdVLP-B
The DEFB-VL mature peptide (RRRRRRPPCVDVNGCQPRGNPCNLRLGACPGRSGCMTPTVAAH) amino acids were assembled manually by stepwise solid-phase synthesis with an in situ neutralization protocol for Boc chemistry (Schnolzer et al. 1992) using benzylation as resin (0.5 mmol, loading 0.49 mmol/g). Boc protected amino acids (2 mmol) were coupled using HBTU and diisopropylethyl amine as an activating agent. The side chain protecting groups were Arg(Tos), Asp(OcHxl), Asn(Xan), Cys-(MeBzl), Gln(Xan), Glu(OcHxl), His(DNP), Ser(Bzl), and Thr(Bzl). Following assembly, the peptide was cleaved from the resin and simultaneously deprotected by treatment with anhydrous HF, using p-cresol and p-thiocresol (50:50) as scavengers at −2°C for 90 min. The crude product was precipitated with cold diethyl ether and then extracted into 50% aqueous acetone containing 0.5% trifluoroacetic acid (TFA) and lyophilized.

Purification of the peptide was achieved by preparative RP-HPLC (Phenomenex Jupiter 10 µ C18 column 250 × 21.20 mm) using a linear gradient of 0%–50% acetone in water and 0.1% TFA over 50 min at a flow rate of 5 mL/min. The purified peptide was characterized by mass spectrometry and oxidized by stirring at 4°C in an aqueous solution of ammonium hydrogen carbonate (0.1 M, pH 8.2) for 3 d. After completion of oxidation, the pH of the solution was adjusted between 3–4 pH units and the oxidized peptide isolated using RP-HPLC. It was then characterized by mass spectrometry. The solution phase (90:10–H2O:D2O, 600 MHz Brucker) NMR spectrum of the fully folded peptide indicated the presence of good structural features that were very similar to OdVLP-B structures previously reported (Torres et al. 2005). The same procedure was used to synthesize the mature peptide of platypus OdVLP-B (IfmFFEIMQACWHSIGVCRDKSERNCK PMAWTYCENRQKCCEY) (Torres et al. 2005).

some (BACs) containing genes of interest. Most procedures, including initiation and maintenance of primary cell cultures, metaphase preparation, C0t-1 suppressor DNA preparation, labeling of BAC DNA, and FISH were performed as previously described (Alsop et al. 2005). Both single-color and two-color hybridizations were performed. For the latter, BACs were labeled differentially with biotin and digoxigenin and coprecipitated with C0t-1 DNA. During the fluorescent signal detection steps, anti-digoxigenin and avidin–FITC antibodies were combined in diluent (1% BSA in 4 × SST). Fluorescein isothiocyanate signals were amplified as previously described (Alsop et al. 2005). The optimal slide denaturation time was −2 min, and 4’,6-diamidino-2-phenylindole (DAPI) in Vectashield was used at 1 µg/mL.
Whittington et al.


Received September 17, 2007; accepted in revised form December 11, 2007.