Published in final edited form as:

Gene. 2014 April 25; 540(1): 37–45. doi:10.1016/j.gene.2014.02.030.

Rem2 in the bullfrog (*Rana catesbeiana*): Patterns of expression within the central nervous system and brain expression at different ontogenetic stages

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1. Introduction

The RGK (Rem, Rad, and Gem/Kir) subfamily of small molecule GTPases belong to the Ras superfamily of GTP binding proteins. The RGK proteins are recognized by a number of characteristics that distinguish them among the Ras superfamily, which includes N-terminal and C-terminal extensions beyond the Ras core as well as a unique G3 motif (Finlin et al., 2000), regulation at the transcriptional level (Maguire et al., 1994; Cohen et al., 1994; Vanhove et al., 1997; Leone et al., 2001; Suyama et al., 2003; Ohsugi et al., 2004; Warton et al., 2004; Finlin et al., 2005; Paradis et al., 2007), a lack of lipid modification for membrane anchorage (Del Villar et al., 1996), and binding of calmodulin and 14-3-3 protein (Béguin et al., 2005a, 2005b, 2006). Functionally, the RGK proteins are associated with modulation of Ca²⁺ currents (Seu and Pitt, 2006), as well as changes in cytoskeletal arrangement (Leone et al., 2001; Béguin et al., 2005a, Fu et al., 2005; Krey et al., 2013).

Among the RGK family, Rem2 is further distinguished by its high expression levels in neural tissue (Finlin et al. 2000). Within neural tissue Rem2 plays a role in very diverse functions including excitatory and inhibitory synapse development (Paradis et al., 2007; Ghiretti and Paradis, 2010), survival of embryonic stem cells (Edel et al., 2010a, 2010b), and regulation of dendrite morphology (Ghiretti and Paradis, 2010, Ghiretti et al., 2014). Like other RGK members, Rem2 has been implicated in Ca²⁺ current modulation (Chen et al., 2005, Finlin et al., 2005, Seu and Pitt, 2006, Leyris et al., 2009, Yang et al., 2012).

In mammals, Rem2 has been well-characterized with the original clone of mammalian *Rem2* described in rat (Finlin et al., 2000). There is a high level of conservation among the Ras core of Rem2 with other members of the RGK subfamily. However, the extended N-terminus of Rem2 appears distinct and may be responsible for functions unique from other RGK subfamily members (Seu and Pitt, 2006). Across vertebrate classes, Rem2 appears

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The origin and development of an individual organism from embryo to adult.

conserved as it has been isolated in two species of fish (Edel et al., 2010b; Hollis et al., 2012). *Rem2* has also been isolated in amphibians *Xenopus laevis*, *X. tropicalis* (Klein et al., 2002). However, unlike in fish, its characterization including tissue specificity in amphibians has yet to be elucidated. A comparative examination of *Rem2* gene expression has shown that in both mammals and fish, the pattern of high *Rem2* expression in the brain relative to other tissues is conserved, yet the general distribution of *Rem2* in the central nervous system between these vertebrate classes is very distinct. In mammals (mice), expression of *Rem2* appears limited to the forebrain (Becker et al., 2008), however, in the brains of adult fish it has been found in every major region of the brain as well as the spinal cord in (Hollis et al., 2012). In both mammals and fish, *Rem2* has been shown to influence the central nervous system regarding neural tissue development, synapse formation, cell proliferation and apoptosis, and stem cell survival (Paradis et al., 2007; Edel et al., 2010a, 2010b; Ghiretti and Paradis, 2010). Unlike mammals, adult fish possess an enormous capacity for neurogenesis and brain repair (Zupanc, 1999) and have zones of cell proliferation throughout the brain and spinal cord (Zupanc et al., 2005). Mammals on the other hand, have zones of proliferation limited to within restricted regions of the telencephalon (reviewed in Ferretti, 2011). Thus, when looking at the comparative brain cell proliferation and plasticity, an important question is what molecular components promote widespread adult neurogenesis in some species rather than others? Whether the distribution of *Rem2* expression has any reflection on these abilities in the vertebrate brain is unknown.

Like fish, adult anuran amphibians are a vertebrate class with known zones of cell proliferation in the telencephalon, diencephalon and mesencephalon and, to a much lesser extent, in the rhombencephalon (Raucci et al., 2006; Almlí and Wilczynski, 2007; Simmons et al., 2008). However, unlike fish (and urodele amphibians), the adult anuran amphibian central nervous system appears to lack neurogenesis in the spinal cord and also lacks the capacity for spontaneous regeneration overall (Yoshino and Tochinali, 2004; Endo et al., 2007). To further examine *rem2* expression for phylogenetic analysis, this study characterized *Rem2* and examined its gene expression in the adult bullfrog (*Rana catesbeiana*). Furthermore, because there is an ontogenetic decline in the ability of the central nervous system to regenerate in anuran amphibians, (Sims, 1962; Forehand & Farel, 1982; Beattie et al., 1990; Ten Donkelaar, 2000), we also examined *rem2* expression in the brain of the bullfrog at different stages of development. The present study characterizes *Rem2* in an anuran amphibian species, the bullfrog. Furthermore, we examine the tissue specificity for the amphibian *rem2* gene as well as its expression profile across major regions of the central nervous system and finally its relative brain expression levels at different stages of development.

2. Materials and methods

2.1 Animals

Bullfrogs at different stages of life were obtained in the spring (mid-March) from The Sullivan Company (Nashville, TN), and additional adults were purchased in the late spring (early June) from both The Sullivan Company and Ward's Science (Rochester, NY). The life stages were based on observations in Ranids (*R. pipiens*) as described in Rugh (1951),

while the boundary for metamorphic climax was based on Duellman and Trueb (1986) and Shi (2000). In brief, metamorphic climax was defined as the period when a dramatic transformation of tadpole organs into their adult form occurs, including resorption and/or degeneration of tadpole-specific structures (such as the tail and larval mouthparts), as well as completed development of adult-specific tissues such as the limbs and jaw. The life stages obtained were divided into tadpole stages before metamorphic climax, which included those with no limbs, or hind limb buds with a foot paddle, but no distinction of digits (between Stages I-VII) and those tadpoles with small hind limbs and distinguishable digits on the foot paddle (Stages X-XV). The latter stages have yet to develop fore legs. The stages after metamorphic climax included small, fully metamorphosed juveniles (mass less than 40g) and large adults (mass between 300 and 575g). Immediately upon arrival, bullfrogs were anesthetized in 10% ethyl 4-aminobenzoate (benzocaine) and then sacrificed by rapid decapitation.

2.2 Tissue collection and RNA isolation

After sacrifice, tissues were collected, weighed, and immediately homogenized in QIAzol® Reagent (QIAGEN) for total RNA isolation using the QIAzol® RNeasy Lipid Tissue Mini Kit (QIAGEN) along with the RNase-Free DNase Set (QIAGEN) to remove genomic DNA. For the isolation of the full-length bullfrog *rem2* gene, whole brain tissue was collected. For tissue specificity of the bullfrog *rem2* gene, tissue was collected from six bullfrogs and included the brain, liver, stomach, intestine, skeletal muscle, spleen, lung, ventricle, conus arteriosus, pancreas, and kidney. For the regional distribution of gene expression of the *rem2* genes in the bullfrog central nervous system, brains were removed from six bullfrogs and the olfactory bulb, cerebrum, diencephalon, midbrain, cerebellum, and hindbrain were isolated from each as was a portion of the spinal cord.

2.3 Molecular cloning and sequencing of full-length *rem2* genes

Bullfrog RNA was reverse transcribed from one microgram of total RNA using Improm II Reverse Transcriptase (Promega). The cycling conditions were 25°C (5 min), 42°C (60 min), and 70°C (15 min). Samples were stored at -20°C. To initially isolate a fragment of the bullfrog *rem2* transcript using end point PCR, primers were designed based on the predicted *rem2* sequence of another anuran amphibian, *Xenopus tropicalis* (GenBank RefSeq XM_002941509.1). Primers used in this and all subsequent assays are listed in Table 1. The cycling conditions for end point PCR were 94°C (5 min) followed by 35 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min). Sequence analysis (described below) of the initial isolated fragment was then used to design bullfrog *rem2* gene-specific primers which were paired with primers that were, again, specific to the same predicted *rem2* sequence of *X. tropicalis* for use in standard end point PCR to acquire a larger bullfrog *rem2* fragment. After isolating this larger fragment of the bullfrog *rem2* gene (verified through sequence analysis), 3' and 5' RACE assays were performed using the RNA ligase-mediated Rapid Amplification of cDNA Ends (RLM-RACE) Amplification kit (BD Biosciences) according to the manufacturer's instructions. Four primers were designed (primary and nested) and the full-length bullfrog *rem2* gene was isolated. The RLM-RACE cycling conditions were 94 °C (3 min) followed by 35 cycles of 94 °C (30 s), 59 °C (30 s) for 3'Race and 60°C (30 s) for 5'Race, 72 °C (1 min), followed by 72 °C (7 min). Finally, verification of the full length

open reading frame (ORF) was performed using primers specific for the extreme 5' and 3' end of the ORF.

For cloning, PCR products were separated on agarose gels, visualized under UV light and the appropriate sized band was cut and cloned into TOPO pCR 2.1 (Invitrogen). Plasmid preparations were made from randomly selected positive colonies which were then prepped for sequencing using the Purelink Quick Plasmid Mini Prep Kit (Invitrogen). Sequencing of the isolated DNA was performed on an automated sequencer (ABI 3730) at the Arizona State University DNA Laboratory (Tempe, AZ, USA). Each product was cloned and sequenced a minimum of 20 times. Four to five additional sequences were also obtained by directly sequencing DNA from PCR reactions when a single product of expected size was acquired. The DNA was purified directly from the PCR reaction using the ExoSAP-It kit (USB/Affymetrix) following the manufacturers' instructions. If multiple transcripts were present in gel electrophoresis analysis, the appropriate sized band was cut and gel purified using the QIAquick Gel Extraction kit (QIAGEN) following the manufacturers' instructions. Direct sequencing of the isolated DNA was also performed on an automated sequencer (ABI 3730) at the Arizona State University DNA Laboratory.

Sequence files were analyzed locally using the Basic Local Alignment Search Tool (BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), specifically, blastx against the NCBI nonredundant (nr) protein database and blastn against the NCBI nucleotide (nt) database. Nucleotide and deduced amino acid alignments were performed using an online ClustalW2 alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

2.4 Gene expression analysis

For tissue specificity, tissue samples, including whole brains, were taken from six individual adult bullfrogs. Likewise, for the central nervous system distribution assay, the brains of six individual adult bullfrogs were each portioned into the major regions previously mentioned. For the ontogenetic assay, whole brains were acquired from three individual tadpoles or frogs from each developmental stage. Gene-specific primers were used for real time PCR (rtPCR) (see Table 1). rtPCR was carried out following the manufacturer's instructions for Power SYBR Green RNA-to-Ct 1-Step kit rtPCR method (Applied Biosystems). A total of 60ng of total RNA was used per 20µl reaction (3ng/µl per reaction). All reactions were performed in triplicate. Reverse transcription was carried out using ArrayScript™ UP Reverse Transcriptase (Applied Biosystems) and the generated cDNA was used as a template for PCR amplification. The cycling conditions were, for the reverse transcription, 48°C (30 min), which was then followed by PCR consisting of 95°C (10 min), then 40 cycles of 95°C (15 sec), and 60°C (1 min). Reverse transcription and PCR amplification were performed on an Eppendorf Mastercycler® ep *realplex*² thermal cycler. Gene expression was normalized using a 154bp fragment of the *ribosomal protein L8 (rpl8)* gene using gene-specific primers following the methods of Hasebe et al. (2008) and Bilesimo et al. (2011). Like *rem2*, expression of *rpl8* was performed using real-time PCR following the manufacturer's instructions for the Power SYBR Green RNA-to-Ct 1-Step kit rtPCR method (Applied Biosystems). The cycling conditions for *rpl8* were as those for *rem2*. Standard curves for both *rem2* and *rpl8* were generated from total RNA of bullfrog brain tissue.

Individual 20 μ l reactions ranged from 10ng to 5000ng in total RNA (2ng/ μ l to 250ng/ μ l) performed in triplicate, which were used to compute expression values for both genes.

Statistical significance for differences in expression levels across tissues, central nervous system regions, and stages of life was evaluated by One-Way ANOVA ($P < 0.05$) followed with either Tukey's multiple comparison test ($P < 0.05$) or post-test for linear trend ($P < 0.05$), as well as t-test ($P < 0.05$). All statistics were performed using GraphPad Prism software.

3. Results

3.1 Molecular cloning and sequencing of the full-length bullfrog rem2 gene

The full-length bullfrog *rem2* gene was isolated by cDNA cloning of PCR products from 5'-RACE and 3'-RACE and had an ORF of 876bp (Fig. 1). BLAST searches (blastx) in the GenBank database resulted in a deduced amino acid sequence having highest identity (80%) with that of the predicted *X. tropicalis* Rem2 protein (GenBank RefSeq XP_002941555.1) followed by the Rem2 proteins of different vertebrates, of which shared between 55% and 63% identity. Thus, the nucleotide sequence coded for an amphibian Rem2 protein and therefore, was named *R. catesbeiana rem2* (GenBank RefSeq KJ026766). The full-length cDNA sequence of bullfrog *rem2* was 1299bp, including a 162bp 5' untranslated region (UTR), an 876bp ORF, and a 261bp 3' UTR (excluding the poly (A)⁺ tail).

3.2 Deduced amino acid sequence alignment

The bullfrog Rem2 protein consisted of 292 deduced amino acids (see Fig. 1). When aligned in pairwise comparisons with the mammalian RGK subfamily members (using those of *Homo sapiens*), the bullfrog Rem2 protein aligned most closely with that of REM2 (61.3% identity. GenBank RefSeq NP_775798.2), as compared to RAD (47.6%. GenBank RefSeq number NP_004156.1), GEM (45.2%. GenBank RefSeq NP_859053.1), or REM (43.8%. GenBank RefSeq NP_054731.2). The bullfrog Rem2 protein shares conservation with the Ras superfamily G1, G3, G4, and G5 GTP binding sites, but like the RGK subfamily, lacks the threonine residue of the G2 GTP binding domain that is conserved throughout the rest of the Ras superfamily (Thr³⁵ in H-RAS; Colicelli, 2004) (Fig. 2). In contrast, RGK subfamily members are distinct from the Ras superfamily in that they possess altered residues (Glu⁸⁴ in GEM, Pro⁸⁹ in REM and Pro¹⁰⁰ in RAD, and Ser¹²³ in REM2) of the residue equivalent to the highly conserved Gly¹² in H-RAS within the G1 domain (Colicelli, 2004; Correll et al. 2008). Consistent with this, rather than a glycine residue, the bullfrog Rem2 protein possesses a threonine (Thr⁸¹) in this position when aligned with mammalian (Human) Rem2, as does the Rem2 proteins of other non-mammalian vertebrates as seen in that of the zebrafish Rem2 protein (Asn⁸³) and the predicted anole Rem2 protein (Ser⁶⁶) (see Fig. 2). Furthermore, like the RGK members, within the G3 box, where the Ras superfamily possess a conserved glycine (Gly⁶⁰ in H-RAS) the bullfrog Rem2 protein possesses a glutamic acid residue (Glu¹³⁰). Thus, while bullfrog Rem2 protein shares the conserved DXWE G3 motif with the rest of the RGK subfamily members, it is divergent from the consensus DTAGQ motif found in the rest of the Ras superfamily. However, the bullfrog does possess the Ras superfamily consensus glutamine (Q: Gln¹³¹) as seen with mammalian Rem2 (Gln¹⁷³) and

Rad (Gln¹⁴⁸). The Rem2 proteins of other vertebrates also follow this pattern (zebrafish; Gln¹⁴³, predicted anole; Gln¹¹⁶; see Fig. 2).

Within the N-terminal extension of the RGK members, the initial four amino acids of the bullfrog, Met¹Thr²Leu³Asn⁴, are conserved with those of the predicted Rem2 protein of *Xenopus* as well as human RAD, GEM, and REM. However, unlike the other members of the RGK family, the corresponding amino acids of human REM2 are preceded by an additional 37 amino acids and only the threonine and leucine (Thr³⁹Leu⁴⁰) are conserved. Furthermore, the predicted anole and zebrafish Rem2 proteins lack homology with regard to the initial four amino acids on the N-terminal end with the exception of the initial methionine. In contrast, in the alignment between the bullfrog Rem2 protein and the Rem2 proteins of the other vertebrates, there is high homology in the N-terminal extension between the bullfrog Rem2 residues Arg²⁷ and Pro⁵¹. Pairwise alignments of this region of the bullfrog Rem2 protein with those of the other vertebrate Rem2 proteins (alignments not shown) indicated higher identity than the pairwise comparisons of the entire proteins (predicted *Xenopus*; 92%, predicted anole; 83%, zebrafish; 72%, human, 96%). On the other hand, identity between this same region of the bullfrog Rem2 protein and the other RGK family members either did not deviate much from the pairwise comparisons of the entire protein (RAD; 46%), or was much lower (GEM; 26%, REM; 23%). On the C-terminal end, the bullfrog Rem2 protein conformed to the conserved CHXLXVL C7 motif with the rest of the RGK subfamily members.

3.3 Tissue specificity of the bullfrog rem2 gene

The pattern of expression of the bullfrog *rem2* gene showed the brain as having significantly greater levels of expression than any other tissue (Fig. 3). Relative to the brain, all tissues were extremely low in *rem2* expression with no other tissue significantly greater or lower than any other tissue (save for the brain). After the brain, the kidney and the pancreas showed the highest levels of *rem2* expression, again, though not significant. The lowest level of expression observed was in the skeletal muscle tissue.

3.4 Central nervous system distribution of the bullfrog rem2 gene

In general, there was differential expression of the *rem2* gene between the different regions of the bullfrog central nervous system ($P < 0.0001$). The level of expression for the *rem2* gene in the central nervous system was highest in the more anterior regions with the cerebrum showing significantly higher levels of expression than any other region ($P < 0.001$; Fig. 4). Apart from the cerebrum, the diencephalon also had significantly greater levels of *rem2* expression than the other regions ($P < 0.001$, excepting the midbrain at $P < 0.05$). In contrast, the cerebellum, hindbrain, and spinal cord had very low levels of *rem2* expression. The midbrain and olfactory bulb had similar levels of *rem2* expression, however, only the midbrain had significantly greater levels when compared to the very low levels in the cerebellum, hindbrain, and spinal cord ($P < 0.05$).

3.5 Brain expression of rem2 at different ontogenetic stages

Because anurans have distinct stages of development, we examined the levels of *rem2* gene expression in the whole brains of these animals at different stages of development (as was

able to be provided) to examine whether brain *rem2* expression levels change as the frogs metamorphose (Fig. 5). A significant change in brain *rem2* gene expression was observed throughout the different stages assayed ($P < 0.05$; Fig. 5A). Furthermore, there was a significant linear trend identified as well ($P < 0.05$; Fig. 5A). In this case, the tadpole stages, which had similar levels of *rem2* expression, had lower levels of *rem2* expression relative to those of the fully metamorphosed juveniles and adults. When the different stages were re-grouped into two separate stages based on stage relative to metamorphic climax, in other words, pre-metamorphic climax (I-VII and X-XV grouped) versus post-metamorphic climax (juveniles and adults grouped), there was a significant increase in *rem2* expression levels at post-metamorphic climax (t-test, $P < 0.05$; Fig. 5B).

4. Discussion

4.1 Molecular cloning and sequencing of the full-length bullfrog *rem2* gene

In this study, we isolated, cloned, and characterized a *rem2* gene from the adult bullfrog brain. The Rem2 protein was first described in mammals (Finlin et al., 2000), and to date, has been identified in a large number of other mammalian species. Furthermore, the *rem2* gene has also been isolated and characterized in teleost fish (Edel et al., 2010b, Hollis et al., 2012). Recent work isolated the *rem2* gene from the brain of another anuran amphibian, *X. laevis* (Ghiretti et al. 2014). Using RACE PCR, we acquired both the 5' and 3' ends of the bullfrog *rem2* gene and to our knowledge, are the first to report the full-length sequence of the *rem2* gene from the bullfrog central nervous system.

4.2 The deduced amino acid sequences of bullfrog Rem2

At the deduced amino acid level, bullfrog Rem2 was highly conserved, sharing conservation with the Ras superfamily G1, G3, G4, and G5 GTP binding sites, and like the RGK subfamily, it lacks the threonine residue of the G2 GTP binding domain that is conserved throughout the rest of the Ras superfamily (Thr³⁵ in H-RAS; Colicelli, 2004). Previous work in non-mammals, specifically fish, has shown a deviation from a very highly conserved alanine found within the G5 GTP binding domain (Edel et al., 2010b, Hollis et al., 2012). This was not the case with the bullfrog (nor is this identified in the predicted proteins of *Xenopus* and the anole). Thus, deviation of this nucleotide in Rem2 proteins deduced from isolated genes so far remains within fish.

The Rem2 protein possesses N-terminal and C-terminal extensions that flank the Ras-homologous G domain (Finlin et al., 2000). These extensions are characteristic of the RGK subfamily. It has been speculated that the effects of mammalian Rem2 upon Ca²⁺ channel kinetics is due to its extended N-terminus and C-terminus (Seu and Pitt, 2006), while the C-terminal end possesses a functional Ca²⁺/CaM binding site (Finlin et al., 2005). Thus, these extensions may provide further functional distinctions among RGK proteins yet to be elucidated. The bullfrog Rem2 protein possessed both extensions. With regard to the N-terminal extension, that of mammals is unique compared to that of other RGK subfamily members in that it is extended with signal transduction binding sites, which likely allows Rem2 to be a transcription regulator as is speculated in its ability to hold human embryonic stem cells in a state of pleuropotency by blocking p53 and cyclinD1 transcription (Edel et

al., 2010a). Within the N-terminus of the bullfrog Rem2 protein there is a specific region of high homology with only the Rem2 proteins of the other vertebrates (from Arg²⁷ to Pro⁵¹), but is lacking with the other RGK members. Within this region resides a phosphorylation site (Correll et al., 2008; Ghiretti et al., 2013), as well as a site of 14-3-3 protein interaction (Béguin et al., 2005a). Whether other functional regions reside in this area remains to be elucidated.

Like the deduced amino acid sequences of Rem2 of other non-mammalian vertebrates, the N-terminal extension of the bullfrog Rem2 deduced amino acid sequence lacks a large portion of the mammalian Rem2 N-terminal extension, as based on Seu and Pitt (2006). In the case of our alignment, the mammalian Rem2 deduced amino acid sequence precedes the bullfrog amino acid sequence on the 5' end by 37 residues. This makes up the largest regional amino acid number difference between the two, as mammalian Rem2 has a total of 48 additional residues compared to the bullfrog. This discrepancy occurs just upstream of the aforementioned, highly conserved regions of the Rem2 proteins.

On the C-terminal end, the bullfrog Rem2 protein conformed to the conserved CHXLXVL C7 motif with the rest of the RGK subfamily members, while the C-terminal phosphorylation sites (Correll et al., 2008; Ghiretti et al., 2013), as well as the site of 14-3-3 protein interaction (Béguin et al. 2005a), were conserved as well. The bullfrog Rem2 protein offers further phylogenetic insight into the RGK subfamily, particularly with regard to areas of conservation in the distinct N-terminal and C-terminal extensions of Rem2. On the other hand, areas of discrepancy may require further comparative studies, particularly involving urodeles and birds, so as to offer a more comprehensive phylogenetic perspective into putative functional domains.

4.3 Tissue specificity of the bullfrog rem2 gene

The bullfrog brain had the highest levels of *rem2* gene expression which is consistent with other vertebrates (Finlin et al., 2000; Becker et al., 2008; Edel et al., 2010b; Hollis et al., 2012). In fact, though *rem2* gene expression was found in all eleven tissues assayed, only the brain showed significant levels of the *rem2* gene as it was exceptionally low in every other tissue. This is a bit of a contrast with other adult vertebrates. For example, in the rat, while *Rem2* expression is highest in the brain, the kidney also has very high basal levels as well (Finlin et al., 2000). In the bullfrog, kidney levels were very low, however, relative to other tissues, it did have a higher level than most (though not significant). Interestingly, in the mouse, *Rem2* expression levels in the kidney are also low (Becker et al., 2008). Thus, the differences in tissue expression level patterns may be more species-specific than simply typical of any one vertebrate class. In another anamniotic vertebrate, the rainbow trout, though the highest levels of *rem2* were in the brain, the spleen also had high levels relative to other tissues (Hollis et al., 2012). This contrasts with the bullfrog where levels of *rem2* gene expression were exceptionally low. Thus, while *rem2* gene expression consistently appears at high levels in neural tissue relative to other tissues, there appears to be greater variation with regard to tissues outside the central nervous system.

4.4 The expression of the bullfrog *rem2* gene in the central nervous system

The relative expression levels of bullfrog *rem2* in the different brain regions varied with the higher expression levels occurring in the more anterior regions. More specifically, there were relatively higher levels of *rem2* in the olfactory bulb, cerebrum, diencephalon and midbrain, whereas the cerebellum, hindbrain, and spinal cord were very low. This is the first such description of the general distribution of *rem2* in an amphibian brain. An overall similar pattern is seen in the distribution of *rem2* in the salmonid brain (Hollis et al., 2012), which also has the higher levels of *rem2* expression occurring in the anterior regions, with the highest levels occurring in the cerebrum. Also similarly, the lower levels of *rem2* expression in the posterior regions are typified with the cerebellum having the very lowest levels. The one exception however, is that in the bullfrog brain, the posterior regions had barely detectable levels of *rem2*, whereas the rainbow trout has higher levels of *rem2* relative to the anterior regions, particularly in the hindbrain and spinal cord. Because both fish and amphibians maintain strong proliferative capacity in their central nervous systems as adults (reviewed in Grandel and Brand, 2013), coupled with the fact that Rem2 can promote cell proliferation (Bierings et al., 2008), maintain stem cell survival (Edel et al., 2010a), and promote neuronal survival (Edel et al., 2010b), it is possible that the distribution of the *rem2* gene in the adult central nervous systems of anamniotes may reflect a role for Rem2 in regional cell proliferative and or neurogenic capacities. For example, in the hyliid brain, the greatest proliferative activity resides within the telencephalon followed by the diencephalon, whereas a tremendous drop off occurs from the midbrain to the hindbrain (Almli and Wilczynski, 2007). A similar pattern has been found in the adult bullfrog where relatively high levels of cell proliferation occur in the telencephalon, diencephalon (hypothalamus), and mesencephalon (Simmons et al., 2008). Furthermore, neurogenesis, which is lacking in the posterior brain of anuran amphibians, remains in fish (reviewed in Ferretti, 2013), which follows the discrepancy pattern seen in the relative *rem2* level differences between the bullfrog and past work in the rainbow trout. However, in contrast, cell proliferation also occurs in the adult anuran (*R. exculenta*) cerebellum (Raucci et al., 2006), which would appear to contradict the idea that *rem2* patterns of expression might reflect proliferative zones, as previously discussed in fish (Hollis et al., 2012). While the involvement of Rem2 in adult anuran amphibian brain cell proliferation capability and neurogenesis is unknown, given the number of different functions that have been attributed to Rem2 in nervous tissue, the differential expression of the *rem2* gene between the different brain regions likely indicates a significant functional role for this small molecule GTPase in the amphibian central nervous system as supported by recent evidence in *X. laevis* (Ghiretti et al., 2014).

4.5 The expression of bullfrog brain *rem2* at different ontogenetic stages

We examined the relative *rem2* expression levels in the brain of the bullfrog across different stages of its development and found increased expression of brain *rem2* as the animals went from tadpole to adult. Most striking is the considerable increase in brain *rem2* expression from pre- to post-metamorphic climax (see Fig. 5B). It is possible that the shift in *rem2* expression observed occurs during metamorphic climax where anurans transition from an aquatic to an amphibious existence. As mentioned, evidence suggests an influence of Rem2

on cell proliferation (Bierings et al., 2008). Shifts in cell proliferation have been observed in the brain of the bullfrog as it moves from the tadpole stages to the postmetamorphic froglet (juvenile) stage. In certain brain regions, such as the torus semicircularis, increases in mitotic index increase significantly from early tadpole stages to metamorphic climax and froglet stages (Simmons et al., 2006). Additionally, the superior olivary nucleus and dorsolateral nucleus experience cell proliferation increases at metamorphic climax as well (Chapman et al., 2006). In this study, the adults we observed maintained high levels of *rem2* expression as seen in the juveniles, however, it has been noted that while there is an overall reduction in proliferative potential of adults, there is, in contrast, a regional increase in the anterior preoptic area (Raucci et al., 2006).

Beyond proliferation, metamorphic climax is characterized by central anatomical changes marked with connection plasticity thought to reflect the development, maturation, and increase in activity from the periphery as described in the anuran auditory path (Horowitz et al., 2007). Recent work on Rem2 has implicated its role in neuronal plasticity, dendritic morphology, and synapse development (Flynn et al., 2012; Ghiretti and Paradis, 2010; Ghiretti et al., 2013; Moore et al., 2013; Ghiretti et al., 2014). In *Xenopus*, for example, high levels of central plasticity in the visual system (binocular inputs) are observed early post-metamorphosis (Udin and Keating, 1981; Titmus et al., 1999). Furthermore, neural plasticity in both early post-metamorphic and adult frog visual systems (pretectal) have been shown to be influenced by NMDA receptors (Cline and Constantine-Paton 1990; Jardon and Bonaventure, 1992, 1997), while in postmetamorphic frogs, there is evidence that within developing retinotectal projections, maintenance of arbor branches requires stable synapses associated with local inhibition within the remodeling terminal (Cline and Constantine-Paton, 1990). Rem2 is known to promote the development of dendritic spines (sites of NMDA clusters) and excitatory synapses and also functions to inhibit dendritic branching (Ghiretti and Paradis, 2010). In fact, it was recently found that transcriptional regulation of Rem2 inhibits dendritic arborization in the visual system of *X. laevis* (Ghiretti et al., 2014). Thus, the changes seen in *rem2* brain expression in bullfrog development may also reflect shifts in its role in regional changes in central plasticity. Furthermore, because Rem2 is known to be regulated at the transcriptional level (Finlin et al., 2005; Bierings et al. 2008), the question arises as to how such changes in *rem2* brain gene expression in development are governed regarding the physiological state of the animal.

4.6 Conclusion

The Rem2 protein is unique among the small molecule GTPases in its tissue specificity and its functional role in different neural processes such as neural development and synapse formation. In this study, we isolated and characterized Rem2 in an adult amphibian, and give the first report of *rem2* gene expression in the amphibian brain at different stages of its development. The tissue specificity of the *rem2* gene in the bullfrog indicates a conserved pattern across vertebrates from the different classes examined thus far, while the distribution of the *rem2* gene in the central nervous system indicates a putatively conserved pattern in anamniotic vertebrates. Finally, the shift in *rem2* expression in the bullfrog brain across different stages of development may be indicative of a shift in its role as a putative mechanism of amphibian brain plasticity and/or proliferative capacity, which vary in the

lifetime of a species. Due to the relatively high cell proliferative capacity of adult anamniotic vertebrates as compared to mammals, the characterization of Rem2 in the bullfrog brain is important as it will allow further investigation into whether this protein is a mechanism intrinsic to the proliferative discrepancies across the central nervous systems of vertebrates.

Acknowledgments

This project was supported by grants from the National Center for Research Resources (5 P20 RR016461) and the National Institute of General Medical Sciences (8 P20 GM103499) from the National Institutes of Health. Further support came from Furman University's Research and Professional Growth (RPG) and Furman Advantage awards. Special thanks to John F. Wheeler, Jason S. Rawlings, Wade B. Worthen, Katrina M. Morgan, and the Arizona State University DNA Lab.

Abbreviations list

bp	base pairs
cDNA	DNA complimentary to RNA
DNase	deoxyribonuclease
NMDA	N-methyl-D-aspartate
ORF	open reading frame
RNase	ribonuclease
UTR	untranslated region(s)
'(prime)	denotes a truncated gene at the indicated side

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Highlights

We acquired the full-length sequence of the *rem2* gene from the bullfrog brain

Bullfrog *rem2* gene expression was much greater in the brain than other tissues

The bullfrog *rem2* gene is differentially expressed between the major brain regions

An increase in *rem2* brain expression occurs after metamorphic climax

Tissue and brain region *rem2* expression patterns appear conserved in adult anamniotes

5'

AAAATGGAGGCGAGCATCTGTACAGAGAAGACCCAGGCAGAAGACAGGGCTTTGGTGTCTAGAGCCAGGGGAGAACATTGAGCAATACA 90

GCTTCAACGACTCTTCTTGGATCAGGCTATATCACACTTCAACCAAATTCTGTTGTGAAAACCCCTACC **ATG** ACA CTA AAC AAA 174

M T L N K 5

ATA GAG AAG CTT GGT GGT CTA CGG CAA CAT CGA GGA AGT CTT CCA ATT TCT GTA GAC TCA CAC CGG CGT 243

I E K L G G L R Q H R G S L P I S V D S H R R 28

GGC AGT ATG CCC CTG CCC TAC AAA CAC CAG TTG AGA CGT GCA CAA GCT GTT GAT GAG CTT GAC TGG CCA 312

G S M P L P Y K H Q L R R A Q A V D E L D W P 51

AGT GTC CAT TCA GGG TCA TCA GAC TCT GTA AGG TCC TCA GAC AGT AAT CCA GAG CCT GGA GCC TAC AAA 381

S V H S G S S D S V R S S D S N P E P G A Y K 74

GTC ATG TTG GTT GGT GAC ACT GGA GTA GGA AAG ACA ACA CTT GCT GGG ATA TTT GGA GGC CTA AAA GAT 450

V M L V G D T G V G K T T L A G I F G G L K D 97

ACT TTC CCA CAT GAA GCT GAA CAT CCA GAG GAT ACA TAT GAG ATA GAT CTG ATG GTA GAT GGA GAA AAG 519

T F P H E A E H P E D T Y E I D L M V D G E K 120

ACA ACA TTA ATT GTA TAT GAT ATC TGG GAA CAG GCA GGT CCC CAC ACT TGG ATG CAA GAA AGC TGT CTA 588

T T L I V Y D I W E Q A G P H T W M Q E S C L 143

GAA ATG GGG GAT GCC TTC CTT CTA ATC TTT TCA GTG ACA GAC AGA AGT AGC TTT CAG CAT CTA CCT GGT 657

E M G D A F L L I F S V T D R S S F Q H L P G 166

CTT CTT CTG AAA CTG CGC TCT GCT CGT CCA CAC CGC CAC ATT CCA GTC ATT TTT GTT GGC AAT AAG GGA 726

L L L K L R S A R P H R H I P V I F V G N K G 189

GAT CTA GCT CGC TCC AGA GAG GTC AGC ATG GAA GAG GGA CGC TCC CTG GCA GGC ATG CTA AGC TGT AAG 795

D L A R S R E V S M E E G R S L A G M L S C K 212

TAC ACA GAG ACA TCT GCT GCC CTT CAC CAT AAT ACA CAG GAG TTG CTT GAG GGG GTT GTC AGA CAG ATT 864

Y T E T S A A L H H N T Q E L L E G V V R Q I 235

CGC CTT CGA AAA GAT GAC GTA CAA CAC AAT CCC CAG CCA GCC ACC CTC CTA CCA CCA GGT CGC AGA GAG 933

R L R K D D V Q H N P Q P A T L L P P G R R E 258

AGT CTG ACC AAA AGA GCA AGG CGA CTT TTG CAG GGG ATT ATG GGA AAA CAT AAA GGT TTC TTC AAG CAG 1002

S L T K R A R R L L Q G I M G K H K G F F K Q 281

AGG TCG AAA TCA TGC CAT GAC CTT TCT GTC CTG TGA CTAGTGAACCTATCTGTCCATGTGCCATAAGGAAGTCGGACTG 1081

R S K S C H D L S V L 292

GCCACATGACCTTGTGCCTGCCACAGAAGTCGGAAAACTTCTTATATGGAACCAAATGAACAGAACATGGGCCTCTTAAACCGATATTTCT 1172

ATGACCCCAATCTGAGACAAAATAACAGCAACACTATGAGAATACTTAGAGATGAAGCACACTACCTGGTATAAATACTTATTGAAAAAT 1263

AATGAATAAAATCTCCAAAGGCGTTAATACTGTAAAAAAT 1309

3'

Fig. 1.

Nucleotide and deduced amino acid sequences of bullfrog *rem2*. The nucleotide sequence is shown 5' to 3' and numbered to the right. The deduced amino acid sequence is in the single letter amino acid code, in boldface, and numbered to the right in italics and boldface. The start and stop codons are shaded in dark gray and underlined. The location of primers used in the Real-Time PCR gene expression assays are underlined.

Bullfrog	-----MTLN-----KIEKLGGLRQHRGSLP	20
Xenopus	-----MTLN-----KKEKLGGLQQRHGSFP	20
Anole	-----MALLP	5
Zebrafish	-----MSDQGYGLT	9
Human REM2	MHTDLDTMDMDTETALCPGSGRRASFPPTPEADATLL-----KKSEKLLAELDRSGLP	57
Human RAD	-----MTLNGGGSGAGSGRGGGQERERRRGSTP	28
Human GEM	-----MTLN-----NVTMRQGTV	13
Human REM	-----MTLN-----TEQEAQTPHLHRRASTP	20
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Bullfrog	ISVDS-HRRGSMPLP---YKQLRR-AQAVDELDP---SVHSGSSDSVRSSDSNPE-----	69
Xenopus	LSSGP-RRRGSMPLP---YKQLRR-TQAVDELDP---SVHSGSSDSIRSSSESPD-----	69
Anole	TLPGAGPRRGSMPLP---IKQLVR-ASAVDELDP---SSPEVGS---HAGVSPAG-----G	54
Zebrafish	LSAAPPLRRGSTPLP---IKQLRREEAVSEEDWTFGLNESATPQISVTNIQDSPSLKPPQKY	71
Human REM2	SAPGAPRRRGSMPLP---YKQLRR-AQAVDELDP---QASSGSSDSLGSGEAFAQ-----K	111
Human RAD	WGPAFLHRRSMFVDERDLQAALTP-GALTAAGTG-TQGP--RLDWPEDESLSGG-SDSD	88
Human GEM	-GMQPPQQRWSIPADGRHLMVQKEP-HQYSHNRHSA-TPEDHCRRSWSSDSDTSDVIS---SESG	72
Human REM	LPLSPRGHQPGRLSTVPSTQSQHPR-LGQSASLNPT-QKPSAPDDWSSSDSEGS-----W	77
<hr/>		
	G1 G2 G3	
Bullfrog	PGAYKVMVLGDTGVGKTTLAGIFGGLKDT---FPHEAHPEDTYEIDLMDVGEKTTLIVYDIWEQ	131
Xenopus	TGVYKVMVLGDSGVGKSTLAGIFGGVEDT---FPHGSEHPEDTYERNLLVDGEKTTLIVYDIWEE	131
Anole	RGPYKVMVLGESGVGKTALAAIFGGLKGS---VHHEESTEDSYERRFCVDDEEVILYDIWDQ	116
Zebrafish	HGPLRIVLLGQNGVGKSSALCLAGLSDRSLSDSETQAADGYPRRVTVDDDESSILVYDNWKQ	136
Human REM2	DGIFKVMVLVGESGVGKSTLAGTFGGLQGD---SAHEPENEDTYERRIMVDEEVLVYDIWEQ	173
Human RAD	ESVYKVLVLGAPGVGKSALARIFGGVED---GPEAEAAG-HTYDRSIVVDGEEASLMVYDIWEQ	148
Human GEM	NTYRVVLLIGEQGVGKSTLANIFAGVHDSM---DSDCEVLGEDTYERTLMVDGESATIIILDMWEN	135
Human REM	EALYRVVLLGDPGVGKSTLASLFAGKQE---RDLHEQLGEDVYERTLTVDGEDTTLVVVDWTWEA	138
	GXXGXGKS DXWE	
<hr/>		
	G4	
Bullfrog	AGP-HTWMQESCLEMGDAFLIFSVTDRSSFQHLPGLLKLRSARPHRHPIVIFVGNKGLDLSR	195
Xenopus	AGS-QSWMQDSCLQMGDAFLIFSVTDRSTFQRLPSLLLQLRTARPHRHPIILVGNKGLDLSR	195
Anole	ADP-GGWMQESCLQMGDAFLMVSVTDRSFTRVPPILRLRAGCPRSDPPILVGNKSDLSR	180
Zebrafish	ELS-----TLQC-EVCLVFSLTDRSFHRIQLRLLLRESLP--HTPIILVGNKSDLSR	190
Human REM2	GDA-GGWLRDHCLQMGDAFLVSVTDRSFQKVPETLLRLRAGRPHHDLVILVGNKSDLSR	237
Human RAD	DG--GRWLPFGHCAMGDAYVIVSVTDKGSFEKASELRVQLRRARQTDVPIILVGNKSDLSR	211
Human GEM	KGE-NEWLHDCMQVGDAYLIVYSITDRASFEEKASELRQLRRARQTEDIPILVGNKSDLSR	199
Human REM	EKLDKSWQESCLQGSAYVIVYSIADRGSFESASELRQLRRTHQADHVPIILVGNKADLSR	203
	NKXD	
<hr/>		
	G5	
Bullfrog	EVSMEGRSLAGMLSKYETSAALHHTQELLEGVVRQIRLRKDDVQHNPQATLLPP-----	254
Xenopus	EVNMEGRSLAGMLNCKYTEISAALHHTHELLEGVVRQIRLRKDEGEHSLQTPILLTP-----	254
Anole	EVSREESRLAVMMNCKHIETSAALHHTTELFEGVVRQIRLRHRRREAAEGLAKEGAG-----	239
Zebrafish	EISTEEAHSSAMFGCLYLELSVSLDHRNTDLLEAAVRAARGHSLGPGWTAEGSPSAAR-----	249
Human REM2	EVSLEEGRHLAGTSLCKHIETSAALHHTRELFEAGVRQIRLRGRNHAGGQRPDPGSPGPAPP	302
Human RAD	EVSVDEGRACAVVFDCKFIETSAALHHTVQALFEAGVRQIRLRDSKEANARRQAGTR-----	269
Human GEM	EVSVEGRACAVVFDCKFIETSAAVQHNVKELFEAGVRQIRLRDSKEKNERRLAYQK-----	257
Human REM	EVSVEGRACAVVFDCKFIETSATLQHNVAELFEAGVRQIRLR--RDSAAKEPPAPR-----	259
	EXSA	
<hr/>		
	* * C7	
Bullfrog	GRRESLTKRARRLLQGLMG-----KHKG--FFKQRSKSCHDLSVL- 292	
Xenopus	GRRESLTKRARRLLQGLMG-----KHKG--FFKQRSKSCHDLSVL- 292	81.9%
Anole	GRRESLTKKAKRFLSLVP-----RNGR--FFKQRSKSCNDLSVL- 277	56.9%
Zebrafish	RESLTSAKRFLSLVPRSHLGRERDREPGRDRDLRSRHRGSRMLBQKRSKCHDLSALM 307	40.1%
Human REM2	ARRESLTKKAKRFLANLVP-----RNAK--FFKQRSKSCHDLSVL- 340	61.3%
Human RAD	RRRESLTGKKAKRFLGRIVA-----RNSRKMAFFAKSKSCHDLSVL- 308	47.6%
Human GEM	RKESMPRKARFVGKIVA-----KNNKNMAFKLKSCHDLSVL- 296	45.2%
Human REM	RPASLAQRARRFLARLTA-----RSARRALKRSKCHNLAVL- 298	43.8%
	CHXLXVL	

Fig. 2.

Amino acid sequence alignment of the bullfrog Rem2 with the Rem2 proteins of other species from different representative vertebrate classes. Included in the alignment are the Rem2 proteins of different representative vertebrate classes, as well as the representatives of the RGK family (from Human). The Rem2 proteins, listed from top to bottom below the bullfrog Rem2 protein are those of *X. tropicalis* (predicted; GenBank RefSeq XP_002941555), the anole (predicted; GenBank RefSeq XP_003229372.1), the zebrafish (GenBank RefSeq NP_001116518.1), and human (GenBank RefSeq NP_775798.2). The RGK members other than Rem2 then includes human RAD (GenBank RefSeq NP_004156.1), human GEM (GenBank RefSeq NP_859053.1), and human REM (GenBank RefSeq NP_054731.2). Amino acids with black background and white letters

indicate 100% identity, while those shaded with gray indicate additional identity with bullfrog Rem2. Asterisks (*) indicate phosphorylation sites (Correll et al., 2008, Ghiretti et al., 2013). Sites of 14-3-3 protein interaction are indicated by the arrow () (Béguin et al., 2005a). Consensus sequences for GTP-binding regions (G1–G5) and the conserved C7 sequence motif are indicated below the alignments (Finlin et al., 2000). The G3 consensus shown is that which is unique to the RGK subfamily. Bold lines below the alignment indicate N-terminal and C-terminal extensions in mammalian Rem2 (Splingard et al. 2007). The boxed amino acids contain the putative C-terminus nuclear localization sequence (NLS) based on Ghiretti et al. (2013). Amino acids are numbered to the right. Percent identity of bullfrog Rem2 with each sequence, indicated at the end of each sequence, is based on individual pairwise alignments.

Fig. 3.

Tissue specificity of bullfrog *rem2* using Real-Time PCR. Relative expression of the bullfrog *rem2* gene was significantly different between the different tissues (One-way ANOVA; $P < 0.0001$). Relative expression of *rem2* in the brain was significantly greater than its expression in all other tissues assayed (*: Tukey's multiple comparison; $P < 0.001$).

Fig. 4.

Regionalization of relative *rem2* expression in the bullfrog central nervous system using Real-Time PCR. The schematic of the bullfrog brain in dorsal view below the figure indicates each region assayed. The bullfrog *rem2* gene showed differential expression in the central nervous system (One-way ANOVA; $P < 0.0001$). The relative expression of *rem2* in the cerebrum was significantly greater than its expression in all other areas assayed (***: Tukey's multiple comparison; $P < 0.001$), as was the case for the diencephalon (**: $P < 0.05$), excepting being lower than the cerebrum. The midbrain had significantly greater *rem2* expression levels than that of the midbrain, hindbrain, and spinal cord (*: $P < 0.05$). Abbreviations: OB = Olfactory bulb, CR = Cerebrum, DI = Diencephalon, MB = Midbrain, CB = Cerebellum, HB = Hindbrain, SC = Spinal cord.

Fig. 5.

Relative *rem2* expression in the bullfrog whole brain at different ontogenetic stages before and after metamorphic climax using Real-Time PCR. Fig. 5A The bullfrog *rem2* gene was differentially expressed in the brain between the different ontogenetic stages of life (One-way ANOVA; $P < 0.05$). Relative expression of brain *rem2* was not significantly different between any one pair of ontogenetic stages (Tukey's multiple comparison). A post test for linear trend in *rem2* relative gene expression was significant ($P < 0.05$). Fig 5B Data from Panel A were grouped into pre-metamorphic climax stages (stages I-XV) and post-metamorphic climax stages (juvenile and adult) and analyzed for significance by t-test. Relative bullfrog brain *rem2* gene expression was significantly higher post-metamorphic climax ($P < 0.05$; asterisk). Stages are defined in the Methods.

Table 1

Primers and the assays in which they were used.

Assay (Usage) Primer Sets	Primer sequence
End point PCR (Isolate bullfrog <i>rem2</i> fragment)	
Degenerate primer (forward)	5' -AGTATGCCCTTGCCCTTACAAGCACCAGCT-3'
Degenerate primer (reverse)	5' -TCACCTTTATTGCCAACAAGGATGATGGG-3'
End point PCR (Extend isolated fragment)	
Gene specific primer (forward)*	5' -ACTGGAGTAGGAAAGACAACACTTGCT-3'
Gene specific primer (reverse)*	5' -TGACAAGACTTCGATCTTTGCTTGAAGA-3'
Nested RLM-RACE PCR (Obtain full-length sequence)	
<i>rem2</i> 3' RACE (forward-outer)	5' -TGTCCATTTCAGGGTCATCAGACTCT-3'
<i>rem2</i> 3' RACE (forward-inner)	5' -AGTAGGAAAGACAACACTTGCTGGGA-3'
<i>rem2</i> 5' RACE (reverse-inner)	5' -TCCCAGCAAGTGTGTCTTTCTACT-3'
<i>rem2</i> 5' RACE (reverse-outer)	5' -AGAGTCTGATGACCCTGAATGGACA-3'
End point PCR (Obtain full-length ORF)	
<i>rem2</i> (forward)	5' -ATCTGTACAGAGAAGACCCAGGCAGAAGA-3'
<i>rem2</i> (reverse)	5' -TGGCATGATTTTCGACCTCTGCTTGAAGA-3'
Real-Time PCR (Relative gene expression levels)	
<i>rem2</i>	
Gene specific primer (forward)	5' -TCTGCTCGTCCACACCGC-3'
Gene specific primer (reverse)	5' -GAGACATCTGCTGCCCTTCACC-3'
<i>rpl8</i> (Reference gene)	
Gene specific primer (forward)	5' -GCTGTTGACTTCGCAGAAAGGCAT-3'
Gene specific primer (reverse)	5' -ATGGATACCCTCAGCCGCAATGA-3'

* Paired with a primer specific to the predicted *X. tropicalis rem2* nucleotide sequence (GenBank RefSeq XM_002941509.1)