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Conservation and Diversity of the Variable Lymphocyte Receptor (VLR) in *Lampetra hubbsi*

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Introduction

A unique adaptive immune system was reported in the sea lamprey (*Petromyzon marinus*), which produces single chain protein effector molecules consisting of imperfect leucine rich repeats, or LRRs (1). These molecules, termed variable lymphocyte receptors (VLRs), are selected polyclonally in response to immunization of the sea lamprey with proteins, oligosaccharides, and whole cells. Since the first reporting in *P. marinus*, the VLR system has been found in other species of lamprey, such as *Lampetra appendix*, *Ichthyomyzon fossor* (2), *Lampetra japonica* (3), *Lampetra planeri* (4), and in the other agnathans, hagfish species *Eptatretus burgeri* and *Eptatretus stoutii* (2), as shown in the phylogenetic tree (Figure 1). Agnathans evolved around half a billion years ago (5) and developed the VLR adaptive immune system independently of immunoglobulin (Ig). The ability to utilize a local lamprey species as an alternative to *P. marinus* would be advantageous as sea lampreys are sourced from the Atlantic Ocean and Lake Michigan. It will also prevent the spread of viruses in *P. marinus* into California waterways originating from housing the species.

The molecular characterization of Lampetra *hubbsi* (also known as the Kern Brook Lamprey) was published previously (6). We hypothesized that *L. hubbsi* would possess the VLR based adaptive immune system and tested our hypothesis in this study. At first, we confirmed the molecular identity of the species, and then examined the evidence of VLR system in them.

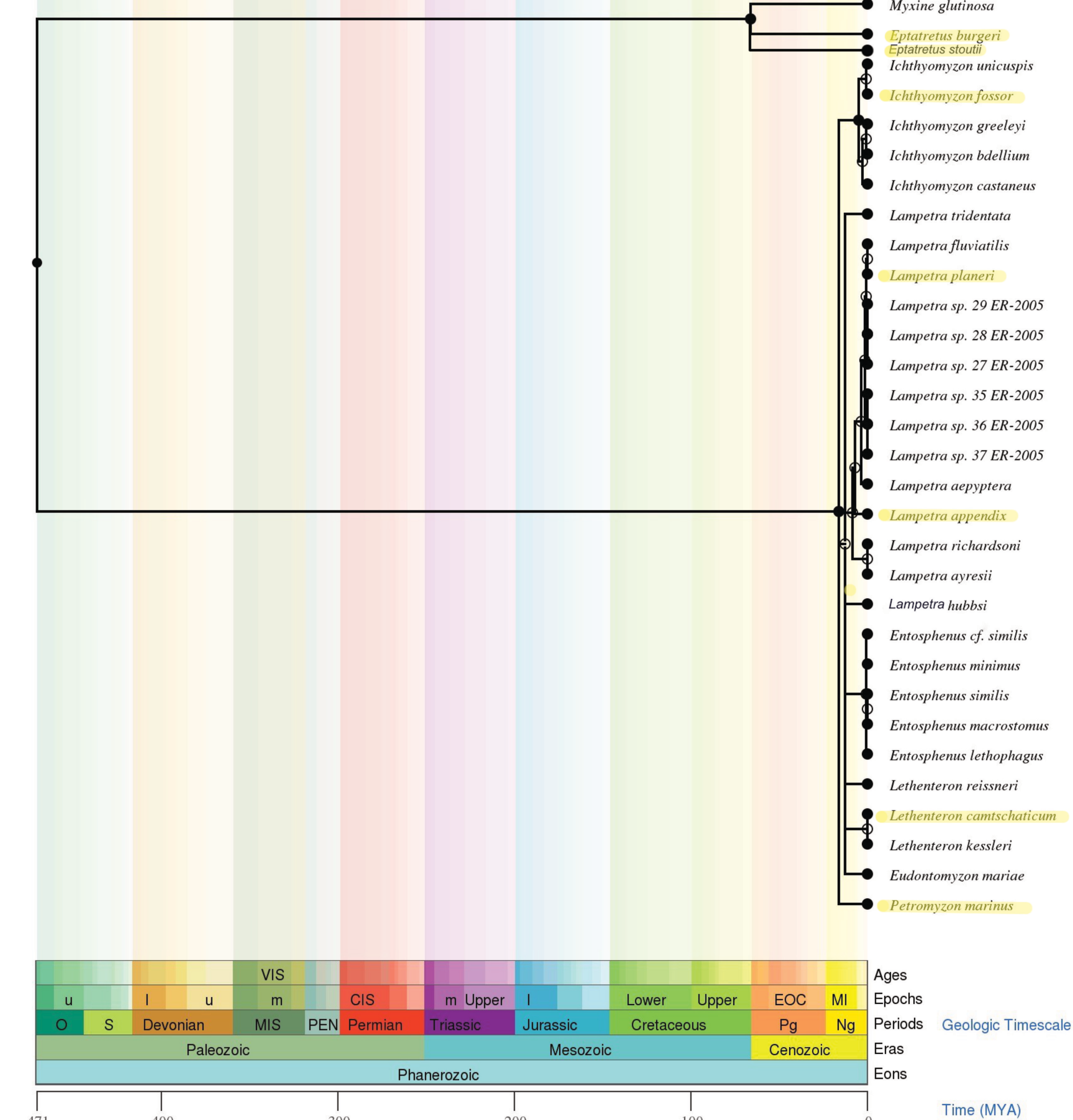


Figure 1: Phylogenetic tree of agnathans. Highlighted species are known to have a Variable Lymphocyte Receptor based adaptive immune system. This includes two hagfish species and five lamprey species.

Figure 2: *L. hubbsi* specimens collected from the Kings River. Top: L. hubbsi adult. Bottom: L. hubbsi ammocoete. *L. hubbsi* grow to approximately 14cm in length.

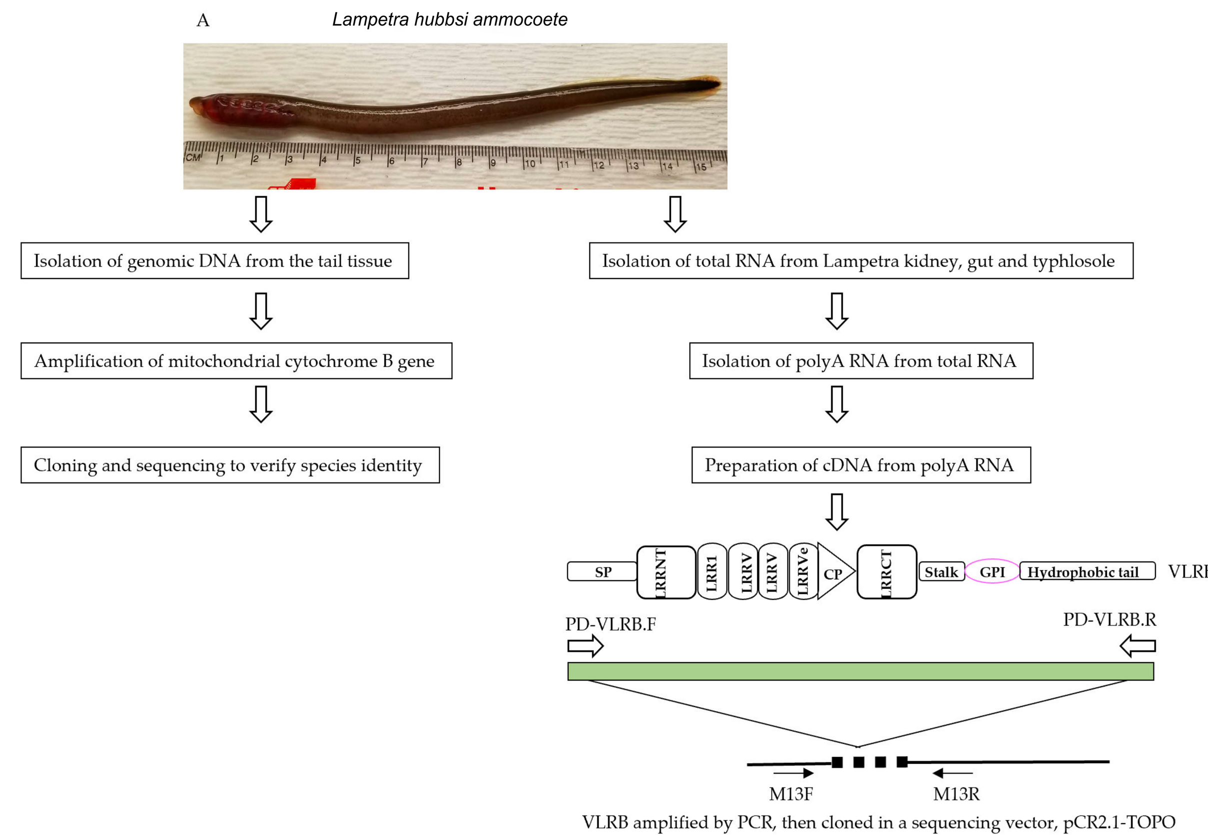


Figure 3: Schematic presentation of cloning of mitochondrial cytochrome *b* gene and VLRB transcripts from the Kern Brook Lamprey in the Kings River, California.

Species Confirmation

Three ammocoetes and two adult *L. hubbsi* were obtained from the Kings River for this study. At first, the species identity of *L. hubbsi* was examined by sequencing the mitochondrial cytochrome *b* gene which is a highly conserved marker for a particular species. The cytochrome *b* gene was amplified from DNA from the tail tissues using previously published degenerate primers, Cytb-196F and Pro-R (6), and sequenced. Alignment of the sequences (Figure 4) from three independent clones from each specimen with the 995 bp published sequence showed that 11 of the 15 sequences were identical. The percentage of bases showing a change was low (0.04%) and it was attributed to PCR artifacts and sequencing errors. Therefore, from the cytochrome *b* sequence data, it was concluded that the specimens studied were members of the *L. hubbsi* species.

Figure 4: Alignment of the cytochrome *b* gene from *L. hubbsi* collected from the Kings River. Three independent sequences from each of the three ammocoetes and two adults were compared with a published sequence (accession number GU120869.1). Base changes and deletions are shaded red with the corresponding base numbers bolded. Asterisks denote matched bases in all the sequences.

Cloning of VLRB Transcripts

Kidney, gut, and the typhlosole were dissected out from unimmunized *L. hubbsi* ammocoetes. A cDNA library was synthesized using *P. Marinus* VLRB specific primers, and individual molecules were sequences. (Figure 3, Figure 5).

Sequencing showed 23 complete and unique VLRB sequences from the library; all of which had extensive homology among themselves (71.53-96.44% identity) and to the published VLRB sequences in the non-redundant database. The LRR cassettes in this set of VLRB molecules were delineated for the number of LRR cassettes; the number of which varied from 2 to 6; however, molecules with 3 LRR cassettes were most abundant (10 of 23). Among the total 73 cassettes, 5 were used twice in the sequenced molecules, resulting in a total number of 67 unique LRRs in this set. Each of these unique cassettes were then blasted against the non-redundant protein database (NCBI); 61% (41 of 67) matched with database, the remaining 39% (26 of 67) were unique to this data set. This result suggests that the LRR cassettes are highly polymorphic in the genome of *L. hubbsi*. A similar observation was made in a naïve *P. marinus* VLRB cDNA library, which contained 35% unique sequences (manuscript submitted). An alignment of the deduced VLRB proteins from the sequenced transcripts is shown in Figure 5.

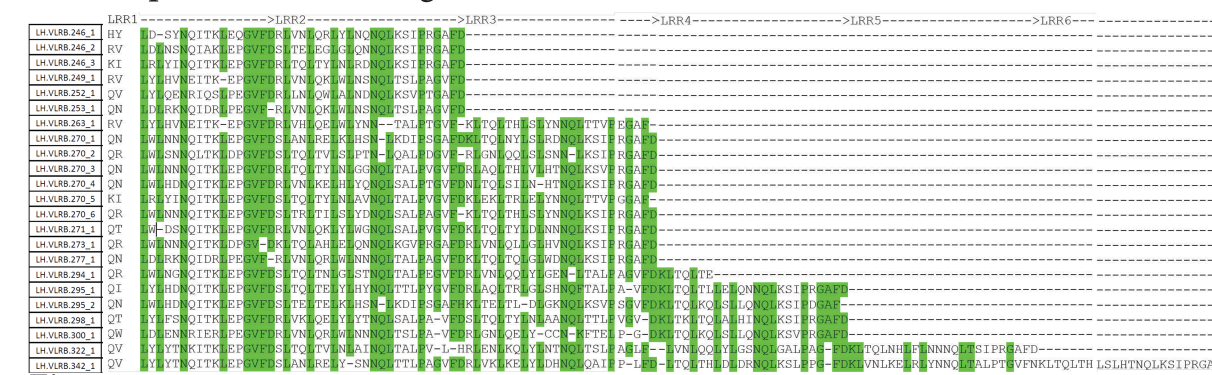
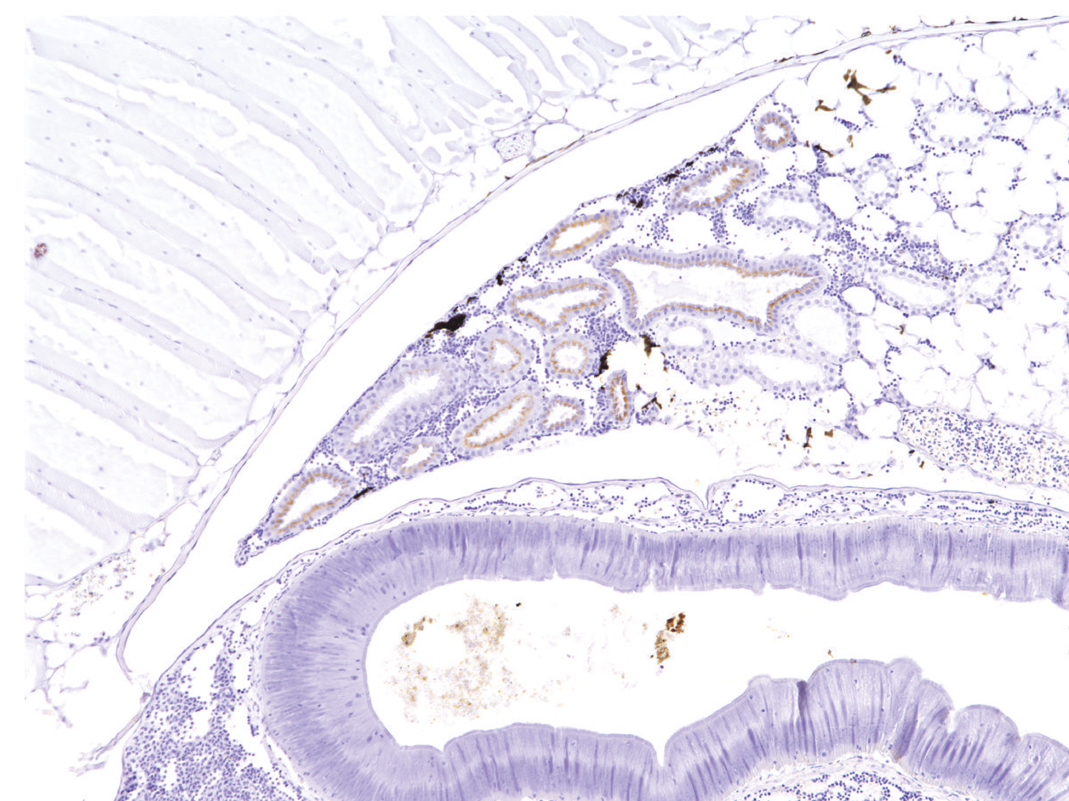


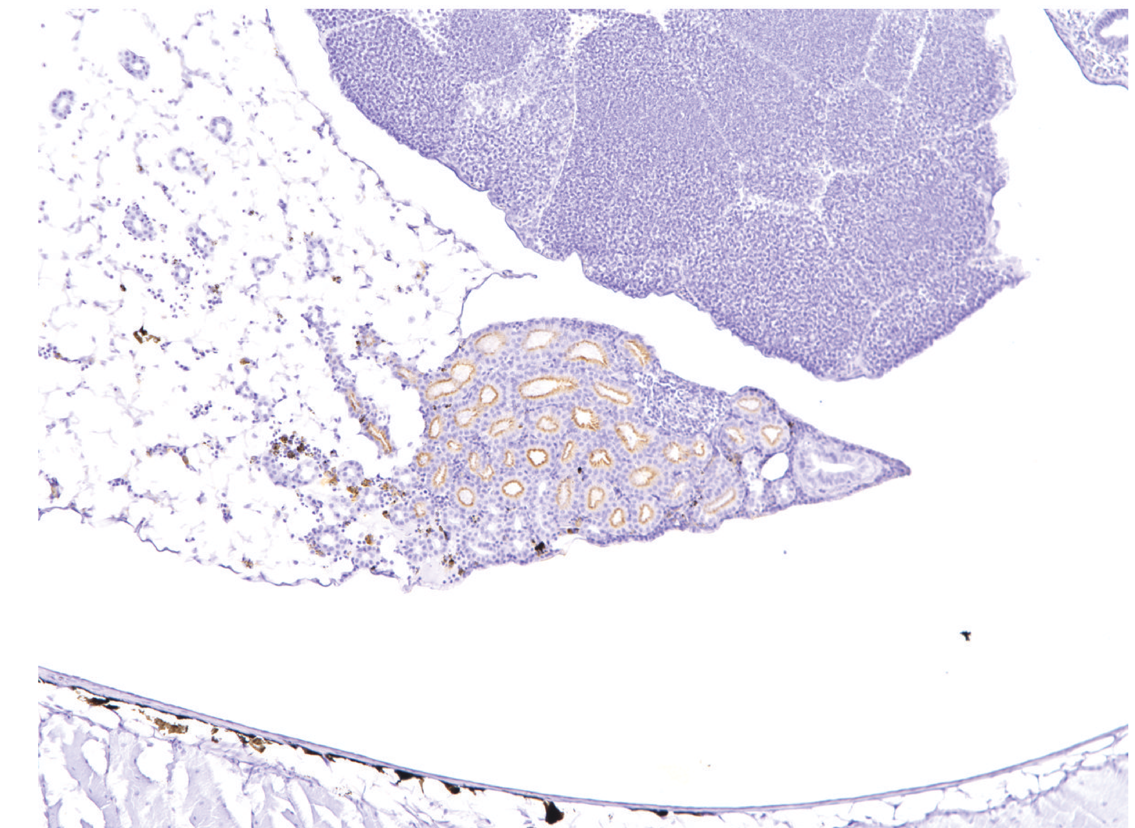
Figure 5: Clustal Omega alignment of the deduced full length VLRB proteins from the sequenced molecules from *L. hubbsi*. Conserved amino acid residues are highlighted in green.

Immunohistochemistry of VLRB

To address if VLRB is produced in *L. hubbsi*, 5 µm sections of formalin fixed and paraffin embedded (FFPE) blocks of tissue were stained with antibody against the conserved C-terminal portion of VLRB protein (4C4, mouse monoclonal). Specific staining was found in the renal tubules in sections from different levels of the body (Figure 6) in both ammocoete and adult stages; similar staining was also observed in *P. marinus* sections (data not shown). The staining was localized to the apical portion of the cytoplasm including the brush border of the renal tubular cells; no staining was seen in the adjacent adipocyte rich areas in the same section. It is also noticeable that the gut and typhlosole complex does not show any staining in these naïve or unimmunized specimens, although the typhlosole is stained in immunized *P. marinus* ammocoetes (7).



LH Ammo 4C4 100X



LH Adult 4C4 100X

Figure 6: Immunohistochemical staining of *L. hubbsi* ammocoete and adult cross section at the central part of the body for VLRB protein. VLRB protein is shown to be present in the kidneys of the lamprey.

Discussion

The molecular identity of the Kern Brook lamprey from the Kings River used in this study was confirmed by sequencing the highly conserved mitochondrial cytochrome *b* gene; the sequences of all five individuals were identical to the published sequence from *L. hubbsi*. VLRB transcripts were amplified, cloned, and sequenced from the cDNA library synthesized from kidney, gut, and typhlosole. The sequences of 23 full length unique VLRBs were obtained, the number of LRR cassettes varied from 2 to 6 with high homology among themselves (77 to 96% identity) and with those in the non-redundant database. The LRR cassettes in the sequences were delineated and a total of 73 unique ones were found, of which 39% were unique to this set. Interestingly, the observation is very similar to that in *P. marinus*. (8). This result is molecular evidence of functional VLR adaptive immune system in *L. hubbsi*. It would be interesting to immunize *L. hubbsi* ammocoetes with different immunogens and isolate target specific VLRB molecules, termed lampribodies.

References

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Acknowledgments

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