RESEARCH ARTICLE

Cloning of nine glucocorticoid receptor isoforms from the slender African lungfish (*Protopterus dolloi*)

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Abstract

We wanted to clone the glucocorticoid receptor (GR) from slender African lungfish (*Protopterus dolloi*) for comparison to the *P. dolloi* mineralocorticoid receptor (MR), which we had cloned and were characterizing, as well as for comparison to the GRs from humans, elephant shark and zebrafish. However, although sequencing of the genome of the Australian lungfish *(Neoceratodus forsteri)*, as well as, that of the West African lungfish (*Protopterus annectens*) were reported in the first three months of 2021, we could not retrieve a GR sequence with a BLAST search of GenBank, when we submitted our research for publication in July 2021. Moreover, we were unsuccessful in cloning the GR from slender African lungfish using a cDNA from the ovary of *P. dolloi* and PCR primers that had successfully cloned a GR from elephant shark, *Xenopus* and gar GRs. On October 21, 2021 the nucleotide sequence of West African lungfish (*P. annectens*) GR was deposited in GenBank. We used this GR sequence to construct PCR primers that successfully cloned the GR from the slender spotted lungfish. Here, we report the sequences of nine *P. dolloi* GR isoforms and explain the basis for the previous failure to clone a GR from slender African lungfish using PCR primers that cloned the GR from elephant shark, *Xenopus* and gar. Studies are underway to determine corticosteroid activation of these slender African lungfish GRs.

Introduction

The glucocorticoid receptor (GR) belongs to the nuclear receptor family, a diverse group of transcription factors that arose in multicellular animals [1–4]. The GR has many key roles in the physiology of humans and other terrestrial vertebrates and fish [5–8]. Important for

understanding the function of the GR is that it is closely related to the mineralocorticoid receptor (MR) [9–11]. These two steroid receptors evolved from a duplication of an ancestral corticoid receptor (CR) in a jawless fish (cyclostome), which has descendants in modern lampreys and hagfish [11–13]. A distinct GR and MR first appear in cartilaginous fishes (Chondrichthyes) [1, 9, 11, 14, 15], which diverged from bony vertebrates about 450 million years ago [16, 17].

Lungfishes are important in the transition of vertebrates from water to land [18–22], and aldosterone activation of the MR is important in this process [11, 22–25]. Aldosterone, the main physiological mineralocorticoid in humans and other terrestrial vertebrates [26–29], first appears in lungfish [21–23]. To investigate the origins of aldosterone signaling, we cloned the MR from slender spotted African lungfish (*P. dolloi*) and studied its activation by aldosterone, other corticosteroids and progesterone [30]. To continue our investigation of early events in the evolution of the GR and MR, we sought to clone the *P. dolloi* GR for comparison with *P. dolloi* MR, as well as with the GR in coelacanths, zebrafish and humans. However, a BLAST search with the sequence of the GR from coelacanth and zebrafish did not retrieve the sequence of *P. dolloi* GR or any other lungfish GR from GenBank. Nor could we clone the *P. dolloi* GR using a cDNA from *P. dolloi* ovary using PCR primers that had successfully cloned a GR from elephant shark GR [15] and chicken, alligator and frog GRs [31]. Fortunately, on October 21, 2021 the nucleotide sequence of African lungfish (*P. annectens*) GR was deposited in GenBank, which gave us sufficient information for PCR primers to clone nine isoforms of *P. dolloi* GR. Here we report the sequences of these nine *P. dolloi* GR isoforms and explain the basis for the previous failure to clone a GR from slender African lungfish using PCR primers that previously cloned the GR from elephant shark, *Xenopus* and gar [15, 31, 32]. Our analysis of these nine GR sequences indicates that they evolved by alternative splicing and gene duplication [33, 34].

Results and discussion

Multiple sequence alignment of nine *P. dolloi* GR isoforms

Fig 1 shows a multiple sequence alignment of the nine isoforms of *P. dolloi* GR. The nine *P. dolloi* GRs cluster into three groups: group I (GR-A1, GR-A2), group II (GR-B1, GR-B2, GR-B3) and group III (GR-C1, GR-C2, GR-C3, GR-C4). GR-A2 begins at "MMDP", a sequence motif that is conserved in all nine GRs.

The multiple alignment reveals that these nine slender African lungfish GRs evolved through alternative splicing and gene duplications (Fig 1). GR-A2 appears to be a product of alternative splicing of GR-A1. GR-C4 appears to be a product of alternative splicing of one or more GR-C isoforms, which supports a GR gene duplication in *P. dolloi* genome. There also is evidence for gene duplications among the *P. dolloi* GRs. MLSE at the beginning of GR-A1 is conserved in GR-B2 and GR-C2. A closely following YAPAD sequence is conserved in all *P. dolloi* GR isoforms. Fifteen of the first sixteen amino acids at the amino terminus of GR-A-1 are conserved in GR-B2 and GR-C2 (Fig 1A). This amino acid sequence is highly conserved in the other seven GRs. The rest of GR-A2 beginning at MMDPAGALNSLNGTQSLNKY is identical in GR-A1, and this amino acid sequence is highly conserved in the other seven GRs. MPFESLKYYAPAD is conserved at the beginning of GR-B3 and GR-C3. Beginning at the conserved MMDP sequence in the N-terminal domain, the two GR-A isoforms differ at 55 positions from the three GR-B and the four GR-C isoforms.

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NTD                                                                              DBD
GR-A1                              MLSEARIARKDYAPADMMDPAGSLNSLNGTQSLKYVERSDKTS 43    GR-A1 439 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRK
GR-A2                                      MMDPAGSLNSLNGTQSLKYVERSDKTS 27            GR-A2 423 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRK
GR-B1 MSVWHESRHDTDWDVCSLKFNRCGLHLDYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS 60              GR-B1 455 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
GR-B2                       MLSEVRITRKDYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS 43          GR-B2 438 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
GR-B3                          MPFESLKYYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS 40          GR-B3 435 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
GR-C1 MSVWHESRHDTDWDVCSPKFNRCGLHLDYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS 60              GR-C1 455 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
GR-C2                       MLSEVRITRKDYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS 43          GR-C2 438 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
GR-C3                          MPFESLKYYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS 40          GR-C3 435 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
GR-C4                                      MMDPAGSLNSLNGTQSLKYAERSDKTS 27            GR-C4 422 CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
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                                                                                                                                   hinge
GR-A1 SGYPFNPICRGGAGATVTISTSPSRQTSAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS 103           GR-A1 499 CLQAGMNLDARKSKKKMKGIQQPNEPSVANPASESTANKALLPTSLPQLTPTIINLLEVI
GR-A2 SGYPFNPICRGGAGATVTISTSPSRQTSAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS 87            GR-A2 483 CLQAGMNLDARKSKKKMKGIQQPNEPSVANPASESTANKALLPTSLPQLTPTIINLLEVI
GR-B1 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 120           GR-B1 515 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
GR-B2 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 103           GR-B2 498 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
GR-B3 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 100           GR-B3 495 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
GR-C1 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 120           GR-C1 515 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
GR-C2 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 103           GR-C2 498 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
GR-C3 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 100           GR-C3 495 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
GR-C4 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 87            GR-C4 482 CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
      ********* ********* * **** ****  ************** * **********                      ******** ************** **  ****** ********* ***********
                                                                                 LBD
GR-A1 MGFYMGEVNSKAAGTDFGSQQQGQQSCVSSAENDFFLLEESLANLNRDAGTEASFLSAET 163           GR-A1 543 EPDVIYAGYDSTSPDTSPRLMSAMNTLGGRQVVAAVKWAKSLPGFRNNMPLDDQMSLIQYS
GR-A2 MGFYMGEVNSKAAGTDFGSQQQGQQSCVSSAENDFFLLEESLANLNRDAGTEASFLSAET 147           GR-A2 513 EPDVIYAGYDSTSPDTSPRLMSAMNTLGGRQVVAAVKWAKSLPGFRNNMPLDDQMSLIQYS
GR-B1 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 180           GR-B1 575 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
GR-B2 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 163           GR-B2 558 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
GR-B3 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 160           GR-B3 555 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
GR-C1 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 180           GR-C1 575 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
GR-C2 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 163           GR-C2 558 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
GR-C3 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 160           GR-C3 555 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
GR-C4 MGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 147           GR-C4 542 EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
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GR-A1 ADSLARGQDFLAMGKTDFPSEQETFSHLGGSDTNGTSRLFSDDQNSFDIFPELSLQTDSP 223           GR-A1 619 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFAQLQIT
GR-A2 ADSLARGQDFLAMGKTDFPSEQETFSHLGGSDTNGTSRLFSDDQNSFDIFPELSLQTDSP 207           GR-A2 603 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFAQLQIT
GR-B1 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 239           GR-B1 635 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
GR-B2 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 223           GR-B2 618 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
GR-B3 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 219           GR-B3 615 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
GR-C1 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 239           GR-C1 635 WMFLMTFGLGWRSYKQSSGTVLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
GR-C2 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 222           GR-C2 618 WMFLMTFGLGWRSYKQSSGTVLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
GR-C3 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 219           GR-C3 615 WMFLMTFGLGWRSYKQSSGTVLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
GR-C4 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 206           GR-C4 602 WMFLMTFGLGWRSYKQSSGTVLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
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GR-A1 GRITDGSPWNLETFCDDEDDGGAGLSPLLIDNAISEPGGLNEECRGLVGNNMFDVKNNEC 283           GR-A1 679 FEEYLCMKALLLLSTIPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-A2 GRITDGSPWNLETFCDDEDDGGAGLSPLLIDNAISEPGGLNEECRGLVGNNMFDVKNNEC 267           GR-A2 663 FEEYLCMKALLLLSTIPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-B1 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 299           GR-B1 695 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-B2 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 283           GR-B2 678 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-B3 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 279           GR-B3 675 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-C1 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 299           GR-C1 695 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-C2 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 282           GR-C2 678 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-C3 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 279           GR-C3 675 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
GR-C4 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 266           GR-C4 662 FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
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GR-A1 HDSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS 343           GR-A1 739 KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 796
GR-A2 HDSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS 327           GR-A2 723 KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 780
GR-B1 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 359           GR-B1 755 KLLDSMHDVVSKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 812
GR-B2 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 342           GR-B2 738 KLLDSMHDVVSKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 795
GR-B3 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 339           GR-B3 735 KLLDSMHDVVSKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 792
GR-C1 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 359           GR-C1 755 KLLDSMHDVVTKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 812
GR-C2 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 342           GR-C2 738 KLLDSMHDVVTKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 795
GR-C3 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 339           GR-C3 735 KLLDSMHDVVTKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 792
GR-C4 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 326           GR-C4 722 KLLDSMHDVVTKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK 779
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GR-A1 TSGGQSYCYGVNSSPRSQHQDQKPVFTFIPPFTAIGNNRSRCQGSSENPSLSPLTTGSYA 403
GR-A2 TSGGQSYCYGVNSSPRSQHQDQKPVFTFIPPFTAIGNNRSRCQGSSENPSLSPLTTGSYA 387
GR-B1 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 419
GR-B2 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 403
GR-B3 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 399
GR-C1 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 419
GR-C2 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 402
GR-C3 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 399
GR-C4 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 386
      *********** *** *** ************* ****** ** *** *** ***** *** ***
GR-A1 GVSGFISSSTGTALKINSSSPPSGTSSSPGPPAKV 438
GR-A2 GVSGFISSSTGTALKINSSSPPSGTSSSPGPPAKV 422
GR-B1 GVSGFISSSTGTAVKTNSNSPPSGTLSSPGPPAKV 454
GR-B2 GVSGFISSSTGTAVKTNSNSPPSGTLSSPGPPAKV 437
GR-B3 GVSGFISSSTGTAVKTNSNSPPSGTLSSPGPPAKV 434
GR-C1 GVSGFISSSTGTAVKTNSNSPPSGTSSSPGPPAKV 454
GR-C2 GVSGFISSSTGTAVKTNSNSPPSGTSSSPGPPAKV 437
GR-C3 GVSGFISSSTGTAVKTNSNSPPSGTSSSPGPPAKV 434
GR-C4 GVSGFISSSTGTAVKTNSNSPPSGTSSSPGPPAKV 421
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Fig 1. Multiple alignment of the amino acid sequences slender African lungfish glucocorticoid receptors. Total RNA was isolated from *P. dolloi* ovary and translated into cDNA. PCR was performed using four primer sets based on the sequence of *P. annectens* GR, as described in the Methods section. The amplified DNA fragments were sub-cloned into a vector for sequence analysis. Similar to other steroid receptors, slender African lungfish GR can be divided into four functional domains [6, 8], consisting of a ligand-binding domain (LBD) at the C-terminus, a DNA-binding domain (DBD) in the center that is joined to the LBD by a short hinge domain (hinge), and a domain at the amino-terminus (NTD). GenBank accession no. BDF84376 for GR-A1, BDF84377 for GR-A2, BDF84378 for GR-B1, BDF84379 for GR-B2, BDF84380 for GR-B3, BDF84381 for GR-C1, BDF84382 for GR-C2, BDF84383 for GR-C3, and BDF84384 for GR-C4. Sequences were aligned with Clustal W [35], as described in the Methods section.

Comparison of slender African lungfish GRs and West African lungfish GRs

To begin to understand sequence conservation and divergence among lungfish GRs, we compared GR-A1, GR-B1 and GR-C1, which are the three longest slender African lungfish GRs, with the four West African lungfish glucocorticoid receptor sequences in GenBank (Fig 2).

The multiple sequence alignment, shown in Fig 2, reveals strong sequence conservation in the DBD, with a difference at only one position containing a semi-conserved phenylalanine-tyrosine. The sequences in the LBD and hinge domains of slender African lungfish GR and
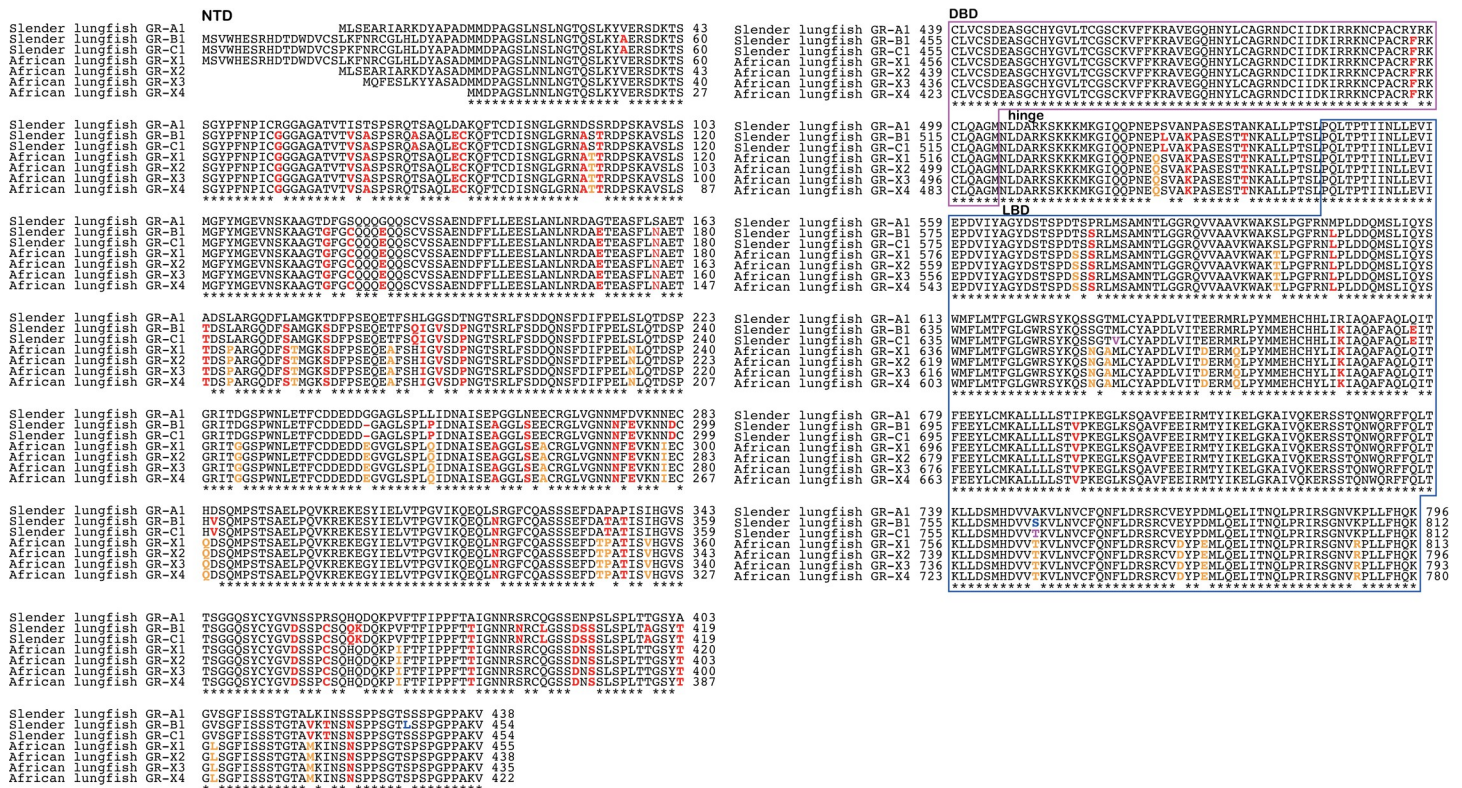
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                              NTD                                                                               DBD
Slender lungfish GR-A1                      MLSEARIARKDYAPADMMDPAGSLNSLNGTQSLKYVERSDKTS  43    Slender lungfish GR-A1 439  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRK
Slender lungfish GR-B1 MSVWHESRHDTDWDVCSLKFNRCGLHLDYAPADMMDPAGSLNSLNGTQSLKYVERSDKTS  60    Slender lungfish GR-B1 455  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
Slender lungfish GR-C1 MSVWHESRHDTDWDVCSPKFNRCGLHLDYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS  60    Slender lungfish GR-C1 455  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
African lungfish GR-X1 MSVWHESRHDTDWDVCSPKFNRCGLHLDYAPADMMDPAGSLNSLNGTQSLKYVERSDKTS  60    African lungfish GR-X1 456  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
African lungfish GR-X2                      MLSEARIARKDYASADMMDPAGSLNNLNGTQSLKYVERSDKTS  43    African lungfish GR-X2 439  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRK
African lungfish GR-X3                         MQFESLKYYASADMMDPAGSLNNLNGTQSLKYVERSDKTS  40    African lungfish GR-X3 436  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
African lungfish GR-X4                           MMDPAGSLNNLNGTQSLKYVERSDKTS  27    African lungfish GR-X4 423  CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRK
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                                                                                                                        hinge
Slender lungfish GR-A1 SGYPFNPICRGGAGATVTISTSPSRQTSAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS 103   Slender lungfish GR-A1 499  CLQAGMNLDARKSKKKMKGIQQPNEPSVANPASESTNKALLPTSLFQLTPTIINLLEVI
Slender lungfish GR-B1 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 120   Slender lungfish GR-B1 515  CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
Slender lungfish GR-C1 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 120   Slender lungfish GR-C1 515  CLQAGMNLDARKSKKKMKGIQQPNEPLVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
African lungfish GR-X1 SGYPFNPICGGGAGATVTVSASPSRQTSAQLECKQFTCDISNGLGRNATTRDPSKAVSLS 120   African lungfish GR-X1 516  CLQAGMNLDARKSKKKMKGIQQPNEQSVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
African lungfish GR-X2 SGYPFNPICGGGAGATVTVSASPSRQTSAQLECKQFTCDISNGLGRNATTRDPSKAVSLS 103   African lungfish GR-X2 499  CLQAGMNLDARKSKKKMKGIQQPNEQSVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
African lungfish GR-X3 SGYPFNPICGGGAGATVTVSASPSRQTSAQLECKQFTCDISNGLGRNATTRDPSKAVSLS 100   African lungfish GR-X3 496  CLQAGMNLDARKSKKKMKGIQQPNEQSVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
African lungfish GR-X4 SGYPFNPICGGGAGATVTVSASPSRQTSAQLECKQFTCDISNGLGRNATTRDPSKAVSLS  87   African lungfish GR-X4 483  CLQAGMNLDARKSKKKMKGIQQPNEQSVAKPASESTTNKALLPTSLPQLTPTIINLLEVI
                       ********* ********    *    **** *  ********** *   ********                                  ****** ****************** *  *** ***** ********* ***********

                                                                                                                        LBD
Slender lungfish GR-A1 MGFYMGEVNSKAAGTDFGSQOGQOGSCVSSAENDFFLLEESLANLNRDAGTEASFLSAET 163   Slender lungfish GR-A1 559  EPDVIYAGYDSTSPDTSPRLMSAMNTLGGRQVVAAVKWAKSLPGFRNMPLDDQMSLIQYS
Slender lungfish GR-B1 MGFYMGEVNSKAAGTGFGCQOQEQOSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 180   Slender lungfish GR-B1 575  EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
Slender lungfish GR-C1 MGFYMGEVNSKAAGTGFGCQOQEQOSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 180   Slender lungfish GR-C1 575  EPDVIYAGYDSTSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYS
African lungfish GR-X1 MGFYMGEVNSKAAGTGFGCQOQEQOSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 180   African lungfish GR-X1 576  EPDVIYAGYDSTSPDSSSRLMSAMNTLGGRQVVAAVKWATLPGFRNLPLDDQMSLIQYS
African lungfish GR-X2 MGFYMGEVNSKAAGTGFGCQOQEQOSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 163   African lungfish GR-X2 559  EPDVIYAGYDSTSPDSSSRLMSAMNTLGGRQVVAAVKWATLPGFRNLPLDDQMSLIQYS
African lungfish GR-X3 MGFYMGEVNSKAAGTGFGCQOQEQOSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 160   African lungfish GR-X3 556  EPDVIYAGYDSTSPDSSSRLMSAMNTLGGRQVVAAVKWATLPGFRNLPLDDQMSLIQYS
African lungfish GR-X4 MGFYMGEVNSKAAGTGFGCQOQEQOSCVSSAENDFFLLEESLANLNRDAETEASFLNAET 147   African lungfish GR-X4 543  EPDVIYAGYDSTSPDSSSRLMSAMNTLGGRQVVAAVKWATLPGFRNLPLDDQMSLIQYS
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Slender lungfish GR-A1 ADSLARGQDFLAMGKTDFPSEQETFSHLGGSDTNGTSRLFSDDQNSFDIFPELSLQTDSP 223   Slender lungfish GR-A1 613  WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFAQLQIT
Slender lungfish GR-B1 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 240   Slender lungfish GR-B1 635  WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
Slender lungfish GR-C1 TDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSLQTDSP 240   Slender lungfish GR-C1 635  WMFLMTFGLGWRSYKQSSGTVLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEIT
African lungfish GR-X1 TDSPARGQDFSTMGKSDFPSEQEAFSHIGVSDPNGTSRLFSDDQNSFDIFPELNLQTDSP 240   African lungfish GR-X1 696  WMFLMTFGLGWRSYKQSNGAMLCYAPDLVITDERMQLPYMMEHCHYLIKIAQAFAQLQIT
African lungfish GR-X2 TDSPARGQDFSTMGKSDFPSEQEAFSHIGVSDPNGTSRLFSDDQNSFDIFPELNLQTDSP 223   African lungfish GR-X2 619  WMFLMTFGLGWRSYKQSNGAMLCYAPDLVITDERMQLPYMMEHCHYLIKIAQAFAQLQIT
African lungfish GR-X3 TDSPARGQDFSTMGKSDFPSEQEAFSHIGVSDPNGTSRLFSDDQNSFDIFPELNLQTDSP 220   African lungfish GR-X3 616  WMFLMTFGLGWRSYKQSNGAMLCYAPDLVITDERMQLPYMMEHCHYLIKIAQAFAQLQIT
African lungfish GR-X4 TDSPARGQDFSTMGKSDFPSEQEAFSHIGVSDPNGTSRLFSDDQNSFDIFPELNLQTDSP 207   African lungfish GR-X4 603  WMFLMTFGLGWRSYKQSNGAMLCYAPDLVITDERMQLPYMMEHCHYLIKIAQAFAQLQIT
                       **  ****** * ***  ****** * * *  ***************** ***** ******                            *****************  * ************** *** * ********* * *******  **

Slender lungfish GR-A1 GRITDGSPWNLETFCDDEDDGGAGLSPLLIDNAISEPGGLNEECRGLVGNNMFDVKNNEC 283   Slender lungfish GR-A1 679  FEEYLCMKALLLLSTIPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
Slender lungfish GR-B1 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 299   Slender lungfish GR-B1 695  FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
Slender lungfish GR-C1 GRITDGSPWNLETFCDDEDD-GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFEVKNNDC 299   Slender lungfish GR-C1 695  FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
African lungfish GR-X1 GRITGSPWNLETFCDDEDDEGVGLSPLQIDNAISEAGGLSEACRGLVGNNNFEVKNIEC 300   African lungfish GR-X1 696  FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
African lungfish GR-X2 GRITGSPWNLETFCDDEDDEGVGLSPLQIDNAISEAGGLSEACRGLVGNNNFEVKNIEC 283   African lungfish GR-X2 679  FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
African lungfish GR-X3 GRITGSPWNLETFCDDEDDEGVGLSPLQIDNAISEAGGLSEACRGLVGNNNFEVKNIEC 280   African lungfish GR-X3 676  FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
African lungfish GR-X4 GRITGSPWNLETFCDDEDDEGVGLSPLQIDNAISEAGGLSEACRGLVGNNNFEVKNIEC 267   African lungfish GR-X4 663  FEEYLCMKALLLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLT
                       **** *********** ****   *****  ***** ***** * ******** ** **                                ************** ****************************************** *******

Slender lungfish GR-A1 HDSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS 343   Slender lungfish GR-A1 739  KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK  796
Slender lungfish GR-B1 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 359   Slender lungfish GR-B1 755  KLLDSMHDVVSKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK  812
Slender lungfish GR-C1 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDATATISIHGVS 359   Slender lungfish GR-C1 755  KLLDSMHDVVTKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNVRPLLFHQK  813
African lungfish GR-X1 QDSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQLNRGFCQASSSEFDTPATISVHGVS 360   African lungfish GR-X1 756  KLLDSMHDVVTKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNVRPLLFHQK  813
African lungfish GR-X2 QDSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQLNRGFCQASSSEFDTPATISVHGVS 343   African lungfish GR-X2 739  KLLDSMHDVVTKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNVRPLLFHQK  796
African lungfish GR-X3 QDSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQLNRGFCQASSSEFDTPATISVHGVS 340   African lungfish GR-X3 736  KLLDSMHDVVTKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNVRPLLFHQK  793
African lungfish GR-X4 QDSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQLNRGFCQASSSEFDTPATISVHGVS 327   African lungfish GR-X4 723  KLLDSMHDVVTKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNVRPLLFHQK  780
                        ******************* ***************** ******** *   ***  ****                             ********* * ********** **** * **  *****************  *******

Slender lungfish GR-A1 TSGGQSYCYGVNSSPRSQHQDQKPVFTFIPPFTAIGNNRSRCQGSSENPSLSPLTTGSYA 403
Slender lungfish GR-B1 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 419
Slender lungfish GR-C1 TSGGQSYCYGVDSSPCSQQKDQKPVFTFIPPFTTIGNNRNRCLGSSDSSSLSPLTAGSYT 419
African lungfish GR-X1 TSGGQSYCYGVDSSPCSQHQDQKPIFTFIPPFTTIGNNRSRCQGSSDNSSLSPLTTGGYT 420
African lungfish GR-X2 TSGGQSYCYGVDSSPCSQHQDQKPIFTFIPPFTTIGNNRSRCQGSSDNSSLSPLTTGSYT 403
African lungfish GR-X3 TSGGQSYCYGVDSSPCSQHQDQKPIFTFIPPFTTIGNNRSRCQGSSDNSSLSPLTTGSYT 400
African lungfish GR-X4 TSGGQSYCYGVDSSPCSQHQDQKPIFTFIPPFTTIGNNRSRCQGSSDNSSLSPLTTGSYT 387
                       *********** ***  **  *** *********** ****** **  ***  *****  **

Slender lungfish GR-A1 GVSGFISSSTGTALKINSSSPPSGTSSSPGPPAKV 438
Slender lungfish GR-B1 GVSGFISSSTGTAVKTNSNSPPSGTLSSPGPPAKV 454
Slender lungfish GR-C1 GVSGFISSSTGTAVKTNSNSPPSGTLSSPGPPAKV 454
African lungfish GR-X1 GLSGFISSSTGTAMKINSNSPPSGTSPSPGPPAKV 455
African lungfish GR-X2 GLSGFISSSTGTAMKINSNSPPSGTSPSPGPPAKV 438
African lungfish GR-X3 GLSGFISSSTGTAMKINSNSPPSGTSPSPGPPAKV 435
African lungfish GR-X4 GLSGFISSSTGTAMKINSNSPPSGTSPSPGPPAKV 422
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Fig 2. Multiple alignment of the amino acid sequences of three African lungfish GRs and four West African lungfish GRs. West African lungfish glucocorticoid receptor sequences were downloaded from GenBank (Accessions XP_043925084 for X1, XP_043925085 for X2, XP_043925087 for X3, XP_043925088 for X4). Sequences were aligned with Clustal W [35], as described in the Methods section.

West African lungfish GR also are highly conserved. There are small segments of sequence divergence in the NTD, but most of the NTD is conserved. Overall slender African lungfish GRs and African lungfish GRs are very similar to each other.

Comparison of the amino acid sequences of slender African lungfish GR, West African lungfish GR, coelacanth GR, zebrafish GR and human GR

To begin to understand the relationship of lungfish GRs to other selected GRs, we constructed a multiple sequence alignment of slender African lungfish GR with West African lungfish GR, coelacanth GR, zebrafish GR and human GR (Fig 3). The DBD and hinge domains are highly conserved in all GRs. There is good sequence conservation of the LBD in all six GRs. However, there is an interesting pattern of sequence conservation in the NTD. There is excellent sequence conservation in the NTD among slender African lungfish GR, West African lungfish GR, coelacanth GR and human GR. The stronger conservation of the NTD in lungfish GRs with human GR than with zebrafish GR, indicates that the NTD in zebrafish GR has diverged from the other GRs.
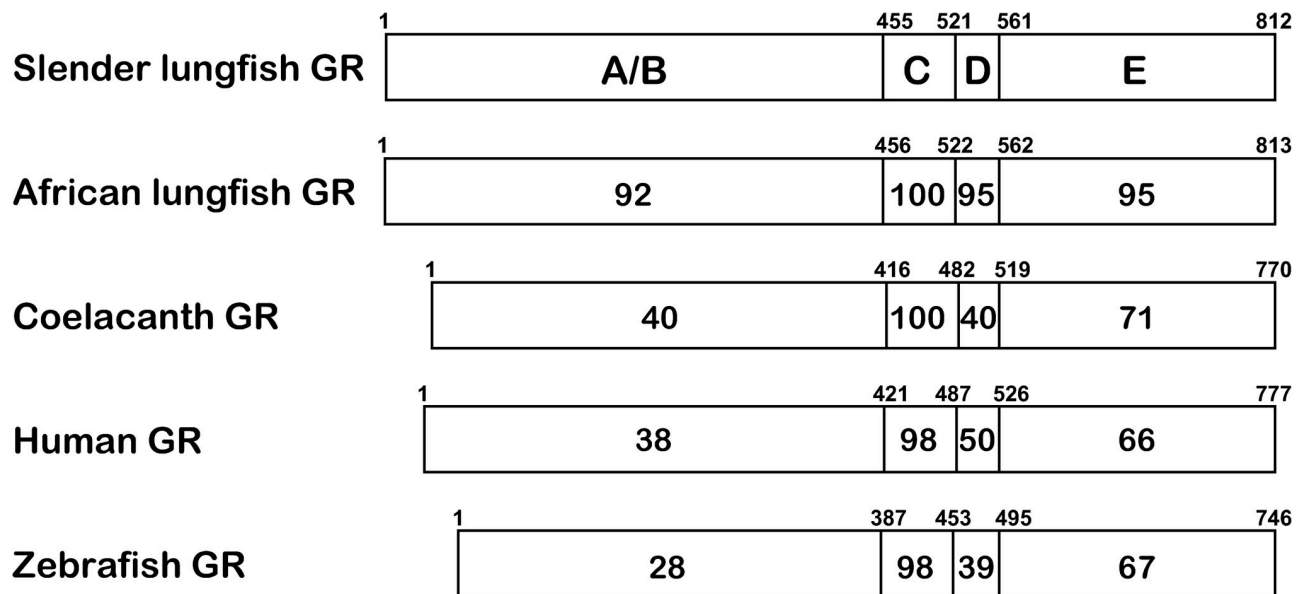
Comparison of functional domains in slender African lungfish GR with domains in West African lungfish GR, coelacanth GR, zebrafish GR and human GR

Fig 4 shows the percent identity in the comparison of the different functional domains on slender African lungfish GR with the GR and MR from other vertebrates.

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Slender lungfish GR  MSVWHESRHDTDWDVCSPKFNRCGLHLDYAPADMMDPAGSLNSLNGTQSLKYAERSDKTS   60
African lungfish GR  MSVWHESRHDTDWDVCSLKFNRCGLHLDYASADMMDPAGSLNNLNGTQSLKYVERSDKTS   60
Coelacanth GR                              MMESKEVLNTSGGD-SLKCVD-YSKRE   25
Human GR                                   MDSKESLTPGREE-NPSSVL-AQERG   24
Zebrafish GR                               MDQGGLENGK---------KRDERL   16

Slender lungfish GR  SGYPFNPICGGGAGA---TVTVSASPSRQ-ASAQLECKQFTCDISNGLGRNASTRDPSKA  116
African lungfish GR  SGYPFNPICGGGAGA---TVTVSASPSRQ-TSAQLECKQFTCDISNGLGRNATTRDPSKA  116
Coelacanth GR        EVYDFNPVFRGGAPI---PLTPASSLLLV-SEADSPRQLASGDTSNGVSGNATPQDLSQA   81
Human GR             DVMDFYKTLRGGATV---KVSASSPSLAVASQSDSKQRRLLVDFPKGSVSNAQQPDLSKA   81
Zebrafish GR         NTLDYNKRATEGILPRRIQSTMSVAPTSMVPQAGPMMQPVSGDIPNGLSNSPTLEEHTSS   76

Slender lungfish GR  VSLSMGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASF-  175
African lungfish GR  VSLSMGFYMGEVNSKAAGTGFGCQQQEQQSCVSSAENDFFLLEESLANLNRDAETEASF-  175
Coelacanth GR        VSESMGLYMGESDSKVIGQKARLQKQGQIS-GLSVETEFALLEESIASLNRSTRIGEVSG  140
Human GR             VSLSMGLYMGETETKVMGNDLGFPQOGQIS-LSSGETDLKLLEESIANLNRSTSVPENPK  140
Zebrafish GR         VS---SIFGDDSELKLLGKEQRALQQQTLV-PFTLGDSLSGLEASIADLNNPSPSMDSLI  132

Slender lungfish GR  LNAETTDSLARGQDFSAMGKSDFPSEQETFSQIGVSDPNGTSRLFSDDQNSFDIFPELSL  235
African lungfish GR  LNAETTDSPARGQDFSTMGKSDFPSEQEAFSHIGVSDPNGTSRLFSDDQNSFDIFPELNL  235
Coelacanth GR        SLAPGQVSL---DNGSGMAKCDLSVEQETFAQT-DSDPNGNLSFFPDDQAAFDILQELDL  196
Human GR             SSASTAVSAAPTEKEFPKTHSDVSSEQQHLKGQ-TGTNGGNVKLYTTDQSTFDILQDLEF  199
Zebrafish GR         GGVDPNLFPLKTEDFSPMIKGDMDLDQDSFGHIGKDVDVGNHKLFSDN--TLDLLQDFEL  190

Slender lungfish GR  QTDSPGRITDGSPWNLETFCDDED-DGAGLSPLP-IDNAISEAGGLSEECRGLVGNNNFE  293
African lungfish GR  QTDSPGRITGGSPWNLETFCDDEDDEGVGLSPLQ-IDNAISEAGGLSEACRGLVGNNNFE  294
Coelacanth GR        TPCSPGKE---NPWSLDPIYDGGGR--GLLSPLA-ADDPFLMAAVANEDCKSSLVTNTSQ  250
Human GR             SSGSPGKETNESPWRSDLLIDE--N--CLLSPLAGEDDSFLLEGNSNEDCKPLILPDTKP  255
Zebrafish GR         DGSPSDFYVAD-----DAFLSTIG-EDALLSELPTN---------LDRDSKAAV--SGSN  233

Slender lungfish GR  VKNNDCHVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEF----DA  349
African lungfish GR  VKNIECQDSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQLNRGFCQASSSEF----DT  350
Coelacanth GR        STNNECGNLFIPDLSSQLSQIKSDKEGYIELLTPGVVKQETLGRSYCQANLTSAAST-TA  309
Human GR             KIKDNGDLVLSSPSNVTLPQVKTEKEDFIELCTPGVIKQEKLGTVYCQASFPGANIIGNK  315
Zebrafish GR         TLNGTASSSLSTANTSILPNIKVEKDSIIQLCTPGVIKQENTGASYCQGGLHS-------  286

Slender lungfish GR  TATISIHGVSTSGGQSYCYGVDS--SPCSQQKDQKPVFTFIPPFTTIGNNNRNRCLGSSDS  407
African lungfish GR  PATISVHGVSTSGGQSYCYGVDS--SPCSQHQDQKPIFTFIPPFTTIGNNNRSRCQGSSDN  408
Coelacanth GR        NSSISIHGVNTSGGQMYHYDVNAGAVSSAQQPDQKPIFNFIPPVSTIAENWNRCQGSGAT  369
Human GR             MSAISVHGVSTSGGQMYHYDMN--TASLSQQQDQKPIFNVIPPIPVGSENWNRCQGSGDD  373
Zebrafish GR         -TPINICGVTTSSGQSFLFGNSSPTAVVGLQKDQKPDFNMYTPLTSSGDGWSRSQGFGNV  345

Slender lungfish GR  SSLSPLTAGSYTGVSGFISSSTGTAVKTNSNSP-PSGTSSSPGPPAKVCLVCSDEASGCH  466
African lungfish GR  SSLSPLTTGSYTGLSGFISSSTGTAVKINSNSP-PSGTSPSPGPPAKVCLVCSDEASGCH  467
Coelacanth GR        NSTPPLGNVNASGRSGFASSYPSPGTRTATPT--PSSSSTSSGPPHKICLVCSDEASGCH  427
Human GR             NL-TSLGTLNFPGRTVFSNGYSSPSMRPDVSSPPSSSTATTGPPPKLCLVCSDEASGCH  432
Zebrafish GR         SGMQQRASLCFSKN--FS---SSPYSRPEDST--ATSSAGGKTGTHKICLVCSDEASGCH  398

Slender lungfish GR  YGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGMNLDARK  526
African lungfish GR  YGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGMNLDARK  527
Coelacanth GR        YGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGMNLEARK  487
Human GR             YGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGMNLEARK  492
Zebrafish GR         YGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLMAGMNLEARK  458

Slender lungfish GR  SKKKMKGIQQPNEP--LVAKPASESTTNKALLPTSLPQLTPTIINLLEVIEPDVIYAGYD  584
African lungfish GR  SKKKMKGIQQPNEQ--SVAKPASESTTNKALLPTSLPQLTPTIINLLEVIEPDVIYAGYD  585
Coelacanth GR        SKKLNKMKGN-LS--S-KEQATPPLPERAVVPASVPQLTPTMISLLEAIEPSILYSGYD  542
Human GR             TKKKIKGIQQATT---GVSQETSENPGNKTIVPATLPQLTPTLVSLLEVIEPEVLYAGYD  549
Zebrafish GR         SKSKARQAGKVIQQQSIPERNLPPLPEARALVPKPMPQLVPTMLSLLKAIEPDTLYAGYD  518

Slender lungfish GR  STSPDTSSRLMSAMNTLGGRQVVAAVKWAKSLPGFRNLPLDDQMSLIQYSWMFLMTFGLG  644
African lungfish GR  STSPDSSSRLMSAMNTLGGRQVVAAVKWAKTLPGFRNLPLDDQMSLIQYSWMFLMTFGLG  645
Coelacanth GR        STIPDTHCRLMTALNKLGGRQVVAAVKWAKALPGFRSLHLDDQMVLLQYSWMFLMAFGLG  602
Human GR             SSVPDSTWRIMTTLNMLGGRQVIAAVKWAKAIPGFRNLHLDDQMTLLQYSWMFLMAFALG  609
Zebrafish GR         STIPDTSVRLMTTLNRLGGRQVISAVKWAKALPGFRNLHLDDQMTLLQCSWLFIMSFGLG  578

Slender lungfish GR  WRSYKQSSGTVLCYAPDLVITEERMRLPYMMEHCHHLIKIAQAFAQLEITFEEYLCMKAL  704
African lungfish GR  WRSYKQSNGAMLCYAPDLVITDERMQLPYMMEHCHYLIKIAQAFAQLQITFEEYLCMKAL  705
Coelacanth GR        WRSYQQANGSMLCFAPDLINEQRMQLPYMYEQCQHMLKIASEFSRLQVSYEEYLCMKAL  662
Human GR             WRSYRQSSANLLCFAPDLIINEQRMTLPCMYDQCKHMLYVSSELHRLQVSYEEYLCMKTL  669
Zebrafish GR         WRSYQHCNGNMLCFAPDLVINEERMKLPYMSDQCEQMLKISNEFVRLQVSTEEYLCMKVL  638

Slender lungfish GR  LLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLTKLLDSMHDVV  764
African lungfish GR  LLLSTVPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWQRFFQLTKLLDSMHDVV  765
Coelacanth GR        LLLSTIPQEGLKSQPVFDEIRMTYIKELGKAIVKKEGNSNQNWQRFYQLTKLLDSMHDVV  722
Human GR             LLLSSVPKDGLKSQELFDEIRMTYIKELGKAIVKREGNSSQNWQRFYQLTKLLDSMHEVV  729
Zebrafish GR         LLLNTVPKDGLKSQSVFDELRMSYIKELGKAIVKREENSSQNWQRFYQLTKLLDSMHDLV  698

Slender lungfish GR  TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK  812
African lungfish GR  TKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNVRPLLFHQK  813
Coelacanth GR        KELLKICFHTFVDKSLSVEFPEMLAEIISNQLPKVTSGSCKALLFHQN  770
Human GR             ENLLNYCFQTFLDKTMSIEFPEMLAEIITNQIPKYSNGNIKKLLFHQK  777
Zebrafish GR         GGLLNFCFYTFVNKSLSVEFPEMLAEIISNQLPKFKDGSVKPLLFHQK  746
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Fig 3. Multiple sequence alignment of slender African lungfish GR, West African lungfish GR, coelacanth GR, zebrafish GR and human GR. Glucocorticoid receptor sequences were downloaded from GenBank (Accession no. NP_000167 for human GR, XP_005996162 for coelacanth GR, and NP_001018547 for zebrafish GR) and aligned with Clustal W [35], as described in the Methods section. The NTD in zebrafish GR has gaps and sequence differences with the other GRs.

Fig 4. Comparison of functional domains of slender lungfish GR with domains in West African lungfish GR, coelacanth GR, zebrafish GR, human GR. Comparison of domains in slender African lungfish GR with GRs from West African lungfish, coelacanths, humans and zebrafish and MRs from slender African lungfish, West African lungfish, humans and zebrafish. The functional NTD (A/B), DBD (C), hinge (D) and LBD (E) domains are schematically represented with the numbers of amino acid residues and the percentage of amino acid identity depicted.

https://doi.org/10.1371/journal.pone.0272219.g004

As shown in Fig 4, the DBD and LBD are highly conserved in all GRs. For example, slender African lungfish GR and human GR have 98% and 66% identity in DBD and LBD, respectively. There are similar % identities between corresponding DBDs and LBDs in lungfish GR and other GRs. This strong conservation of the DBD and LBD contrasts with the lower sequence identity between the NTD of slender African lungfish GR and human GR (38%) and even lower sequence identity with the NTD in zebrafish GR (28%).

Phylogenetic analysis

To better understand the relationships among the nine *P. dolloi* GRs and four *P. annectens* GRs, we constructed the phylogenetic tree, shown in Fig 5. In this phylogeny, the four African lungfish GRs cluster into one group. Slender African lungfish GR-A1 and GR-A2 are in a separate branch from the other slender African lungfish GRs. GR-A2 appears to be formed by alternative splicing of GR-A1. GR-B1, GR-B2 and GR-B3 cluster. GR-C3 and GR-C4 cluster, and GR-C4 appears to be formed by alternative splicing of GR-C3.

Basis for the failure to clone *P. dolloi* GR

Fig 6 shows the location of the PCR primers that we used to successfully clone GRs from chicken, alligator and frog [31]. Due to the strong conservation of the GR and MR these PCR primers retrieved partial sequences from both the GR and MR in chicken, alligator and frog. The full sequences of these GRs and MRs was achieved in the next step using RACE. Our failure to clone *P. dolloi* GR was due using WQRF**Y**Q instead of WQRF**F**Q for the 1st/2nd-reverse primer. When we used WQRF**F**Q we were able to clone *P. dolloi* GR.

Summary

P. dolloi contains nine GR isoforms, in contrast to *P. annectens*, which contains four GR isoforms. We do not know how many GR isoforms are in Australian lungfish (*Neoceratodus*
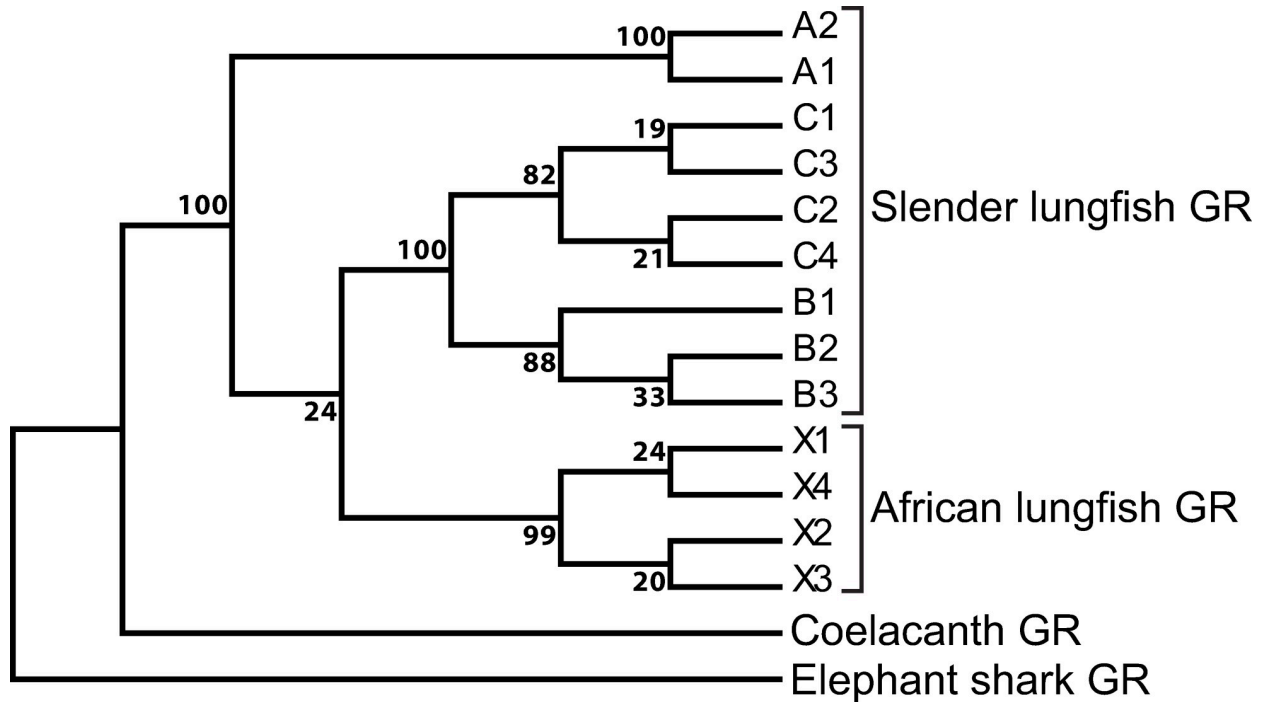
Fig 5. Phylogeny of slender African lungfish glucocorticoid receptors, West African lungfish glucocorticoid receptors, coelacanth GR and elephant shark GR. MEGA5 [36] was used to construct this phylogeny. Statistics are based on 1,000 runs.

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forsteri) because their GR sequences have not been deposited in GenBank. The availability of sequences of *P. dolloi* GRs and *P. annectens* GRs should permit using PCR to clone *N. forsteri* GRs, which would elucidate the number GR isoforms in this lungfish and the relationship of their GRs to the GRs of *P. dolloi* and *P. annectens*.

The response to corticosteroids of any lungfish GR is not known, nor are the functions of the multiple GR isoforms in *P. dolloi* GRs and *P. annectens* GRs. We have initiated studies to

```
1st-forward primer site              2nd-forward primer site
human GR          430 GCHYGV 435     human GR          441 CKVFFK 446
chicken GR        425 GCHYGV 430     chicken GR        436 CKVFFK 441
Xenopus GR        429 GCHYGV 434     Xenopus GR        440 CKVFFK 445
zebrafish GR      396 GCHYGV 401     zebrafish GR      407 CKVFFK 412
coelacanth GR     425 GCHYGV 430     coelacanth GR     436 CKVFFK 441
P.a lungfish GR   465 GCHYGV 470     P.a lungfish GR   476 CKVFFK 481
P.d lungfish GR   464 GCHYGV 469     P.d lungfish GR   475 CKVFFK 480

1st/2nd-reverse primer site
human GR          712 WQRFYQ 717
chicken GR        707 WQRFYQ 712
Xenopus GR        711 WQRFYQ 716
zebrafish GR      681 WQRFYQ 686
coelacanth GR     705 WQRFYQ 710
P.a lungfish GR   748 WQRFFQ 753
P.d lungfish GR   747 WQRFFQ 752
```

Fig 6. Location of PCR primers used for cloning of slender African lungfish GR, coelacanth GR, elephant shark GR, zebrafish GR and human GR. The correct 1st/2nd-reverse primer for PCR cloning of *P. dolloi* GR is WQRFFQ instead of WQRFYQ.

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determine corticosteroid activation of *P. dolloi* GRs to begin to elucidate the functions of slender African lungfish GRs. It is interesting that there are multiple isoforms of human GR, due to alternative splicing of human GR, and these isoforms are important in achieving functional diversity of human GR [6, 8, 34, 37]. A similar scenario is likely for *P. dolloi* GRs and *P. annectens* GRs.

Materials and methods

Animals

African lungfish (*Protopterus dolloi*) were purchased from a local commercial supplier. Lungfish were anesthetized in freshwater containing 0.02% ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich Corp., St. Louis, MO), and tissue samples were quickly dissected and frozen in liquid nitrogen. We used two individuals of lungfish. All experiments in this study were carried out under the guidelines specified by the Institutional Animal Care and Use Committee at the Hokkaido University (Chairman: Prof. Masahiko Watanabe, permission No. 12–0015). The Institutional Animal Care and Use Committee at the Hokkaido University prospectively approved this research.

Molecular cloning of lungfish *P. dolloi* glucocorticoid receptor

For P. dolloi GR cloning, we designed 4 types of forward N-terminal primers:

F-X1: `5'-GTCATTTTCCCCGTGCTTAACGAA-3'`,

F-X2: `5'-GTCTGCAGCTTGAAACTTTGTAAC-3'`,

F-X3: `5'-GACGAACATGCTGACCGGATCATAA-3'`, and

F-X4: `5'-CATACTGCATTTACCAGAATAGAC-3'`

and one C-terminal Reverse primer: R: `5'-GTTAAGGCAAATTTCTGATATTAAGGCAG-3'` based on the sequences of *P. annectens* GR (X1: XM_044069149, X2: XM_044069150, X3: XM_044069152, X4: XM_044069153). PCR was performed using four primer sets (F-X1xR, F-X2xR, F-X3xR, and F-X4xR) with ovary cDNA of *P. dolloi*, and the amplified DNA fragments with KOD-plus- DNA polymerase were subcloned into a cloning vector, pCR-BluntII-TOPO, and sequence analysis was performed for 10 or more clones for each primer sets.

Database and sequence analysis

GRs for phylogenetic analysis were collected with Blast searches of Genbank. A phylogenetic tree for GRs was constructed by Maximum Likelihood analysis based on the JTT + G model after sequences were aligned by Clustal W [35]. Statistical confidence for each branch in the tree was evaluated by the bootstrap methods [38] with 1000 replications. Evolutionary analyses were conducted in MEGA5 program [36].

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Supervision: Yoshinao Katsu, Michael E. Baker.

Writing – original draft: Yoshinao Katsu, Laurent Bianchetti, Michael E. Baker.

Writing – review & editing: Yoshinao Katsu, Laurent Bianchetti, Michael E. Baker.

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