

1 Cloning of Nine Glucocorticoid Receptor Isoforms from the Slender African lungfish

2 (*Protopterus dolloi*)

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

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47 Key words: glucocorticoid receptor, lungfish, evolution, slender spotted African lungfish.

48

## 49 **1. INTRODUCTION**

50 The glucocorticoid receptor (GR) belongs to the nuclear receptor family, a diverse group  
51 of transcription factors that arose in multicellular animals [1–4]. The GR has many key roles in  
52 the physiology of humans and other terrestrial vertebrates and fish [5–8]. Important for  
53 understanding the function of the GR is that it is closely related to the mineralocorticoid receptor  
54 (MR) [9–11]. These two steroid receptors evolved from a duplication of an ancestral corticoid  
55 receptor (CR) in a jawless fish (cyclostome), which has descendants in modern lampreys and  
56 hagfish [11–13]. A distinct GR and MR first appear in cartilaginous fishes (Chondrichthyes)  
57 [1,9,11,14,15], which diverged from bony vertebrates about 450 million years ago [16,17].

58 Lungfish are important in the transition of vertebrates from water to land [18–22], and  
59 aldosterone activation of the MR is important in this process [11,22–25]. Aldosterone, the main  
60 physiological mineralocorticoid in humans and other terrestrial vertebrates [26–29], first appears

61 in lungfish [21–23]. To investigate the origins of aldosterone signaling, we cloned the MR from  
62 slender spotted African lungfish (*P. dolloi*) and studied its activation by aldosterone, other  
63 corticosteroids and progesterone [30]. To continue our investigation of early events in the  
64 evolution of the GR and MR, we sought to clone the *P. dolloi* GR for comparison with *P. dolloi*  
65 MR, as well as with the GR in coelacanth, zebrafish and humans. However, a BLAST search  
66 with the sequence of the GR from coelacanth and zebrafish did not retrieve the sequence of *P.*  
67 *dolloi* GR or any other lungfish GR from GenBank. Nor could we clone the *P. dolloi* GR using a  
68 cDNA from *P. dolloi* ovary using PCR primers that had successfully cloned a GR from elephant  
69 shark GR [15] and chicken, alligator and frog GRs [31]. Fortunately, on October 21, 2021 the  
70 nucleotide sequence of African lungfish (*P. annectens*) GR was deposited in GenBank, which  
71 gave us sufficient information for PCR primers to clone nine isoforms of *P. dolloi* GR. Here we  
72 report the sequences of these nine *P. dolloi* GR isoforms and explain the basis for the previous  
73 failure to clone a GR from slender African lungfish using PCR primers that previously cloned  
74 the GR from elephant shark, *Xenopus* and gar [15,31,32]. Our analysis of these nine GR  
75 sequences indicates that they evolved by alternative splicing and gene duplication [33,34].

76

## 77 **2. Results and Discussion.**

### 78 **2A. Multiple sequence alignment of nine *P. dolloi* GR isoforms.**

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

83 The multiple alignment reveals that these nine slender African lungfish GRs evolved  
84 through alternative splicing and gene duplications (Figure 1). GR-A2 appears to be a product of  
85 alternative splicing of GR-A1. GR-C4 appears to be a product of alternative splicing of one or

86 more GR-C isoforms, which supports a GR gene duplication in *P. dolloi* genome. There also is  
87 evidence for gene duplications among the *P. dolloi* GRs. MLSE at the beginning of GR-A1 is  
88 conserved in GR-B2 and GR-C2. A closely following YAPAD sequence is conserved in all *P.*  
89 *dolloi* GR isoforms. Fifteen of the first sixteen amino acids at the amino terminus of GR-A-1 are  
90 conserved in GR-B2 and GR-C2 (Figure 1A). This amino acid sequence is highly conserved in  
91 the other seven GRs. The rest of GR-A2 beginning at MMDPAGALNSLNGTQSLNKY is  
92 identical in GR-A1, and this amino acid sequence is highly conserved in the other seven GRs.  
93 MPFESLKYYAPAD is conserved at the beginning of GR-B3 and GR-C3. Beginning at the  
94 conserved MMDP sequence in the N-terminal domain, the two GR-A isoforms differ at 55  
95 positions from the three GR-B and the four GR-C isoforms.  
96

**NTD**

GR-A1 MLSEARIARKDYAPADMMDPAGSLNSLNGTQSLKYVERS DKTS 43  
 GR-A2 MMDPAGSLNSLNGTQSLKYVERS DKTS 27  
 GR-B1 MSVWHE SRHDTDWDVCSLKFNR CGLHLDYAPADMMDPAGSLNSLNGTQSLKY AERSDKTS 60  
 GR-B2 MLSEVRITRKDYAPADMMDPAGSLNSLNGTQSLKY AERSDKTS 43  
 GR-B3 MPFESLKYYPADMMMDPAGSLNSLNGTQSLKY AERSDKTS 40  
 GR-C1 MSVWHE SRHDTDWDVCSPKFNR CGLHLDYAPADMMDPAGSLNSLNGTQSLKY AERSDKTS 60  
 GR-C2 MLSEVRITRKDYAPADMMDPAGSLNSLNGTQSLKY AERSDKTS 43  
 GR-C3 MPFESLKYYPADMMMDPAGSLNSLNGTQSLKY AERSDKTS 40  
 GR-C4 MMDPAGSLNSLNGTQSLKY AERSDKTS 27  
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GR-A1 SGYPFNPICRGGAGATVTIISTSPSRQ TSAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS 103  
 GR-A2 SGYPFNPICRGGAGATVTIISTSPSRQ TSAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS 87  
 GR-B1 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 120  
 GR-B2 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 103  
 GR-B3 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 100  
 GR-C1 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 120  
 GR-C2 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 103  
 GR-C3 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 100  
 GR-C4 SGYPFNPICGGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS 87  
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GR-A1 MGFYMGEVNSKAAGTDFGSOQQOQSCVSSAENDFFLLEESLANLNRDAGTEASFLSAET 163  
 GR-A2 MGFYMGEVNSKAAGTDFGSOQQOQSCVSSAENDFFLLEESLANLNRDAGTEASFLSAET 147  
 GR-B1 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 180  
 GR-B2 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 163  
 GR-B3 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 160  
 GR-C1 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 180  
 GR-C2 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 163  
 GR-C3 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 160  
 GR-C4 MGFYMGEVNSKAAGT GFGCQQQEQQSCVSSAENDFFLLEESLANLNRDA ETEASFLNAET 147  
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GR-A1 ADSLARGQDFLAMGKTDFPSEQETFSHLGGSDTNGTSRFLSDDQNSFDIFPELSLQTDSP 223  
 GR-A2 ADSLARGQDFLAMGKTDFPSEQETFSHLGGSDTNGTSRFLSDDQNSFDIFPELSLQTDSP 207  
 GR-B1 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 239  
 GR-B2 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 223  
 GR-B3 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 219  
 GR-C1 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 239  
 GR-C2 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 222  
 GR-C3 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 219  
 GR-C4 TDSLARGQDF SAMGKSDFPSEQETFS QIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP 206  
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GR-A1 GRITDGSPWNLETFCDDEDDGGAGLSPLLDNAISEPGLNNEECRGLVGNNMFDVKNNEC 283  
 GR-A2 GRITDGSPWNLETFCDDEDDGGAGLSPLLDNAISEPGLNNEECRGLVGNNMFDVKNNEC 267  
 GR-B1 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 299  
 GR-B2 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 283  
 GR-B3 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 279  
 GR-C1 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 299  
 GR-C2 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 282  
 GR-C3 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 279  
 GR-C4 GRITDGSPWNLETFCDDEDD -GAGLSPLPIDNAISEAGGLSEECRGLVGNNNFVKNND 266  
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GR-A1 HDSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS 343  
 GR-A2 HDSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS 327  
 GR-B1 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 359  
 GR-B2 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 342  
 GR-B3 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 339  
 GR-C1 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 359  
 GR-C2 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 342  
 GR-C3 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 339  
 GR-C4 HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRFQASSSEFDA TATISIHGVS 326  
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GR-A1 TSGGQSYCYGVNNSPRSQHODQKPVFTFIPFPTAIGNNRSRCQGSSENPSLSPLTTGSYA 403  
 GR-A2 TSGGQSYCYGVNNSPRSQHODQKPVFTFIPFPTAIGNNRSRCQGSSENPSLSPLTTGSYA 387  
 GR-B1 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 419  
 GR-B2 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 403  
 GR-B3 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 399  
 GR-C1 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 419  
 GR-C2 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 402  
 GR-C3 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 399  
 GR-C4 TSGGQSYCYGV DSSPCSQQKDKQKPVFTFIPFPTAIGNNRSRCLGSSDSSSLSPLTAGSYT 386  
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GR-A1 GVSGFISSTGTALKINSSPPSGTSSSPGPPAKV 438  
 GR-A2 GVSGFISSTGTALKINSSPPSGTSSSPGPPAKV 422  
 GR-B1 GVSGFISSTGTATA VKTNSNSPPSGTLSSPGPPAKV 454  
 GR-B2 GVSGFISSTGTATA VKTNSNSPPSGTLSSPGPPAKV 437  
 GR-B3 GVSGFISSTGTATA VKTNSNSPPSGTLSSPGPPAKV 434  
 GR-C1 GVSGFISSTGTATA VKTNSNSPPSGTSSSPGPPAKV 454  
 GR-C2 GVSGFISSTGTATA VKTNSNSPPSGTSSSPGPPAKV 437  
 GR-C3 GVSGFISSTGTATA VKTNSNSPPSGTSSSPGPPAKV 434  
 GR-C4 GVSGFISSTGTATA VKTNSNSPPSGTSSSPGPPAKV 421  
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**DBD**

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GR-A1 439 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACRYRK  
GR-A2 423 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACRYRK  
GR-B1 455 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
GR-B2 438 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
GR-B3 435 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
GR-C1 455 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
GR-C2 438 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
GR-C3 435 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
GR-C4 422 CLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKCPACR FRK  
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**hinge**

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GR-A1 499 CLOAGMNL D A R K S K K K M K G I Q O P N E P S V A N P A S E T A N K A L L P T S L P Q L T P T I I N L L E V I  
GR-A2 483 CLOAGMNL D A R K S K K K M K G I Q O P N E P S V A N P A S E T A N K A L L P T S L P Q L T P T I I N L L E V I  
GR-B1 515 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
GR-B2 498 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
GR-B3 495 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
GR-C1 515 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
GR-C2 498 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
GR-C3 495 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
GR-C4 482 CLOAGMNL D A R K S K K K M K G I Q O P N E P L V A K P A S E S T T N K A L L P T S L P Q L T P T I I N L L E V I  
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**LBD**

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GR-A1 543 EPDVIYAGYDSTSPDTS PRLMSAMNTLGGROVVAAVKWAKSLPGFRNMP LDDQMSLIQYS  
GR-A2 513 EPDVIYAGYDSTSPDTS PRLMSAMNTLGGROVVAAVKWAKSLPGFRNMP LDDQMSLIQYS  
GR-B1 575 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
GR-B2 558 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
GR-B3 555 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
GR-C1 575 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
GR-C2 558 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
GR-C3 555 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
GR-C4 542 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGROVVAAVKWAKSLPGFRN LPLDDQMSLIQYS  
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GR-A1 619 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFQLOIT  
GR-A2 603 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFQLOIT  
GR-B1 635 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
GR-B2 618 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
GR-B3 615 WMFLMTFGLGWRSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
GR-C1 635 WMFLMTFGLGWRSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
GR-C2 618 WMFLMTFGLGWRSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
GR-C3 615 WMFLMTFGLGWRSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
GR-C4 602 WMFLMTFGLGWRSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFQLEIT  
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GR-A1 679 FEEYLCMKALLLLSTIPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-A2 663 FEEYLCMKALLLLSTIPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-B1 695 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-B2 678 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-B3 675 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-C1 695 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-C2 678 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-C3 675 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
GR-C4 662 FEEYLCMKALLLLSTVPKEGLKSOAVFEEIRMTYIKELGKAI VOKERSSTQNWORFFOLT  
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GR-A1 739 KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 796  
GR-A2 723 KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 780  
GR-B1 755 KLLDSMHDVV SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 812  
GR-B2 738 KLLDSMHDVV SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 795  
GR-B3 735 KLLDSMHDVV SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 792  
GR-C1 755 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 812  
GR-C2 738 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 795  
GR-C3 735 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 792  
GR-C4 722 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLL FHQK 779  
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101  
102  
103

**Fig 1b**



104 **Legend for**

105 **Figure 1A and 1B. Multiple Alignment of the amino acid sequences slender African**  
106 **lungfish glucocorticoid receptors.**

107 Total RNA was isolated from *P. dolloi* ovary and translated into cDNA. PCR was performed  
108 using four primer sets based on the sequence of *P. annectens* GR, as described in the Methods  
109 section. The amplified DNA fragments were sub-cloned into a vector for sequence analysis.  
110 Similar to other steroid receptors, slender African lungfish GR can be divided into four  
111 functional domains [6,8], consisting of a ligand-binding domain (LBD) at the C-terminus, a  
112 DNA-binding domain (DBD) in the center that is joined to the LBD by a short hinge domain  
113 (hinge), and a domain at the amino-terminus (NTD). GenBank accession no. BDF84376 for GR-  
114 A1, BDF84377 for GR-A2, BDF84378 for GR-B1, BDF84379 for GR-B2, BDF84380 for GR-  
115 B3, BDF84381 for GR-C1, BDF84382 for GR-C2, BDF84383 for GR-C3, and BDF84384 for  
116 GR-C4. Sequences were aligned with Clustal W [35], as described in the Methods section.

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118

119 **2B. Comparison of slender African lungfish GRs and West African lungfish GRs.**

120 To begin to understand sequence conservation and divergence among lungfish GRs, we  
121 compared GR-A1, GR-B1 and GR-C1, which are the three longest slender African lungfish GRs,  
122 with the four West African lungfish glucocorticoid receptor sequences in GenBank (Figure 2).  
123 The multiple sequence alignment, shown in Figure 2, reveals strong sequence conservation in the  
124 DBD, with a difference at only one position containing a semi-conserved phenylalanine-tyrosine.  
125 The sequences in the LBD and hinge domains of slender African lungfish GR and West African  
126 lungfish GR also are highly conserved. There are small segments of sequence divergence in the  
127 NTD, but most of the NTD is conserved. Overall slender African lungfish GRs and African  
128 lungfish GRs are very similar to each other.

			<b>NTD</b>	
Slender lungfish	GR-A1		MLSEARIARKDYAPADMMDPAGSLNSLNGTQSLKYVERSDKTS	43
Slender lungfish	GR-B1		MSVWHESRHDTDWVCSLKFNRCLHLDYAPADMMDPAGSLNSLNGTQSLKY <b>A</b> ERSDKTS	60
Slender lungfish	GR-C1		MSVWHESRHDTDWVCSLKFNRCLHLDYAPADMMDPAGSLNSLNGTQSLKY <b>A</b> ERSDKTS	60
African lungfish	GR-X1		MSVWHESRHDTDWVCSLKFNRCLHLDYASADMMDPAGSLNNLNGTQSLKYVERSDKTS	60
African lungfish	GR-X2		MLSEARIARKDYASADMMDPAGSLNNLNGTQSLKYVERSDKTS	43
African lungfish	GR-X3		MQFESLKYASADMMDPAGSLNNLNGTQSLKYVERSDKTS	40
African lungfish	GR-X4		MMDPAGSLNNLNGTQSLKYVERSDKTS	27
*****				
Slender lungfish	GR-A1		SGYPFNPICRGGAGATVTITSPSPRQSAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS	103
Slender lungfish	GR-B1		SGYPFNPIC <b>GGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS</b>	120
Slender lungfish	GR-C1		SGYPFNPIC <b>GGGAGATVTVSASPSRQASAQLECKQFTCDISNGLGRNASTRDPSKAVSLS</b>	120
African lungfish	GR-X1		SGYPFNPIC <b>GGGAGATVTVSASPSRQSAQLECKQFTCDISNGLGRNASTRDPSKAVSLS</b>	120
African lungfish	GR-X2		SGYPFNPIC <b>GGGAGATVTVSASPSRQSAQLECKQFTCDISNGLGRNASTRDPSKAVSLS</b>	103
African lungfish	GR-X3		SGYPFNPIC <b>GGGAGATVTVSASPSRQSAQLECKQFTCDISNGLGRNASTRDPSKAVSLS</b>	100
African lungfish	GR-X4		SGYPFNPIC <b>GGGAGATVTVSASPSRQSAQLECKQFTCDISNGLGRNASTRDPSKAVSLS</b>	87
*****				
Slender lungfish	GR-A1		MGFYMGEVNSKAAGTDFGSO QOQQQSCVSSAENDFLLLEESLANLNRDAGTEASFLSAET	163
Slender lungfish	GR-B1		MGFYMGEVNSKAAGT <b>GFGCQQQEQQSCVSSAENDFLLLEESLANLNRDAETASFLNAET</b>	180
Slender lungfish	GR-C1		MGFYMGEVNSKAAGT <b>GFGCQQQEQQSCVSSAENDFLLLEESLANLNRDAETASFLNAET</b>	180
African lungfish	GR-X1		MGFYMGEVNSKAAGT <b>GFGCQQQEQQSCVSSAENDFLLLEESLANLNRDAETASFLNAET</b>	180
African lungfish	GR-X2		MGFYMGEVNSKAAGT <b>GFGCQQQEQQSCVSSAENDFLLLEESLANLNRDAETASFLNAET</b>	163
African lungfish	GR-X3		MGFYMGEVNSKAAGT <b>GFGCQQQEQQSCVSSAENDFLLLEESLANLNRDAETASFLNAET</b>	160
African lungfish	GR-X4		MGFYMGEVNSKAAGT <b>GFGCQQQEQQSCVSSAENDFLLLEESLANLNRDAETASFLNAET</b>	147
*****				
Slender lungfish	GR-A1		ADSLARGQDFLAMGKTDFFS EQETFSLHGGSDTNGTSRFLSDDQNSFDIFPELSLQTDSP	223
Slender lungfish	GR-B1		<b>TDSLARGQDFSAMGKSDFFPSEQETFSQIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP</b>	240
Slender lungfish	GR-C1		<b>TDSLARGQDFSAMGKSDFFPSEQETFSQIGVSDPNGTSRFLSDDQNSFDIFPELSLQTDSP</b>	240
African lungfish	GR-X1		<b>TDSPARGQDFSTMGKSDFFPSEQEAFFSHIGVSDPNGTSRFLSDDQNSFDIFPELNLQTDSP</b>	240
African lungfish	GR-X2		<b>TDSPARGQDFSTMGKSDFFPSEQEAFFSHIGVSDPNGTSRFLSDDQNSFDIFPELNLQTDSP</b>	223
African lungfish	GR-X3		<b>TDSPARGQDFSTMGKSDFFPSEQEAFFSHIGVSDPNGTSRFLSDDQNSFDIFPELNLQTDSP</b>	220
African lungfish	GR-X4		<b>TDSPARGQDFSTMGKSDFFPSEQEAFFSHIGVSDPNGTSRFLSDDQNSFDIFPELNLQTDSP</b>	207
*****				
Slender lungfish	GR-A1		GRITDGPWNLETFCDDEDD GGAGLSPLLDNAISEPGGLNEECRGLVGNMFMFVKNNEC	283
Slender lungfish	GR-B1		GRITDGPWNLETFCDDEDD <b>-GAGLSPLPIDNAISEAGGLSEECRGLVGNMNFVKNNDC</b>	299
Slender lungfish	GR-C1		GRITDGPWNLETFCDDEDD <b>-GAGLSPLPIDNAISEAGGLSEECRGLVGNMNFVKNNDC</b>	299
African lungfish	GR-X1		GRIT <b>GGS</b> PWNLETFCDDEDD <b>EGVGLSPLQIDNAISEAGGLSEACRGLVGNMNFVKNI</b> EC	300
African lungfish	GR-X2		GRIT <b>GGS</b> PWNLETFCDDEDD <b>EGVGLSPLQIDNAISEAGGLSEACRGLVGNMNFVKNI</b> EC	283
African lungfish	GR-X3		GRIT <b>GGS</b> PWNLETFCDDEDD <b>EGVGLSPLQIDNAISEAGGLSEACRGLVGNMNFVKNI</b> EC	280
African lungfish	GR-X4		GRIT <b>GGS</b> PWNLETFCDDEDD <b>EGVGLSPLQIDNAISEAGGLSEACRGLVGNMNFVKNI</b> EC	267
*****				
Slender lungfish	GR-A1		HDSQMPSTSAELPQVKREKE SYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS	343
Slender lungfish	GR-B1		<b>HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDA</b> TATISIHGVS	359
Slender lungfish	GR-C1		<b>HVSQMPSTSAELPQVKREKESYIELVTPGVIKQEQLNRGFCQASSSEFDA</b> TATISIHGVS	359
African lungfish	GR-X1		<b>QDSQMPSTSAELPQVKREKEYIELVTPGVIKQEQLNRGFCQASSSEFD</b> TPATISVHGVS	360
African lungfish	GR-X2		<b>QDSQMPSTSAELPQVKREKEYIELVTPGVIKQEQLNRGFCQASSSEFD</b> TPATISVHGVS	343
African lungfish	GR-X3		<b>QDSQMPSTSAELPQVKREKEYIELVTPGVIKQEQLNRGFCQASSSEFD</b> TPATISVHGVS	340
African lungfish	GR-X4		<b>QDSQMPSTSAELPQVKREKEYIELVTPGVIKQEQLNRGFCQASSSEFD</b> TPATISVHGVS	327
*****				
Slender lungfish	GR-A1		TSGGQSYCYGVNSSPRSOHQ DQKPVFTFIPPFITAIGNNRSRCQGSSSNPLSPLTTGSYA	403
Slender lungfish	GR-B1		TSGGQSYCYGV <b>DSSPCSQKQDKQPVFTFIPPF</b> TIGNNRSRCLGSS <b>DSS</b> LSPLT <b>AGSY</b> T	419
Slender lungfish	GR-C1		TSGGQSYCYGV <b>DSSPCSQKQDKQPVFTFIPPF</b> TIGNNRSRCLGSS <b>DSS</b> LSPLT <b>AGSY</b> T	419
African lungfish	GR-X1		TSGGQSYCYGV <b>DSSPCSQKQDKQPVFTFIPPF</b> TIGNNRSRCLGSS <b>DSS</b> LSPLT <b>AGSY</b> T	420
African lungfish	GR-X2		TSGGQSYCYGV <b>DSSPCSQKQDKQPVFTFIPPF</b> TIGNNRSRCLGSS <b>DSS</b> LSPLT <b>AGSY</b> T	403
African lungfish	GR-X3		TSGGQSYCYGV <b>DSSPCSQKQDKQPVFTFIPPF</b> TIGNNRSRCLGSS <b>DSS</b> LSPLT <b>AGSY</b> T	400
African lungfish	GR-X4		TSGGQSYCYGV <b>DSSPCSQKQDKQPVFTFIPPF</b> TIGNNRSRCLGSS <b>DSS</b> LSPLT <b>AGSY</b> T	387
*****				
Slender lungfish	GR-A1		GVSGFISSTGTALKINSSPPSGTSSSPGPPAKV	438
Slender lungfish	GR-B1		GVSGFISSTGT <b>VKTNSNSPPSGTSSSPGPPAKV</b>	454
Slender lungfish	GR-C1		GVSGFISSTGT <b>VKTNSNSPPSGTSSSPGPPAKV</b>	454
African lungfish	GR-X1		<b>G</b> LSGFISSTGT <b>MKINSNSPPSGTSSSPGPPAKV</b>	455
African lungfish	GR-X2		<b>G</b> LSGFISSTGT <b>MKINSNSPPSGTSSSPGPPAKV</b>	438
African lungfish	GR-X3		<b>G</b> LSGFISSTGT <b>MKINSNSPPSGTSSSPGPPAKV</b>	435
African lungfish	GR-X4		<b>G</b> LSGFISSTGT <b>MKINSNSPPSGTSSSPGPPAKV</b>	422
*****				

129

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**FIGURE 2A**



				DBD	
Slender lungfish	GR-A1	439	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACRYRK
Slender lungfish	GR-B1	455	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACR FRK
Slender lungfish	GR-C1	455	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACR FRK
African lungfish	GR-X1	456	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACR FRK
African lungfish	GR-X2	439	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACR FRK
African lungfish	GR-X3	436	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACR FRK
African lungfish	GR-X4	423	CLVCSDEASGCHYGVLTC	GSCKVFVKRAVEGOHNYLCAGRND	CIIDKIRRKNCPCACR FRK
*****					
				hinge	
Slender lungfish	GR-A1	499	CLOAGMNLDAKSKKKMK	GIQOPNEPSVANPASESTANKALPTSL	POLTPTTIINLLEVI
Slender lungfish	GR-B1	515	CLOAGMNLDAKSKKKMK	GIQOPNEPLVAKPASESTTNKALLPTSL	POLTPTTIINLLEVI
Slender lungfish	GR-C1	515	CLOAGMNLDAKSKKKMK	GIQOPNEPLVAKPASESTTNKALLPTSL	POLTPTTIINLLEVI
African lungfish	GR-X1	516	CLOAGMNLDAKSKKKMK	GIQOPNEQSVAKPASESTTNKALLPTSL	POLTPTTIINLLEVI
African lungfish	GR-X2	499	CLOAGMNLDAKSKKKMK	GIQOPNEQSVAKPASESTTNKALLPTSL	POLTPTTIINLLEVI
African lungfish	GR-X3	496	CLOAGMNLDAKSKKKMK	GIQOPNEQSVAKPASESTTNKALLPTSL	POLTPTTIINLLEVI
African lungfish	GR-X4	483	CLOAGMNLDAKSKKKMK	GIQOPNEQSVAKPASESTTNKALLPTSL	POLTPTTIINLLEVI
*****					
				LBD	
Slender lungfish	GR-A1	559	EPDVIYAGYDSTSPDTS	RLMSAMNTLGGQVVAAVKWA	SLPGFRNPLDDQMSLIQYS
Slender lungfish	GR-B1	575	EPDVIYAGYDSTSPDTS	SSRLMSAMNTLGGQVVAAVKWA	SLPGFRNLPPLDDQMSLIQYS
Slender lungfish	GR-C1	575	EPDVIYAGYDSTSPDTS	SSRLMSAMNTLGGQVVAAVKWA	SLPGFRNLPPLDDQMSLIQYS
African lungfish	GR-X1	576	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWA	TLPGFRNLPPLDDQMSLIQYS
African lungfish	GR-X2	559	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWA	TLPGFRNLPPLDDQMSLIQYS
African lungfish	GR-X3	556	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWA	TLPGFRNLPPLDDQMSLIQYS
African lungfish	GR-X4	543	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWA	TLPGFRNLPPLDDQMSLIQYS
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Slender lungfish	GR-A1	613	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLI	RIAQAFAQLQIT
Slender lungfish	GR-B1	635	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLI	KIAQAFAQLLEIT
Slender lungfish	GR-C1	635	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLI	KIAQAFAQLLEIT
African lungfish	GR-X1	636	WMFLMTFGLGWRSYKQS	NGAMLCYAPDLVITDERMQLPYMMEHCHYLI	KIAQAFAQLQIT
African lungfish	GR-X2	619	WMFLMTFGLGWRSYKQS	NGAMLCYAPDLVITDERMQLPYMMEHCHYLI	KIAQAFAQLQIT
African lungfish	GR-X3	616	WMFLMTFGLGWRSYKQS	NGAMLCYAPDLVITDERMQLPYMMEHCHYLI	KIAQAFAQLQIT
African lungfish	GR-X4	603	WMFLMTFGLGWRSYKQS	NGAMLCYAPDLVITDERMQLPYMMEHCHYLI	KIAQAFAQLQIT
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Slender lungfish	GR-A1	679	FEEYLCMKALLLLSTIPK	EGLKKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
Slender lungfish	GR-B1	695	FEEYLCMKALLLLST	VPKEGLKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
Slender lungfish	GR-C1	695	FEEYLCMKALLLLST	VPKEGLKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
African lungfish	GR-X1	696	FEEYLCMKALLLLST	VPKEGLKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
African lungfish	GR-X2	679	FEEYLCMKALLLLST	VPKEGLKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
African lungfish	GR-X3	676	FEEYLCMKALLLLST	VPKEGLKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
African lungfish	GR-X4	663	FEEYLCMKALLLLST	VPKEGLKSQAVFEEIRMTYIKELGKAI	VOKERSSTONWQRFQLT
*****					
Slender lungfish	GR-A1	739	KLLDSMHDVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK	796
Slender lungfish	GR-B1	755	KLLDSMHDVV	SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK	812
Slender lungfish	GR-C1	755	KLLDSMHDVV	TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK	812
African lungfish	GR-X1	756	KLLDSMHDVV	TKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNV	RPLLFHQK 813
African lungfish	GR-X2	739	KLLDSMHDVV	TKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNV	RPLLFHQK 796
African lungfish	GR-X3	736	KLLDSMHDVV	TKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNV	RPLLFHQK 793
African lungfish	GR-X4	723	KLLDSMHDVV	TKVLNVCFQNFLDRSRCVDYPEMLQELITNQLPRIRSGNV	RPLLFHQK 780
*****					

Figure 2B

Figure 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.

## 2C. 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

145 lungfish GR, coelacanth GR, zebrafish GR and human GR (Figure 3). The DBD and hinge  
146 domains are highly conserved in all GRs. There is good sequence conservation of the LBD in all  
147 six GRs. However, there is an interesting pattern of sequence conservation in the NTD. There is  
148 excellent sequence conservation in the NTD among slender African lungfish GR, West African  
149 lungfish GR, coelacanth GR and human GR. The stronger conservation of the NTD in lungfish  
150 GRs with human GR than with zebrafish GR, indicates that the NTD in zebrafish GR has  
151 diverged from the other GRs.  
152

Slender lungfish GR	MSVWHESRHD	TWDVCS	PKFNRC	GLHLDY	APADM	PAGS	LNSLNGTQ	SLKYAERS	SDKTS	60
African lungfish GR	MSVWHESRHD	TWDVCS	PKFNRC	GLHLDY	ASADM	PAGS	LNNLNGTQ	SLKYVERS	SDKTS	60
Coelacanth GR							MMESKEV	LNTSGGD-	SLKVD-	YSKRE
Human GR							MDSKES	LTPGEE-	NPSSVL-	AQERG
Zebrafish GR							MDQGLENGK	-----	KRDERL	16
Slender lungfish GR	SGYP	FNPI	CGGAGA	---	TVTVSAS	PSRQ-	ASAQLECK	QFTCDI	SNGLGR	NASTRDP
African lungfish GR	SGYP	FNPI	CGGAGA	---	TVTVSAS	PSRQ-	TSAQLECK	QFTCDI	SNGLGR	NATTRDP
Coelacanth GR	EVYD	FNPF	RGCAPI	---	PLTPASS	LLLV-	SEADSPROL	ASGDT	SNVSG	NATPODLS
Human GR	DVMD	FYKTL	RGCAPI	---	KVSASSP	LAVASQ	SDSKORRL	LVDFPK	GSVS	NAQQPDL
Zebrafish GR	NTLD	YNKR	ATEG	IL	PRRIQ	STMSV	APTSMV	PQAGP	PMQPV	SGDIPNGL
Slender lungfish GR	VSL	SMGF	YMGE	VNSK	AAGT	GFGC	QQQE	QSCVSS	SAEND	FFLLES
African lungfish GR	VSL	SMGF	YMGE	VNSK	AAGT	GFGC	QQQE	QSCVSS	SAEND	FFLLES
Coelacanth GR	VSE	SMGL	YMGE	SDSK	VIGOK	ARLQ	QKQIS	-GLSV	TEFAL	LESIAS
Human GR	VSL	SMGL	YMGE	TETK	VMGND	LGFP	QQQIS	-LSSGE	TDLKL	LESIAS
Zebrafish GR	VSL	--SIF	GDDSEL	LLGK	EQRAL	QQQTLV	-PFTL	GDSL	SGL	EASLAD
Slender lungfish GR	LNAETD	SLARG	QDFS	AMKSD	FPS	EQ	ETFSQ	IGVSD	PNGT	SRLFS
African lungfish GR	LNAETD	SPARG	QDFS	TMKSD	FPS	EQ	EAFSH	IGVSD	PNGT	SRLFS
Coelacanth GR	SLAPQV	SL--	DNGS	GMKAC	DL	SV	EQ	ETFAQ	T-DSD	PNGLS
Human GR	SSASTAV	SAAPTE	KEKFP	KTHSD	VSS	EQ	QHLKG	Q-TGT	NGN	VKLYTT
Zebrafish GR	GGVD	PNL	FLPK	TEDF	SPM	IKGD	MDL	QDS	FGH	GKDV
Slender lungfish GR	QTD	SPGR	ITDGS	PWNLET	FC	DD	EDGAG	LSP	LP-	IDNAISE
African lungfish GR	QTD	SPGR	ITDGS	PWNLET	FC	DD	EDGAG	LSP	LP-	IDNAISE
Coelacanth GR	TPC	SPGKE	--	NPWS	LDPI	YD	GGGR--	GLLS	P	LA-ADD
Human GR	SSG	SPGKET	NES	PWR	SDLL	IE-	-N--	CLLS	P	LAGE
Zebrafish GR	DGSP	SPDFY	VAD----	DAFL	STIG-	EDALL	SEL	ETN-----	LDR	SKAAV--
Slender lungfish GR	VKNND	CHVS	QMPST	SAELP	QV	KRE	KEYI	IEL	VTP	PGVI
African lungfish GR	VKNI	ECQD	SQMPST	SAELP	QV	KRE	KEYI	IEL	VTP	PGVI
Coelacanth GR	STNNE	CGN	LFT	PDL	SSQLS	QIK	SDKE	GYI	IEL	TPGV
Human GR	KIKD	NDL	VLSS	SPSN	VTL	QV	TEK	EDFI	IEL	CTPG
Zebrafish GR	TLNG	TASS	SL	STANT	SII	ENIK	VEK	DSII	IQ	CTPG
Slender lungfish GR	TAT	SIH	GV	TS	GGQ	S	YCYG	VDS--	SPCS	QK
African lungfish GR	PATI	SVH	GV	TS	GGQ	S	YCYG	VDS--	SPCS	QH
Coelacanth GR	NSSI	SIH	GV	TS	GGQ	MYH	VDN	AGAV	SSA	QQ
Human GR	MSAI	SVH	GV	TS	GGQ	MYH	DMN--	--	TASL	QQ
Zebrafish GR	--	TPIN	IC	GV	TS	GGQ	S	YCYG	VDS--	SPCS
Slender lungfish GR	SSLSP	L	TAGSY	T	GVSG	L	SSST	G	TAVK	TNSNP-
African lungfish GR	SSLSP	L	TAGSY	T	GVSG	L	SSST	G	TAVK	TNSNP-
Coelacanth GR	NSTPP	L	GNV	NAS	G	RSG	F	ASSY	SPG	TRTAT
Human GR	NL-TS	L	GT	LNF	P	GRTV	PS	NGY	SSP	MRP
Zebrafish GR	SGM	Q	RAS	L	C	F	S	KN--	FS---	SS
Slender lungfish GR	YGV	L	TCG	S	CKV	V	FK	R	A	V
African lungfish GR	YGV	L	TCG	S	CKV	V	FK	R	A	V
Coelacanth GR	YGV	L	TCG	S	CKV	V	FK	R	A	V
Human GR	YGV	L	TCG	S	CKV	V	FK	R	A	V
Zebrafish GR	YGV	L	TCG	S	CKV	V	FK	R	A	V
Slender lungfish GR	SK	K	K	M	G	I	Q	P	N	E
African lungfish GR	SK	K	K	M	G	I	Q	P	N	E
Coelacanth GR	SK	K	L	N	K	M	G	N	-	L
Human GR	TK	K	K	I	G	I	Q	A	T	---
Zebrafish GR	SK	K	A	R	Q	A	G	V	I	Q
Slender lungfish GR	ST	S	P	D	T	S	S	R	L	S
African lungfish GR	ST	S	P	D	T	S	S	R	L	S
Coelacanth GR	ST	I	P	D	T	H	C	R	L	M
Human GR	SSV	P	D	S	T	W	R	I	M	T
Zebrafish GR	ST	I	P	D	T	S	V	R	L	M
Slender lungfish GR	WRSYK	Q	S	S	G	T	V	L	C	Y
African lungfish GR	WRSYK	Q	S	S	G	T	V	L	C	Y
Coelacanth GR	WRSYQ	Q	S	S	G	T	V	L	C	Y
Human GR	WRSYR	Q	S	S	A	N	L	C	F	A
Zebrafish GR	WRSYQ	H	C	N	G	N	M	L	C	F
Slender lungfish GR	LLL	STV	P	K	E	G	L	K	S	O
African lungfish GR	LLL	STV	P	K	E	G	L	K	S	O
Coelacanth GR	LLL	STI	P	O	E	G	L	K	S	O
Human GR	LLL	SSV	P	K	D	G	L	K	S	O
Zebrafish GR	LLL	N	T	V	P	K	D	G	L	K
Slender lungfish GR	TKV	L	N	V	C	F	O	N	E	L
African lungfish GR	TKV	L	N	V	C	F	O	N	E	L
Coelacanth GR	KELL	K	I	C	F	H	T	F	V	D
Human GR	ENL	N	Y	C	F	O	T	F	L	D
Zebrafish GR	GGL	L	N	F	C	F	Y	T	F	V

153

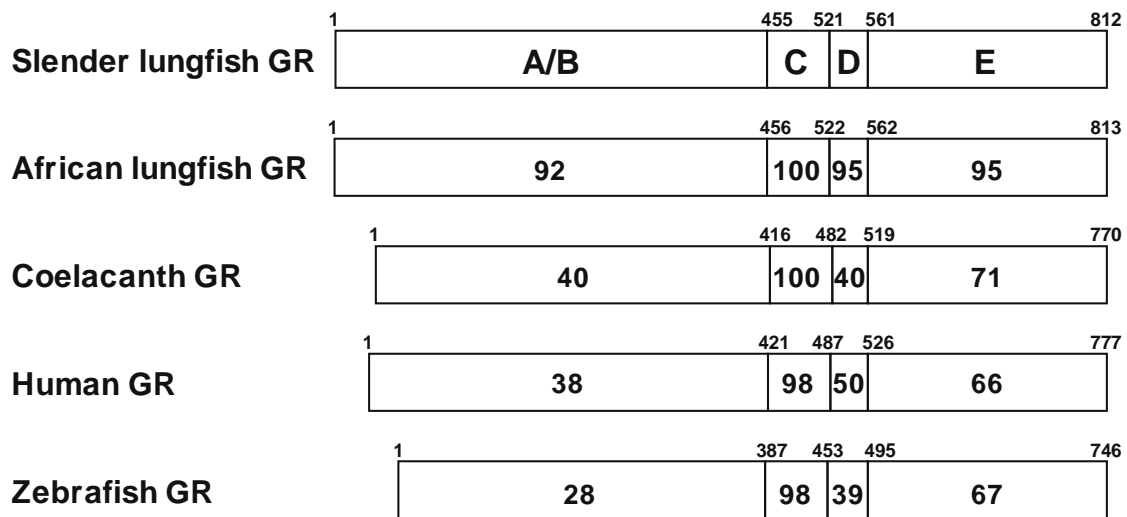
154

155 **Figure 3. Multiple Sequence Alignment of slender African lungfish GR, West African**  
 156 **lungfish GR, coelacanth GR, zebrafish GR and human GR.**

157 Glucocorticoid receptor sequences were downloaded from GenBank (Accession no.  
 158 NP\_000167 for human GR, XP\_005996162 for coelacanth GR, and NP\_001018547 for  
 159 zebrafish GR) and aligned with Clustal W [35], as described in the Methods section. The NTD  
 160 in zebrafish GR has gaps and sequence differences with the other GRs.

162 **2D. Comparison of functional domains in slender African lungfish GR with domains in**  
 163 **West African lungfish GR, coelacanth GR, zebrafish GR and human GR.**

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



169  
 170  
 171 **Figure 4. Comparison of functional domains of slender lungfish GR with domains in West**  
 172 **African lungfish GR, coelacanth GR, zebrafish GR, human GR.**

173 Comparison of domains in slender African lungfish GR with GRs from West African lungfish,  
 174 coelacanths, humans and zebrafish and MRs from slender African lungfish, West African  
 175 lungfish, humans and zebrafish. The functional NTD (A/B), DBD (C), hinge (D) and LBD (E)

176 domains are schematically represented with the numbers of amino acid residues and the  
177 percentage of amino acid identity depicted.

178

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

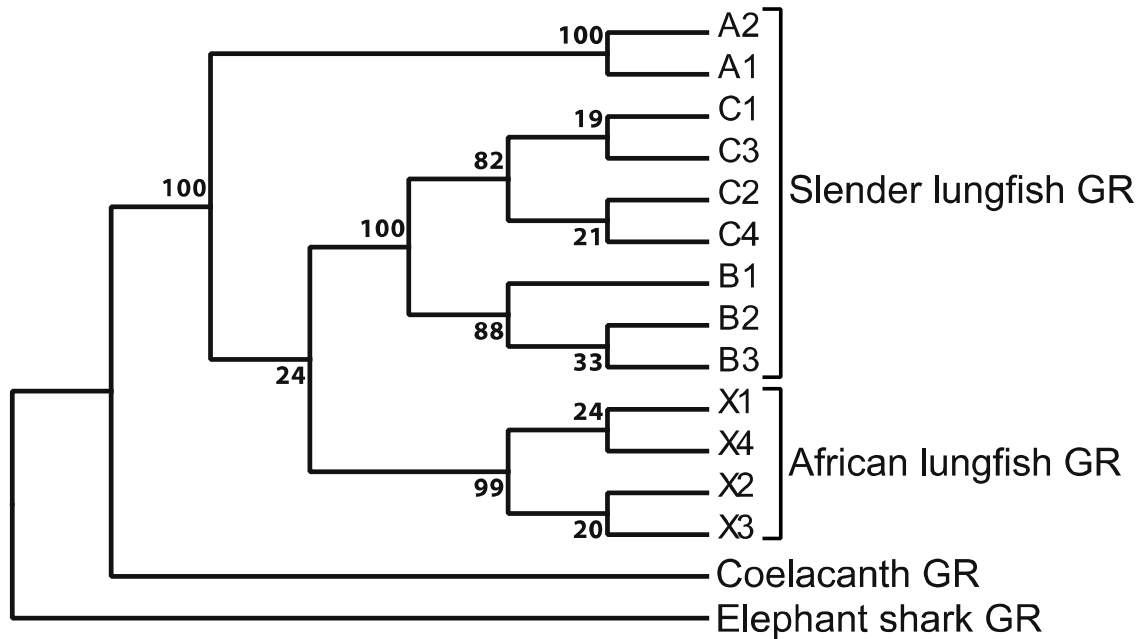
185

## 186 **2E. Phylogenetic Analysis.**

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

193

194



195

196

197 **Figure 5. Phylogeny of slender African lungfish Glucocorticoid Receptors, West African**  
198 **lungfish Glucocorticoid Receptors, Coelacanth GR and Elephant Shark GR.** MEGA5 [36]  
199 was used to construct this phylogeny. Statistics are based on 1,000 runs.

200

## 201 **2F. Basis for the failure to clone *P. dolloi* GR.**

202 Figure 6 shows the location of the PCR primers that we used to successfully clone GRs  
203 from chicken, alligator and frog [31]. Due to the strong conservation of the GR and MR these  
204 PCR primers retrieved partial sequences from both the GR and MR in chicken, alligator and  
205 frog. The full sequences of these GRs and MRs was achieved in the next step using RACE. Our  
206 failure to clone *P. dolloi* GR was due using WQRFYQ instead of WQRFFQ for the 1<sup>st</sup>/2<sup>nd</sup>-  
207 reverse primer. When we used WQRFFQ we were able to clone *P. dolloi* GR.



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	WQRF <u>F</u> Q	753
P.d lungfish GR	747	WQRF <u>F</u> Q	752

208  
209 P.a. = *Protopterus annectens*, P. d. = *Protopterus dolloi*

210

211 **Figure 6. Location of PCR primers used for cloning of slender African lungfish GR,**  
212 **coelacanth GR, elephant shark GR, zebrafish GR and human GR.**

213 The correct 1<sup>st</sup>/2<sup>nd</sup>-reverse primer for PCR cloning of *P. dolloi* GR is WQRFFQ instead  
214 of WQRFYQ.

215

## 216 **2G. Summary**

217 *P. dolloi* contains nine GR isoforms, in contrast to *P. annectens*, which contains four GR  
218 isoforms. We do not know how many GR isoforms are in Australian lungfish (*Neoceratodus*  
219 *forsteri*) because their GR sequences have not been deposited in GenBank. The availability of  
220 sequences of *P. dolloi* GRs and *P. annectens* GRs should permit using PCR to clone *N. forsteri*  
221 GRs, which would elucidate the number GR isoforms in this lungfish and the relationship of  
222 their GRs to the GRs of *P. dolloi* and *P. annectens*.

223 The response to corticosteroids of any lungfish GR is not known, nor are the functions of  
224 the multiple GR isoforms in *P. dolloi* GRs and *P. annectens* GRs. We have initiated studies to  
225 determine corticosteroid activation of *P. dolloi* GRs to begin to elucidate the functions of slender  
226 African lungfish GRs. It is interesting that there are multiple isoforms of human GR, due to  
227 alternative splicing of human GR, and these isoforms are important in achieving functional  
228 diversity of human GR [6,8,34,37]. A similar scenario is likely for *P. dolloi* GRs and *P.*  
229 *annectens* GRs.

230

### 231 **3. Methods**

#### 232 **3A. Animal**

233 A slender spotted African lungfish, *Protopterus dolloi*, was purchased from a local  
234 commercial supplier. Lungfish were anesthetized in freshwater containing 0.02% ethyl 3-  
235 aminobenzoate methane-sulfonate from Sigma-Aldrich, and tissue samples were quickly  
236 dissected and frozen in liquid nitrogen. Animal handling procedures conformed to the guidelines  
237 set forth by the Institutional Animal Care and Use Committee at the University of Tokyo.

238

#### 239 **3B. Molecular cloning of lungfish *P. dolloi* glucocorticoid receptor.**

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

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

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

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

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

245 and one C-terminal Reverse primer: R: 5'-GTTAAGGCAAATTTCTGATATTAAGGCAG-3'

246 based on the sequences of *P. annectens* GR (X1: XM\_044069149, X2: XM\_044069150, X3:

247 XM\_044069152, X4: XM\_044069153). PCR was performed using four primer sets (F-X1xR, F-

248 X2xR, F-X3xR, and F-X4xR) with ovary cDNA of *P. dolloi*, and the amplified DNA fragments

249 with KOD-plus- DNA polymerase were subcloned into a cloning vector, pCR-BluntII-TOPO,

250 and sequence analysis was performed for 10 or more clones for each primer sets.

251

#### 252 **3C. Database and sequence analysis.**

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

258

#### 259 **4. AUTHOR CONTRIBUTIONS**

260 Y.K., S.O., L.B. and M.E.B. carried out the research and analyzed data. S.H. aided in the  
261 collection of animals. X.L. constructed plasmid DNAs used in this study. Y.K. and M.E.B.  
262 conceived and designed the experiments. Y.K., L.B. and M.E.B. wrote the paper. All authors  
263 gave final approval for publication.

264

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