

Cloning of Nine Glucocorticoid Receptor Isoforms from the Slender African lungfish
(*Protopterus dolloi*)

Yoshinao Katsu^{1,2}, Shin Oana², Xiaozhi Lin², Susumu Hyodo³,
Laurent Bianchetti⁴, Michael E. Baker^{5,6}

¹Graduate School of Life Science

Hokkaido University

Sapporo, Japan

²Faculty of Science

Hokkaido University

Sapporo, Japan

³Laboratory of Physiology

Atmosphere and Ocean Research Institute

University of Tokyo

Chiba, Japan

⁴Institute of Genetics and Molecular and Cellular Biology (IGBMC),

CNRS UMR 7104 - Inserm U1258

Université de Strasbourg

67404 Illkirch, France

⁵Division of Nephrology-Hypertension

Department of Medicine, 0693

University of California, San Diego

9500 Gilman Drive

La Jolla, CA 92093-0693

⁶Center for Academic Research and Training in Anthropogeny (CARTA)

University of California, San Diego

La Jolla, CA 92093

Correspondence to:

Y. Katsu: ykatsu@sci.hokudai.ac.jp

M. E. Baker: mbaker@health.ucsd.edu

ABSTRACT. We wanted to clone the glucocorticoid receptor (GR) from slender African lungfish (*Protopterus dolloi*) for comparison to *P. dolloi* 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.

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

1. 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].

Lungfish 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].

2. Results and Discussion.

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

Figure 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 (Figure 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 (Figure 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.

DBD

GR-A1 439 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACRYRK
 GR-A2 423 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACRYRK
 GR-B1 455 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK
 GR-B2 438 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK
 GR-B3 435 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK
 GR-C1 455 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK
 GR-C2 438 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK
 GR-C3 435 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK
 GR-C4 422 CLVCSDEASGCHYGVLTCGSKCVFFKRAVEGQHNYLCAGRNDICI IDKIRRNKNCAPACR FRK

hinge

GR-A1 499 CLQAGMNLDAKSSKKMKGIQQPNEPSVANPASESTANKALLPTSLSLPOLTPPTIINLLEVI
 GR-A2 483 CLQAGMNLDAKSSKKMKGIQQPNEPSVANPASESTANKALLPTSLSLPOLTPPTIINLLEVI
 GR-B1 515 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI
 GR-B2 498 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI
 GR-B3 495 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI
 GR-C1 515 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI
 GR-C2 498 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI
 GR-C3 495 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI
 GR-C4 482 CLQAGMNLDAKSSKKMKGIQQPNEP LVAKPASESTTNKALLPTSLSLPOLTPPTIINLLEVI

LBD

GR-A1 543 EPDVIYAGYDSTSPDTSPLRLMSAMNTLGGRQVVAAVKWAKSLPGFRNMPPLDDQMSLIQYS
 GR-A2 513 EPDVIYAGYDSTSPDTSPLRLMSAMNTLGGRQVVAAVKWAKSLPGFRNMPPLDDQMSLIQYS
 GR-B1 575 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS
 GR-B2 558 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS
 GR-B3 555 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS
 GR-C1 575 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS
 GR-C2 558 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS
 GR-C3 555 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS
 GR-C4 542 EPDVIYAGYDSTSPDTS SRLMSAMNTLGGRQVVAAVKWAKSLPGFRN LPLDDQMSLIQYS

GR-A1 619 WMFLMTFGLGWRYSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
 GR-A2 603 WMFLMTFGLGWRYSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
 GR-B1 635 WMFLMTFGLGWRYSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT
 GR-B2 618 WMFLMTFGLGWRYSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT
 GR-B3 615 WMFLMTFGLGWRYSYKQSSGTMLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT
 GR-C1 635 WMFLMTFGLGWRYSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT
 GR-C2 618 WMFLMTFGLGWRYSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT
 GR-C3 615 WMFLMTFGLGWRYSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT
 GR-C4 602 WMFLMTFGLGWRYSYKQSSGT VLCYAPDLVITEERMRLPYMMEHCHHLI KIAQAFALQIT

GR-A1 679 FEEYLCMKALLLLSTIPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-A2 663 FEEYLCMKALLLLSTIPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-B1 695 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-B2 678 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-B3 675 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-C1 695 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-C2 678 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-C3 675 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT
 GR-C4 662 FEEYLCMKALLLLST VPKEGLKSQAVFEEIRMTYIKELGKAIVQKERSSTQNWORFFOLT

GR-A1 739 KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 796
 GR-A2 723 KLLDSMHDVVAKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 780
 GR-B1 755 KLLDSMHDVV SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 812
 GR-B2 738 KLLDSMHDVV SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 795
 GR-B3 735 KLLDSMHDVV SKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 792
 GR-C1 755 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 812
 GR-C2 738 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 795
 GR-C3 735 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 792
 GR-C4 722 KLLDSMHDVV TKVLNVCFQNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLPHQK 779

Fig 1b

Legend for

Figure 1A and 1B. 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.

2B. 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 (Figure 2). The multiple sequence alignment, shown in Figure 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 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.

NTD			
Slender lungfish	GR-A1	MLSEARIARKDYAPADMMDPAGSLNSLNGTQSLKYVERSDKTS	43
Slender lungfish	GR-B1	MSVWHESRHDTDWVCSLKFNRCLHLDYAPADMMDPAGSLNSLNGTQSLKY A ERSDKTS	60
Slender lungfish	GR-C1	MSVWHESRHDTDWVCSLKFNRCLHLDYAPADMMDPAGSLNSLNGTQSLKY A 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	SGYPFNPICRGGAGATVTITSTSPSRQTSQAQLDAKQFTCDISNGLGRNDSSRDPSKAVSLS	103
Slender lungfish	GR-B1	SGYPFNPIC G GGAGATVT V SA S PSRQ A SAQ L EC K QFTCDISNGLGRN A STRDPSKAVSLS	120
Slender lungfish	GR-C1	SGYPFNPIC G GGAGATVT V SA S PSRQ A SAQ L EC K QFTCDISNGLGRN A STRDPSKAVSLS	120
African lungfish	GR-X1	SGYPFNPIC G GGAGATVT V SA S PSRQTSQAQL E C K QFTCDISNGLGRN A STRDPSKAVSLS	120
African lungfish	GR-X2	SGYPFNPIC G GGAGATVT V SA S PSRQTSQAQL E C K QFTCDISNGLGRN A STRDPSKAVSLS	103
African lungfish	GR-X3	SGYPFNPIC G GGAGATVT V SA S PSRQTSQAQL E C K QFTCDISNGLGRN A STRDPSKAVSLS	100
African lungfish	GR-X4	SGYPFNPIC G GGAGATVT V SA S PSRQTSQAQL E C K QFTCDISNGLGRN A STRDPSKAVSLS	87
*****			*****
Slender lungfish	GR-A1	MGFYMGEVNSKAAGTDFGSQ QQGQSCVSSAENDFFLLEESLANLNRDAGTEASFLSAET	163
Slender lungfish	GR-B1	MGFYMGEVNSKAAGT G FG C QQQ E QQSCVSSAENDFFLLEESLANLNRD A ETASFL N AE T	180
Slender lungfish	GR-C1	MGFYMGEVNSKAAGT G FG C QQQ E QQSCVSSAENDFFLLEESLANLNRD A ETASFL N AE T	180
African lungfish	GR-X1	MGFYMGEVNSKAAGT G FG C QQQ E QQSCVSSAENDFFLLEESLANLNRD A ETASFL N AE T	180
African lungfish	GR-X2	MGFYMGEVNSKAAGT G FG C QQQ E QQSCVSSAENDFFLLEESLANLNRD A ETASFL N AE T	163
African lungfish	GR-X3	MGFYMGEVNSKAAGT G FG C QQQ E QQSCVSSAENDFFLLEESLANLNRD A ETASFL N AE T	160
African lungfish	GR-X4	MGFYMGEVNSKAAGT G FG C QQQ E QQSCVSSAENDFFLLEESLANLNRD A ETASFL N AE T	147
*****			*****
Slender lungfish	GR-A1	ADSLARGQDFLAMGKTDFFS EQETFSHLGGSDTNGTSRLFSDDQNSFDIFPELSLQTDSP	223
Slender lungfish	GR-B1	TD S LARGQDF S AMG K SDFPSEQ E TFS Q IG V S D PNGTSRLFSDDQNSFDIFPELSLQTDSP	240
Slender lungfish	GR-C1	TD S LARGQDF S AMG K SDFPSEQ E TFS Q IG V S D PNGTSRLFSDDQNSFDIFPELSLQTDSP	240
African lungfish	GR-X1	TD S PARQDF S TMG K SDFPSEQ E A F SH I GV S D P NGTSRLFSDDQNSFDIFPEL N LQTDSP	240
African lungfish	GR-X2	TD S PARQDF S TMG K SDFPSEQ E A F SH I GV S D P NGTSRLFSDDQNSFDIFPEL N LQTDSP	223
African lungfish	GR-X3	TD S PARQDF S TMG K SDFPSEQ E A F SH I GV S D P NGTSRLFSDDQNSFDIFPEL N LQTDSP	220
African lungfish	GR-X4	TD S PARQDF S TMG K SDFPSEQ E A F SH I GV S D P NGTSRLFSDDQNSFDIFPEL N LQTDSP	207
** *****			*****
Slender lungfish	GR-A1	GRITDGSPWNLETFCDDEDD GGAGLSPLLDIDNAISEPGGLNNEECRGLVGNNMFVKNNEC	283
Slender lungfish	GR-B1	GRITDGSPWNLETFCDDEDD -GAGLSPL P IDNAISEAGGL S EECRGLVGNN N FEVKN N D	299
Slender lungfish	GR-C1	GRITDGSPWNLETFCDDEDD -GAGLSPL P IDNAISEAGGL S EECRGLVGNN N FEVKN N D	299
African lungfish	GR-X1	GRIT G GSPWNLETFCDDEDD E GVGLSPL Q IDNAISEAGGL S EACRGLVGNN N FEVKN I E C	300
African lungfish	GR-X2	GRIT G GSPWNLETFCDDEDD E GVGLSPL Q IDNAISEAGGL S EACRGLVGNN N FEVKN I E C	383
African lungfish	GR-X3	GRIT G GSPWNLETFCDDEDD E GVGLSPL Q IDNAISEAGGL S EACRGLVGNN N FEVKN I E C	280
African lungfish	GR-X4	GRIT G GSPWNLETFCDDEDD E GVGLSPL Q IDNAISEAGGL S EACRGLVGNN N FEVKN I E C	267
**** *****			*****
Slender lungfish	GR-A1	HDSQMPSTSAELPQVKREKE SYIELVTPGVIKQEQLSRGFCQASSSEFDAPAPISIHGVS	343
Slender lungfish	GR-B1	H V SQMPSTSAELPQVKREKESYIELVTPGVIKQEQL N RGFCQASSSEFD A T A TISIHGVS	359
Slender lungfish	GR-C1	H V SQMPSTSAELPQVKREKESYIELVTPGVIKQEQL N RGFCQASSSEFD A T A TISIHGVS	359
African lungfish	GR-X1	Q DSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQL N RGFCQASSSEFD T P A TISVHGVS	360
African lungfish	GR-X2	Q DSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQL N RGFCQASSSEFD T P A TISVHGVS	343
African lungfish	GR-X3	Q DSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQL N RGFCQASSSEFD T P A TISVHGVS	340
African lungfish	GR-X4	Q DSQMPSTSAELPQVKREKEGYIELVTPGVIKQEQL N RGFCQASSSEFD T P A TISVHGVS	327
*****			*****
Slender lungfish	GR-A1	TSGGQSYCYGVNSSPSRQHQ DQKPVFTFIPPFITAIGNNRSRCQGSSSNPSLSPLTTGSYA	403
Slender lungfish	GR-B1	TSGGQSYCYGV D SSP C SQ Q KDQKPVFTFIPPF T IGNNR N R L GSS D SSLSPLT A GSY T	419
Slender lungfish	GR-C1	TSGGQSYCYGV D SSP C SQ Q KDQKPVFTFIPPF T IGNNR N R L GSS D SSLSPLT A GSY T	419
African lungfish	GR-X1	TSGGQSYCYGV D SSP C SQ Q KDQK I FTFIPPF T IGNNR N R S RCQGSS D SSLSPLT A GSY T	420
African lungfish	GR-X2	TSGGQSYCYGV D SSP C SQ Q KDQK I FTFIPPF T IGNNR N R S RCQGSS D SSLSPLT A GSY T	403
African lungfish	GR-X3	TSGGQSYCYGV D SSP C SQ Q KDQK I FTFIPPF T IGNNR N R S RCQGSS D SSLSPLT A GSY T	400
African lungfish	GR-X4	TSGGQSYCYGV D SSP C SQ Q KDQK I FTFIPPF T IGNNR N R S RCQGSS D SSLSPLT A GSY T	387
*****			*****
Slender lungfish	GR-A1	GVSGFISSTGTALKINSSPPSGTSSSPGPPAKV	438
Slender lungfish	GR-B1	GVSGFISSTGT A V K T NS N SPSGT L SSPGPPAKV	454
Slender lungfish	GR-C1	GVSGFISSTGT A V K T NS N SPSGT L SSPGPPAKV	454
African lungfish	GR-X1	G LSGFISSTGT A M K INS N SPSGTSPSPGPPAKV	455
African lungfish	GR-X2	G LSGFISSTGT A M K INS N SPSGTSPSPGPPAKV	438
African lungfish	GR-X3	G LSGFISSTGT A M K INS N SPSGTSPSPGPPAKV	435
African lungfish	GR-X4	G LSGFISSTGT A M K INS N SPSGTSPSPGPPAKV	422
* *****			*****

FIGURE 2A

				DBD
Slender lungfish	GR-A1	439	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACRYRK
Slender lungfish	GR-B1	455	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACR FRK
Slender lungfish	GR-C1	455	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACR FRK
African lungfish	GR-X1	456	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACR FRK
African lungfish	GR-X2	439	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACR FRK
African lungfish	GR-X3	436	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACR FRK
African lungfish	GR-X4	423	CLVCSDEASGCHYGVLTC	GSCKVFFKRAVEGQHNYLCAGRNDICIIDKIRRKNCPCACR FRK

				hinge
Slender lungfish	GR-A1	499	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI
Slender lungfish	GR-B1	515	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI
Slender lungfish	GR-C1	515	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI
African lungfish	GR-X1	516	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI
African lungfish	GR-X2	499	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI
African lungfish	GR-X3	496	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI
African lungfish	GR-X4	483	CLOAGMNLDAKSKKKMK	GIOQPNESPANPAESTANKALLPTSLPOLPTTIINLLEVI

				LBD
Slender lungfish	GR-A1	559	EPDVIYAGYDSTSPDTS	RLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS
Slender lungfish	GR-B1	575	EPDVIYAGYDSTSPDTS	RLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS
Slender lungfish	GR-C1	575	EPDVIYAGYDSTSPDTS	RLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS
African lungfish	GR-X1	576	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS
African lungfish	GR-X2	559	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS
African lungfish	GR-X3	556	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS
African lungfish	GR-X4	543	EPDVIYAGYDSTSPD	SSRLMSAMNTLGGQVVAAVKWAWSLPGFRNLPLDDQMSLIQYS

Slender lungfish	GR-A1	613	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
Slender lungfish	GR-B1	635	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
Slender lungfish	GR-C1	635	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
African lungfish	GR-X1	636	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
African lungfish	GR-X2	619	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
African lungfish	GR-X3	616	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT
African lungfish	GR-X4	603	WMFLMTFGLGWRSYKQSS	GTMLCYAPDLVITEERMRLPYMMEHCHHLIRIAQAFALQIT

Slender lungfish	GR-A1	679	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT
Slender lungfish	GR-B1	695	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT
Slender lungfish	GR-C1	695	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT
African lungfish	GR-X1	696	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT
African lungfish	GR-X2	679	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT
African lungfish	GR-X3	676	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT
African lungfish	GR-X4	663	FEEYLCMKALLLLSTIPK	EGLKSQAVFEEIRMTYIKELGKAIQKERSSTONWQRFQOLT

Slender lungfish	GR-A1	739	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK
Slender lungfish	GR-B1	755	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK
Slender lungfish	GR-C1	755	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK
African lungfish	GR-X1	756	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK
African lungfish	GR-X2	739	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK
African lungfish	GR-X3	736	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK
African lungfish	GR-X4	723	KLDSMDHVVAKVLNVCF	QNFLDRSRCVEYPDMLQELITNQLPRIRSGNVKPLLFHQK

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

lungfish GR, coelacanth GR, zebrafish GR and human GR (Figure 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.

Slender lungfish GR	MSVWHESRHD TDWVCSPKFNRCGLHLDYAPADMM	PAGS	LNSLNGTQSLKYAERSDKTS	60
African lungfish GR	MSVWHESRHD TDWVCSLKFNRCGLHLDYASADMM	PAGS	LNNLNGTQSLKYVERSDKTS	60
Coelacanth GR		MMESEKEV	LNTSGGD-SLKVD-YSKRE	25
Human GR		MDSKES	LTPGREE-NPSSVL-AQERG	24
Zebrafish GR		MDQGLENGK	-----KRDERL	16
Slender lungfish GR	SGYP FNPICGGGAGA---TVTVSASPSRQ-ASAQLECKQFTCD	ISNGLGR	NASTRDPSCA	116
African lungfish GR	SGYP FNPICGGGAGA---TVTVSASPSRQ-TSAQLECKQFTCD	ISNGLGR	NATTRDPSCA	116
Coelacanth GR	EVYD FNPVFRGGAPI---PLTPASSLLLV-SEADSPQLASGDTSNVSG	NATPQDLSCA		81
Human GR	DVMD FYKTLRGCA TV---KVSASSPLAVASQSDSKQRLLVDFPKGSVS	NAQQPDLSCA		81
Zebrafish GR	NTLDYNKRATEGILPRRIQSTMSVAPTSMVPQAGPMMPVSGD	IPNGLSNSPTLEEHTSS		76
Slender lungfish GR	VSL SMGFYMGENVNSKAAGTGFQCQOE QQSCVSSAENDFFLLEESLANLN	RDAETEASF-		175
African lungfish GR	VSL SMGFYMGENVNSKAAGTGFQCQOE QQSCVSSAENDFFLLEESLANLN	RDAETEASF-		175
Coelacanth GR	VSE SMGLYMGESDSKVIGOKARLQKQGIS-GLSVETEFALLEESIASLN	RSTRIVEGVS		140
Human GR	VSL SMGLYMGETETKVMGNLDGFPQOGQIS-LSSGETDLKLEESIANLN	RSTRIVEGVS		140
Zebrafish GR	VSL--SIFGDDSELKLLGKEQRLAQCTLV-PFTLGDLSLGLASLADLN	PNPSPSMDSLI		132
Slender lungfish GR	LNAETTD SLARGQDFSAMGKSLFPS E QETFSQIGVSDPNC	TSRLFSD	DQNSFDIFPELSL	235
African lungfish GR	LNAETTD SPARGQDFSTMKGSLFPS E QETFSQIGVSDPNC	TSRLFSD	DQNSFDIFPELSL	235
Coelacanth GR	SLAPQV SL---DNGSGMAKCDLSV E QETFAQT-DSDPNCNLSFFPD	DQAAFDILQELDL		196
Human GR	SSASTAV SAAPTEKEFPKTHSDVSS E QOHLKGQ-TGTNGGNVKLYTT	DQSTFDILQDFEL		199
Zebrafish GR	GGVDPNLFLPKTEDFSPMIGKMDLDQDSFGHIGKDVVDVGNHKLFS	SDN---TLDLQDFEL		190
Slender lungfish GR	QTD SPGRITDGS PWNLETFCDDED-DGAGLSPLP-IDNAISEAGGLS	EECRGLVGNNNFE		293
African lungfish GR	QTD SPGRITDGS PWNLETFCDDEDDEGVGLSPLQ-IDNAISEAGGLS	EACRGLVGNNNFE		294
Coelacanth GR	TPC SPGKE---NPWSLDPIYDGGR---GLLSPLA-ADDPFLMAAVAN	EDCKSSLVTNTSQ		250
Human GR	SSG SPGKETNES PWRSDLLIDG--N-CLLSPLAGEDDSFLLEGNSN	EDCKPLILPDTKP		255
Zebrafish GR	DGSPSDFYVAD----DAFLSTIE-EDALLSELFTN-----LDRDSKAAV	---SGSN		233
Slender lungfish GR	VKNNDCHVSQMPSTSAELP QVKREKESYIELVTPGVIKOEQLNRGF	COASSSEF----	DA	349
African lungfish GR	VKNIECQDSQMPSTSAELP QVKREKESYIELVTPGVIKOEQLNRGF	COASSSEF----	DT	350
Coelacanth GR	STNNECNLFIPDLSSQLS QIKSDKEGYIELVTPGVVKQETLGRSY	COANLTSAAST-TA		309
Human GR	KIKDNGDLVSSPSNVTL P QVKTEKEDFIELCTPGVIKQELGTVY	COASFPGANIGNK		315
Zebrafish GR	TLNGTASSLSSTANTSIILNIVKEKDSIIQLCTPGVIKQENTGASYC	QGLHS-----		286
Slender lungfish GR	TATL SIHGVSTSGGQSYCYGVDS--SPCSQQKDOKPVTFTFIP	FTTIGNNRNRCLGSSDS		407
African lungfish GR	PATL SVHGVSTSGGQSYCYGVDS--SPCSQHODQKPIFTFTFIP	FTTIGNNRNRCLGSSDN		408
Coelacanth GR	NSSI SIHGVNTSGGQMYHYDVNAGAVSSAQQPDOKPIFNFIPI	PVSTIAENWNRCCSGAT		369
Human GR	MSAI SVHGVSTSGGQMYHDMN---TASLSQQODQKPIFNFIPI	PVSGENWNRCCSGGD		372
Zebrafish GR	-TPI NICGVTTSSGQSLFGNSSPTAVVGLQDKDPFNMYTLP	TSSGDGWSRQGRNV		345
Slender lungfish GR	SSLSP LTAGSYTGVSGLISSSTGTAVKTNNSNP-P SGTSSSPGPPAKV	CLVCSDEASGCH		466
African lungfish GR	SSLSP LTTGSYTGLSGLISSSTGTAMKINSNP-P SGTSPSPGPPAKV	CLVCSDEASGCH		467
Coelacanth GR	NSTPP LGNVNASGRSGFASSYSPGTRTATPT--P SSSSTSGPPHKI	CLVCSDEASGCH		427
Human GR	NL-TS LGTLNFPGRTVPSNGYSPPMRPDVSSPPS SSSATTGPPPKL	CLVCSDEASGCH		431
Zebrafish GR	SGMQQRASLCFSKN--FS---SSPYSRPEDST--ATSSAGGKTGTHK	CLVCSDEASGCH		398
Slender lungfish GR	YGVLTGCSCKVFFKRAVEGQHNYLCAGRNDICI	IDKIRRNKCPACFRKCL	QAGMNLDAK	526
African lungfish GR	YGVLTGCSCKVFFKRAVEGQHNYLCAGRNDICI	IDKIRRNKCPACFRKCL	QAGMNLDAK	527
Coelacanth GR	YGVLTGCSCKVFFKRAVEGQHNYLCAGRNDICI	IDKIRRNKCPACFRKCL	QAGMNLDAK	487
Human GR	YGVLTGCSCKVFFKRAVEGQHNYLCAGRNDICI	IDKIRRNKCPACFRKCL	QAGMNLDAK	491
Zebrafish GR	YGVLTGCSCKVFFKRAVEGQHNYLCAGRNDICI	IDKIRRNKCPACFRKCL	QAGMNLDAK	458
Slender lungfish GR	SKKKMKGIQQPNEP--LVAKPASESTTNKALLPTSLPQL	TPTIINLLEVIEPDVYAGYD		584
African lungfish GR	SKKKMKGIQQPNEQ--SVAKPASESTTNKALLPTSLPQL	TPTIINLLEVIEPDVYAGYD		585
Coelacanth GR	SKKLNKMGKN-LS---S-KEQATPPLPERAVVPASVPQL	TPTMISLLEAIEPSILYAGYD		542
Human GR	TKKKIKGIQQAATT---GVSQETSENPGNKTIPTATLPQL	TPTLVSLLEVIEPEVLYAGYD		548
Zebrafish GR	SKSKARQAGKVIQQQSIPERNLPLPEARALVPKMPQLVPT	MLSLKAIPEPTLYAGYD		518
Slender lungfish GR	STSPDTSSRLMSAMNTLGGROVV AAVKWALESFGFRNLPLDDOMSL	QYSWMLMTFGLG		644
African lungfish GR	STSPDSSRLMSAMNTLGGROVV AAVKWALESFGFRNLPLDDOMSL	QYSWMLMTFGLG		645
Coelacanth GR	STIPDTHCRIMTALNKLGGROVV AAVKWALESFGFRNLPLDDOMVLL	QYSWMLMAFGLG		602
Human GR	SSVPDSTWRIMTTLNMLGGROVI AAVKWALESFGFRNLPLDDOMTLL	QYSWMLMAFGLG		608
Zebrafish GR	STIPDTSVRIMTTLNMLGGROVIAAVKWALESFGFRNLPLDDOMTLL	QYSWMLMAFGLG		578
Slender lungfish GR	WRSYK QSSGTVLCYAPDLVITEERMILPYMMEHCHHLIKIAQAFAQL	EITFEEYLCMKAL		704
African lungfish GR	WRSYK QSNGAMLCYAPDLVITDERMOLPYMMEHCHYLKIAQAFAQL	EITFEEYLCMKAL		705
Coelacanth GR	WRSYK QANGSMLCFAPDLIINEQRMOLPYMYEQCHMLKIASEFSRL	QVSYYEYLCMKAL		662
Human GR	WRSYK QSSANLFCFAPDLIINEQRMOLPYMYEQCHMLKIASEFSRL	QVSYYEYLCMKAL		668
Zebrafish GR	WRSYK QHCNGNMLCFAPDLVINEERMILPYMSDQCEQMLKISNEFVRL	QVSTEEYLCMKAL		638
Slender lungfish GR	LLL STVPKEGLKSQAVFEEIRMTYIKELGKAIVOKERSSTONWQREF	FOLTKLLDSMHD	VV	764
African lungfish GR	LLL STVPKEGLKSQAVFEEIRMTYIKELGKAIVOKERSSTONWQREF	FOLTKLLDSMHD	VV	765
Coelacanth GR	LLL STIPQEGLSQPVFDEIRMTYIKELGKAIVKKEGNSNONWQREF	FOLTKLLDSMHD	VV	722
Human GR	LLL SSVPKDGLKSQELFDEVRMTYIKELGKAIVKKEGNSNONWQREF	FOLTKLLDSMHD	VV	728
Zebrafish GR	LLLNTVPKDLKSQSVFDEIRMSYIKELGKAIVKKEGNSNONWQREF	FOLTKLLDSMHD	VV	698
Slender lungfish GR	TKVLNVCFQNELDRSRCVEYDMLQELITNQLPRIRSGNVKPLLFHQK			812
African lungfish GR	TKVLNVCFQNELDRSRCVDYDMLQELITNQLPRIRSGNVKPLLFHQK			813
Coelacanth GR	KELLKICFHTFV DKSLSVEFPEMLAETISNOLPKVTSGSCKALLFHQK			770
Human GR	ENLLNYCFQTFPLDKTMSIEFPEMLAETISNOLPKYSNGNIKLLFHQK			776
Zebrafish GR	GGLLNFCFYTFVFNKSLSVEFPEMLAETISNOLPKFKDGSVKPLLFHQK			746

153

154

Figure 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.

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

Figure 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.

Slender lungfish GR	1	455	521	561	812
	A/B	C	D	E	
African lungfish GR	1	456	522	562	813
	92	100	95	95	
Coelacanth GR	1	416	482	519	770
	40	100	40	71	
Human GR	1	421	487	526	777
	38	98	50	66	
Zebrafish GR	1	387	453	495	746
	28	98	39	67	

Figure 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.

As shown in figure 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%).

2E. 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 Figures 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.

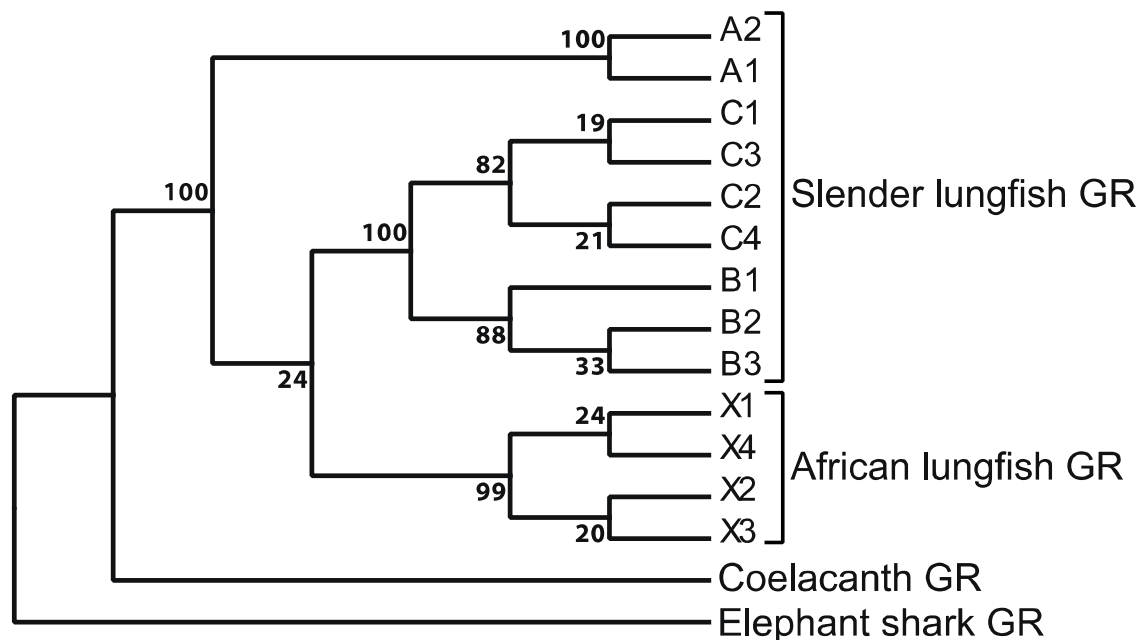


Figure 5. Phylogeny of slender African lungfish Glucocorticoid Receptors, West African lungfish Glucocorticoid Receptors, Coelacanth GR and Elephant Shark GR. MEGA5 [36] was used to construct this phylogeny. Statistics are based on 1,000 runs.

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

Figure 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 WQRFYQ instead of WQRFFQ for the 1st/2nd-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	WQRFYQ	753				
P.d lungfish GR	747	WQRFYQ	752				

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

Figure 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.

2G. 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 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 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.

3. Methods

3A. Animal

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

3B. 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.

3C. 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].

4. AUTHOR CONTRIBUTIONS

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

5. FUNDING

Y.K. was supported in part by Grants-in-Aid for Scientific Research [19K067309] from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Takeda Science Foundation. L.B. was supported by the INSERM (Institut National de la Santé et de la Recherche Médicale). M.E.B. was supported by UC San Diego Research fund #3096.

6. REFERENCES

1. Baker ME, Nelson DR, Studer RA. Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors. *J Steroid Biochem Mol Biol.* 2015;151:12-24. doi:10.1016/j.jsbmb.2014.10.020.
2. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science.* 1988;240(4854):889-895. doi:10.1126/science.3283939.
3. Bridgham JT, Eick GN, Larroux C, et al. Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. *PLoS Biol.* 2010;8(10):e1000497. Published 2010 Oct 5. doi:10.1371/journal.pbio.1000497.
4. Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M. Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived

- 282 endocrine systems. *Mol Biol Evol.* 2004 Oct;21(10):1923-37. doi: 10.1093/molbev/msh200.
283 Epub 2004 Jun 30. PMID: 15229292.
- 284 5. Baker ME, Funder JW, Kattoula SR. Evolution of hormone selectivity in glucocorticoid and
285 mineralocorticoid receptors [published correction appears in *J Steroid Biochem Mol Biol.*
286 2014 Jan;139:104]. *J Steroid Biochem Mol Biol.* 2013;137:57-70.
287 doi:10.1016/j.jsbmb.2013.07.009.
- 288 6. Weikum ER, Knuesel MT, Ortlund EA, Yamamoto KR. Glucocorticoid receptor control of
289 transcription: precision and plasticity via allostery. *Nat Rev Mol Cell Biol.* 2017;18(3):159-
290 174. doi:10.1038/nrm.2016.152. doi:doi:10.1038/nrm.2016.152
- 291 7. de Kloet ER. From receptor balance to rational glucocorticoid therapy. *Endocrinology.*
292 2014;155(8):2754-2769. doi:10.1210/en.2014-1048.
- 293 8. Gross KL, Cidlowski JA. Tissue-specific glucocorticoid action: a family affair. *Trends*
294 *Endocrinol Metab.* 2008;19(9):331-339. doi:10.1016/j.tem.2008.07.009.
- 295 9. Bridgham JT, Carroll SM, Thornton JW. Evolution of hormone-receptor complexity by
296 molecular exploitation. *Science.* 2006;312(5770):97-101. doi:10.1126/science.1123348.
- 297 10. Arriza JL, Weinberger C, Cerelli G, et al. Cloning of human mineralocorticoid receptor
298 complementary DNA: structural and functional kinship with the glucocorticoid receptor.
299 *Science.* 1987;237(4812):268-275. doi:10.1126/science.3037703.
- 300 11. Baker ME, Katsu Y. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR:
301 Evolution of the mineralocorticoid receptor: sequence, structure and function. *J Endocrinol.*
302 2017;234(1):T1-T16. doi:10.1530/JOE-16-0661.
- 303 12. Thornton JW. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor
304 by ligand exploitation and serial genome expansions. *Proc Natl Acad Sci U S A.* 2001 May
305 8;98(10):5671-6. doi: 10.1073/pnas.091553298. Epub 2001 May 1. PMID: 11331759;
306 PMCID: PMC33271.
- 307 13. Osório J, Rétaux S. The lamprey in evolutionary studies. *Dev Genes Evol.* 2008
308 May;218(5):221-35. doi: 10.1007/s00427-008-0208-1. Epub 2008 Feb 15. PMID:
309 18274775.
- 310 14. Carroll SM, Bridgham JT, Thornton JW. Evolution of hormone signaling in elasmobranchs
311 by exploitation of promiscuous receptors. *Mol Biol Evol.* 2008;25(12):2643-2652.
312 doi:10.1093/molbev/msn204.
- 313 15. Katsu Y, Shariful IMD, Lin X, Takagi W, Urushitani H, Kohno S, Hyodo S, Baker ME. N-
314 terminal Domain Regulates Steroid Activation of Elephant Shark Glucocorticoid and
315 Mineralocorticoid Receptors. *J Steroid Biochem Mol Biol.* 2021 Feb 27:105845. doi:
316 10.1016/j.jsbmb.2021.105845. Epub ahead of print. PMID: 33652098.

16. Inoue JG, Miya M, Lam K, et al. Evolutionary origin and phylogeny of the modern holocephalans (Chondrichthyes: Chimaeriformes): a mitogenomic perspective. *Mol Biol Evol.* 2010;27(11):2576-2586. doi:10.1093/molbev/msq147.
17. Venkatesh B, Lee AP, Ravi V, et al. Elephant shark genome provides unique insights into gnathostome evolution [published correction appears in *Nature*. 2014 Sep 25;513(7519):574]. *Nature.* 2014;505(7482):174-179. doi:10.1038/nature12826.
18. Biscotti MA, Gerdol M, Canapa A, Forconi M, Olmo E, Pallavicini A, Barucca M, Scharl M. The Lungfish Transcriptome: A Glimpse into Molecular Evolution Events at the Transition from Water to Land. *Sci Rep.* 2016 Feb 24;6:21571. doi: 10.1038/srep21571. PMID: 26908371; PMCID: PMC4764851.
19. Meyer A, Schloissnig S, Franchini P, Du K, Woltering JM, Irisarri I, Wong WY, Nowoshilow S, Kneitz S, Kawaguchi A, Fabrizius A, Xiong P, Dechaud C, Spaink HP, Volff JN, Simakov O, Burmester T, Tanaka EM, Scharl M. Giant lungfish genome elucidates the conquest of land by vertebrates. *Nature.* 2021 Feb;590(7845):284-289. doi: 10.1038/s41586-021-03198-8. Epub 2021 Jan 18. PMID: 33461212; PMCID: PMC7875771.
20. Wang K, Wang J, Zhu C, Yang L, Ren Y, Ruan J, Fan G, Hu J, Xu W, Bi X, Zhu Y, Song Y, Chen H, Ma T, Zhao R, Jiang H, Zhang B, Feng C, Yuan Y, Gan X, Li Y, Zeng H, Liu Q, Zhang Y, Shao F, Hao S, Zhang H, Xu X, Liu X, Wang D, Zhu M, Zhang G, Zhao W, Qiu Q, He S, Wang W. African lungfish genome sheds light on the vertebrate water-to-land transition. *Cell.* 2021 Feb 4:S0092-8674(21)00090-8. doi: 10.1016/j.cell.2021.01.047. Epub ahead of print. PMID: 33545087.
21. Joss JM. Lungfish evolution and development. *Gen Comp Endocrinol.* 2006 Sep 15;148(3):285-9. doi: 10.1016/j.ygcen.2005.10.010. Epub 2005 Dec 7. PMID: 16337631.
22. Rossier BC. Osmoregulation during Long-Term Fasting in Lungfish and Elephant Seal: Old and New Lessons for the Nephrologist. *Nephron.* 2016;134(1):5-9. doi: 10.1159/000444307. Epub 2016 Feb 23. PMID: 26901864.
23. Joss JM, Itahara Y, Watanabe TX, Nakajima K, Takei Y. Teleost-type angiotensin is present in Australian lungfish, *Neoceratodus forsteri*. *Gen Comp Endocrinol.* 1999;114(2):206-212. doi:10.1006/gcen.1999.7246.
24. Joss, J. M., D. Arnold-Reed and R. Balment. "The steroidogenic response to angiotensin II in the Australian lungfish, *Neoceratodus forsteri*." *Journal of Comparative Physiology B* 164 (2004): 378-382.
25. Baker ME. Steroid receptors and vertebrate evolution. *Mol Cell Endocrinol.* 2019;496:110526. doi:10.1016/j.mce.2019.110526.
26. Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. *Cell.* 2001;104(4):545-556. doi:10.1016/s0092-8674(01)00241-0.

27. Shibata S. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: Mineralocorticoid receptor and NaCl transport mechanisms in the renal distal nephron. *J Endocrinol.* 2017;234(1):T35-T47. doi:10.1530/JOE-16-0669.
28. Jaisser F, Farman N. Emerging Roles of the Mineralocorticoid Receptor in Pathology: Toward New Paradigms in Clinical Pharmacology. *Pharmacol Rev.* 2016;68(1):49-75. doi:10.1124/pr.115.011106.
29. Joëls M, de Kloet ER. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: The brain mineralocorticoid receptor: a saga in three episodes. *J Endocrinol.* 2017 Jul;234(1):T49-T66. doi: 10.1530/JOE-16-0660. PMID: 28634266.
30. Katsu Y, Oana S, Lin X, Hyodo S, Baker ME. Aldosterone and dexamethasone activate African lungfish mineralocorticoid receptor: Increased activation after removal of the amino-terminal domain. *J Steroid Biochem Mol Biol.* 2022 Jan;215:106024. doi: 10.1016/j.jsbmb.2021.106024. Epub 2021 Nov 10. PMID: 34774724.
31. Katsu Y, Kohno S, Oka K, Baker ME. Evolution of corticosteroid specificity for human, chicken, alligator and frog glucocorticoid receptors. *Steroids.* 2016;113:38-45. doi:10.1016/j.steroids.2016.06.005.
32. Oka K, Hoang A, Okada D, Iguchi T, Baker ME, Katsu Y. Allosteric role of the amino-terminal A/B domain on corticosteroid transactivation of gar and human glucocorticoid receptors. *J Steroid Biochem Mol Biol.* 2015;154:112-119. doi:10.1016/j.jsbmb.2015.07.025.
33. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol Cell.* 2005 Apr 29;18(3):331-42. doi: 10.1016/j.molcel.2005.03.025. PMID: 15866175.
34. Gross KL, Lu NZ, Cidlowski JA. Molecular mechanisms regulating glucocorticoid sensitivity and resistance. *Mol Cell Endocrinol.* 2009;300(1-2):7-16. doi:10.1016/j.mce.2008.10.001.
35. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994 Nov 11;22(22):4673-80. doi: 10.1093/nar/22.22.4673. PMID: 7984417; PMCID: PMC308517.
36. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011 Oct;28(10):2731-9. doi: 10.1093/molbev/msr121. Epub 2011 May 4. PMID: 21546353; PMCID: PMC3203626.
37. Lu NZ, Cidlowski JA. Glucocorticoid receptor isoforms generate transcription specificity. *Trends Cell Biol.* 2006 Jun;16(6):301-7. doi: 10.1016/j.tcb.2006.04.005. Epub 2006 May 11. PMID: 16697199.

391 38. Felsenstein J. CONFIDENCE LIMITS ON PHYLOGENIES: AN APPROACH USING
 392 THE BOOTSTRAP. *Evolution*. 1985 Jul;39(4):783-791. doi: 10.1111/j.1558-
 393 5646.1985.tb00420.x. PMID: 28561359.

394