Cartography of rhodopsin-like G protein-coupled receptors across vertebrate genomes

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We conduct a cartography of rhodopsin-like non-olfactory G protein-coupled receptors in the Ensembl database. The most recent genomic data (releases 90–92, 90 vertebrate genomes) are analyzed through the online interface and receptors mapped on phylogenetic guide trees that were constructed based on a set of ~14,000 amino acid sequences. This snapshot of genomic data suggest vertebrate genomes to harbour 142 clades of GPCRs without human orthologues. Among those, 69 have not to our knowledge been mentioned or studied previously in the literature, of which 28 are distant from existing receptors and likely new orphans. These newly identified receptors are candidates for more focused evolutionary studies such as chromosomal mapping as well for in-depth pharmacological characterization. Interestingly, we also show that 37 of the 72 human orphan (or recently deorphanized) receptors included in this study cluster into nineteen closely related groups, which implies that there are less ligands to be identified than previously anticipated. Altogether, this work has significant implications when discussing nomenclature issues for GPCRs.

G protein-coupled receptors (GPCRs) are signalling proteins activated by for example neurotransmitters and neuromodulators and as such are involved in many physiological processes. Their location as a cellular gateway makes them key targets for drug discovery and chemical biology¹⁻³, and 30–50% of drugs on the market have been reported to target GPCRs directly or indirectly⁴.

GPCRs are well known for sharing a three-dimensional architecture characterized by seven transmembrane α-helical segments⁵,⁶ connected by loops. In the rhodopsin family, a well conserved disulphide bridge often connects the second extracellular loop and the third transmembrane segment. This architecture is reflected at the sequence level by conserved sets of amino acids that serve structurally or as determinants of signal transduction, e.g. to name a few the E/DRY, CWxP, NPxxY motifs in the transmembrane segments 3, 6 and 7 of the rhodopsin family⁷,⁸. Additional family-specific motifs have been identified for examples in connecting loops⁹. Within each transmembrane segment, the most conserved amino acid will be referred to here as pivots (pivot amino acids are not always fully conserved: N1.50, 98% conservation; D2.50, 90%; R3.50, 95%; W4.50, 97%; P5.50, 78%; P6.50, 99%; P7.50, 88% see ⁷). These amino acids are used as a basis of the widely used Ballesteros-Weinstein numbering¹⁰, where equivalent positions are reported by transmembrane segment number followed by relative distance to pivot, itself assigned the index 50.

The International Union of Basic and Clinical Pharmacology (IUPHAR) is responsible for the international classification of GPCRs and issues regular recommendations. GPCRs have been divided into five main families based on phylogenetic analyses¹¹⁻¹³. The largest, Rhodopsin, in human ~700 members, subdivides into four branches (α, β, γ and δ) and 13 sub-branches¹¹ and has originated through local duplications about 1400–1100 million years ago¹⁴. Families are further divided into subtypes¹⁵ that arouse, for many, 350–500 million years ago from two rounds of whole genome duplication (2R)¹⁶⁻²². In ray-finned fish (not in lobe-finned or jawless fishes), a third specific genome duplication took place about ~250 million years ago, leading to fish-specific duplicates²³⁻²⁵. In tunicates, GPCRs originated before the 2R and after the separation of invertebrates from chordates²⁶⁻²⁸. In lamprey, it remains unclear whether the cyclostome-gnathostome split at the origin of jawed vertebrates happened before or after the second round of genome duplication²⁷⁻²⁹.
As consequence of the origin of vertebrate GPCRs through the 2R/3R, in an ideal scenario, for each ancestral receptor we expect four/eight paralogues (that can be referred to as "ohnologues"), many ohnologues are simply equivalent to subtypes). Nonetheless, local duplications and deletions (pseudogenes) may make the current day picture complex and, consequently, the expect four/eight ohnologues are most often not seen. Furthermore, ligand binding preferences do not necessarily indicates the closest possible evolutionary relationships; some receptors have acquired the same ligand specificity several times, for example $\alpha_1$-, $\alpha_2$-, and $\beta$-adrenoceptors that are parts of the amine family, or e.g. the cannabinoid receptors CB1 and CB2, and the recently deorphanized GPR55 that are activated by the same endogenous ligand 2-Arachidonoylglycerol.

GPCRs have been identified and characterized in the 70's and cloned in the 80's, e.g. the well-studied rhodopsin and $\beta$-adrenergic receptor. The apparition of genomics in the 2000's has brought an explosion in the amount of discovered GPCRs, yet GPCRs are in most cases named according to their human orthologues. This leads to complex nomenclature issues especially when new clades without human orthologues are identified. In addition, the repertoires in non-human vertebrate species are much less explored, and new subtypes or orphan receptors are often not annotated at all. The only way to date to tackle the issue of GPCRs in non-vertebrate species is the tedious manual annotation based on phylogenetic information.

Here, we conducted this manual annotation based on the online Ensembl database. The transcripts predicted in Ensembl are based on automated alignments matched to curated homologues sequences or ESTs. Ensembl gene trees are based on a consensus of five tree reconstruction methods: a maximum likelihood based on two types or orphan receptors are often not annotated at all. The only way to date to tackle the issue of GPCRs in non-vertebrate species is the tedious manual annotation based on phylogenetic information. For this purpose the suggested genes symbols were reevaluated against the Ensembl.R94 (December 2018) and Ensembl.R95 (February 2019) releases. They will be submitted to the other relevant committees on publication (MGNC, Mouse Genomic Nomenclature Committee; RGNC, Rat Genome and Nomenclature Committee, ZNC, Zebrafish Nomenclature Committee; XGC, Xenopus Gene Nomenclature Committee; CGNC, Chicken Gene Nomenclature Consortium). Therefore all gene symbols presented in this manuscript should be regraded as tentative until approved by the relevant committees.

All data used for this study are available online and can be easily accessed using the codes provided (see the Experimental section).

This study was conducted based on the Ensembl genomic data twice with a five-year interval: 2012–2013, Ensembl release 67 (referred to as Ensembl.R67); and 2017–2018, Ensembl.R90-Ensembl.R.92. The text is organized according to the Ensembl.R91 trees (December 2017, available as an archive, see Experimental section). Some of the groupings may slightly differ in future releases. Ensembl.R92 (April 2018) became available during the final preparation of this manuscript and was used to solve a few ambiguities, as mentioned in text. The proposed new gene symbols were submitted to HUGO (Human Genome Nomenclature Committee) for initial consideration; for this purpose the suggested genes symbols were reevaluated against the Ensembl.R94 (December 2018) and Ensembl.R95 (February 2019) releases. They will be submitted to the other relevant committees on publication (MGNC, Mouse Genomic Nomenclature Committee; RGNC, Rat Genome and Nomenclature Committee, ZNC, Zebrafish Nomenclature Committee; XGC, Xenopus Gene Nomenclature Committee; CGNC, Chicken Gene Nomenclature Consortium). Therefore all gene symbols presented in this manuscript should be regraded as tentative until approved by the relevant committees.

Altogether, in Ensembl.R92 we identified 142 clusters of genes corresponding to non-olfactory rhodopsin-like GPCRs without human orthologues (Tables 1, 2), of which 69 have not been to our best knowledge previously described. Twenty-eight are distant from any group of receptors and likely orphan, and the others probable subtypes of existing receptors (Table 2). Not surprisingly, most of the new receptors are present in ray-finned fishes and only a few in placental mammals (Table 1). Nonetheless, most (23) of the new orphan receptors are found in two or more species clades (considering separately ray-finned and lobe-finned fishes, amphibians, birds, reptiles, monotreme, marsupial and placental mammals). In addition, families that are evolutionary more recent and faster evolving such as purine (PUR) and chemokine (CHEM) contains a larger number of previously unidenti- fied receptors (Table 2). We furthermore identify groups of ambiguous branching that need to wait for more and/or increased quality sequence data to be characterized (not annotated, see text below).

Table 1. Species-specific counts of non-olfactory GPCRs clades without human orthologues in Ensembl.R91.

| Species | Lampry | Fishes (Actinopterygii) | Fishes (Sarcopterygii) | Amphibian | Reptiles | Birds | Mammals |
|---------|--------|------------------------|------------------------|-----------|----------|-------|---------|
| Whole genome duplication | 1R/2R | 3R | 3R, except Lepisosteus oculatus 2R | 2R | 2R | 2R | 2R |
| Pictogram | | | | | | | |
| Number of genomes | 1 | 2 | 9 | 1 | 1 | 2 | 5 | 1 | 3 | 65 |
| Paralogous GPCR clades identified | 22 | 118 | 134 | 95 | 69 | 74 | 52 | 16 | 19 | 11 |

Results and Discussion

Overview. All data used for this study are available online and can be easily accessed using the codes provided (see the Experimental section).
ADRA2D subtype was also found9,47. Near the histamine receptor H2 (HRH2), rooted by two lampreys, a gene subtypes: 5-HT7B (HTR7B) found in gar/fishes(10)/coelacanth, see ENSLACP00000008875, that has likely been ADRA1A,1B,1C; HTR1A,1B,1C,1D,1E; HTR5,7.

Subtypes have been previously reported in zebrafish55, which should correspond here to a set of fishes(17)/coelacanth/reptile(1) and to S1PR3a in fishes(10)/coelacanth.

Table 2. Family-specific counts of non-olfactory GPCRs clades without human orthologues in Ensembl.R91.

| AMIN | MECAM | PTGERM | MLTM | OPP | PEP | CHEM | SOG & MCH | LGR | MRG | PURIN | Others | Total |
|------|-------|--------|------|-----|-----|------|-----------|-----|-----|-------|--------|-------|
| Subtypes | 13 | 5 | 1 | 20 | 3 | 24 | 12 | 13 | 2 | 3 | 20 | 7 | 123 |
| Orphans | 2 | 1 | 1 | 0 | 0 | 1 | 5 | 2 | 0 | 0 | 10 | 2 | 24 |
| Total orphan + subtypes | 15 | 7 | 2 | 20 | 3 | 25 | 17 | 15 | 2 | 3 | 30 | 9 | 147 |
| Including, novel | 6 | 3 | 1 | 1 | 0 | 9 | 7 | 3 | 0 | 0 | 27 | 8 | 66 |

Amine and trace amine receptors (AMIN). In the AMIN family (Fig. 1a,b) we suggest one new orphan receptor and four new subtypes that to our best knowledge have not been previously reported. This family was among the first discovered and its evolution has been comprehensively studied (see e.g.45), in particular, for dopamine receptors46,47.

Gene tree ADRA2A,2B, 2C; ADRB1,2,3; DRD1,5; DRD2,3,4; HTR2A,2B,2C; HTR6; HRH2. In the dopamine D2,3,4 receptor family, there are two new fish-specific clades found in coelacanth and ray-finned fishes: see ENSDARP00000127653, named here D21 (gene: DRD2L)(not to be confused with the long splice variant of D2); and see ENSAMXP00000016690, named D4L. Previous work characterized the expression in zebrafish of a single D2 and three D3 receptors46, whereas this study suggest seven zebras­fish receptors in the D2,3,4 family: D2a, D2b, D2s, D3a, D3b, and D3c. A previously cloned and pharmacologically characterized α2-adrenoceptor D subtype (ADRA2D) subtype was also found47. Near the histamine receptor H1 (HRH1), rooted by two lampreys, a gene cluster from gar/coelacanth/amphibian/sauropsids(7), see ENSMAP00000006842, was named GPR185.

ADRA1A,1B,1C; HRTR1A,1B,1C,1D,1E; HTR5,7. We identified at least two unannotated 5-hydroxytryptamine subtypes: 5-HT3B (HTR7B) found in gar/fishes(10)/coelacanth, see ENSLACP00000008875, that has likely been cloned in zebras­fish44, and a set of genes in gar/coelacanth/sauropsids(7), see ENSLACP00000011078, that we name 5-HT3C (HTR7C). A cluster of five genes from gar/coelacanth/birds(3), see ENSLOC00000003684, may be orthologues of the mammalianian 5-HT3B. The 5-HT3B is pseudogenic in humans, but well characterized in mice49.

HRH1,3,4; CHRM1,2,3,4,5. In the histamine H1,3,4 subtree, a set of genes from gar/fishes(2)/amphibian/sauropsids(7)/mammals(4, 2 marsupials), see ENSLOC00000006664, was named H1 (HRH1). Near the muscarinic cholinergic receptors 4 and 2, a group of ray-finned fish genes (8), see ENSDARP00000127653, named here D2L (gene: DRD2L)(not to be confused with the long splice variant of D2); and see ENSAMXP00000016690, named D4L. Previous work characterized the expression in zebrafish of a single D4 and three D2 receptors46, whereas this study suggest seven zebras­fish receptors in the D2,3,4 family: D2a, D2b, D2s, D3a, D3b, and D3c. A previously cloned and pharmacologically characterized α2-adrenoceptor D subtype (ADRA2D) subtype was also found47. Near the histamine receptor H1 (HRH1), rooted by two lampreys, a gene cluster from gar/coelacanth/amphibian/sauropsids(7), see ENSMAP00000006842, was named GPR185.

HTR4: TAAR. Equally distant from 5-HT, and trace amine (TAAR) receptors, a monophyletic group containing fishes(9)/coelacanth/gar/amphibian/reptiles(2), see ENSLOC00000014144, was left unannotated. Near the 5-HT3 subtree, a set containing gar/fishes(10)/coelacanth genes, see ENSLACP00000004821, was named 5-HT2B (gene HTR4B; not orthologous to human 5-HT, since this later has spotted gar). The TAAR have been studied in mouse, rat, human and chimpanzee31 and fishes, where their repertoire is substantially larger than in human. A set of ray-finned fish genes, see ENSLOC00000022119, may belong to a previously unannotated subtype.

Melanocortin/EDG/Cannabinoid/Adenosine (MECA) receptors. In the MECA family (Fig. 2a) we identified one new orphans, a new subtype, and a fish-specific receptor clade.

ADORA1,2,3. Two sets of genes are likely subtypes of a new orphan receptor, GPR119, rooted by lamprey and ascidian sequences, see ENSCINP00000025465. The first set contains gar/coelacanth/duplicated)/fishes(9), see ENSLOC00000022091; and the second includes fishes(2)/coelacanth/amphibian/reptiles(2), see ENSGMP00000016757. The duplicated coelacanth sequences open the possibility of a third subtype, but not conclusively.

MC1,2,3,4,5R; GPR119. The orphan receptor GPR119 has a potential subtype named GPR119B, found in gar/sauropsids(5), see ENSLOC00000002170.

SIP1R,1,2,3; LPAR1,2,3; GRP3,6,12; CNR1,2. A cluster of ray-finned fishes (9) genes suggest a new receptor close to GPR3, see ENSAMXP00000025494. Nearby, another set has genes of gar/fishes(9)/coelacanth/amphibian/sauropsids(3)/marsupial(1)/monotreme(1), see ENSLOC00000021577, and corresponds to GPR145; this receptor has been cloned in Xenopus laevis (named also GPR35). Two novel sphingosine-1-phosphate receptor subtypes have been previously reported in zebras­fish, which should correspond here to a set of fishes(17)/coelacanth/reptile(1) and to SIP1R in fishes(10)/coelacanth.
Opsin (OPN) receptors. In the OPN family (Fig. 2b), one new fish-specific opsin was identified. OPN has been extensively studied, in particular cone visual pigments, UV-sensitive photoreceptors, and melanopsins.

Zebrafish has 10 classical visual photo pigments and 32 non-visual opsins. Near OPN 8a, receptors from a set found in gar/fishes, see ENSLOCP00000020544, were named OPN8b. They come in addition to the OPN4–9 in zebrafish.
Prostaglandin (PTGER) receptors. In the PTGER family, one new subtype was identified (Fig. 2c). PTGER1,2,3,4; PTGDR; PTGIR; PTGFR; TBXA2R. Near PTGER4, a group of fishes/coelacanth/spotted gar, see ENSLACP00000020254, is a probable new subtype, PTGER4D that has been previously characterized in zebrafish⁶⁰. In addition, a group of gar/fishes/coelacanth genes, rooted by lamprey, see ENSAMXP00000018684, suggests a new subtype PTGER4C.

Melatonin (MLT) receptors. In the melatonin tree, no new receptors could be identified (Fig. 2d). MLT, GPR50. Ambiguities in this tree were lifted in Ensembl.R92. A complete set containing gar/fishes/coelacanth/amphibian/duplicated/sauropsids/platypus, see ENSLOCP00000018152, clusters with GPR50 (data from R.95). Another set includes lamprey/gar/fishes/amphibian/sauropsids/platypus, see ENSLOCP00000014362, was named MTNR1al. A third set of fishes, see ENSDARP00000070419, was named MTNR1Ba/1Bb. Three MLT subtypes have been previously cloned in zebrafish⁶¹.

Peptide (PEP) receptors. In the PEP family (Fig. 3a–c), we identified ten vertebrate receptors without human orthologues, one of which is likely a new orphan. PEP is generally conserved, retaining many lamprey sequences at the root of receptor clades.

NTSR, GPR39, MLNR, GHR, NMUR, TRHR. Near GPR39 and neurotensin receptors (gene: NTSR), rooted by lamprey, a set of gar/fishes/coelacanth/amphibian genes, see ENSXETP00000007421, suggest a new orphan, GPR189.

The motilin receptor (gene: MLNR) has one novel subtype, MLNRa that contains coelacanth/amphibian/reptiles, see ENSLACP0000009190. A closely branched set of gar/fishes genes, see ENSLOCP00000011352, may additionally belong to MLNRa.
The growth hormone secretagogue receptor (GHSR) tree includes three groups of fishes: one containing gar/zebrafish, see ENSLOC00000009072, likely orthologous to human GHSR; one containing gar/fishes(10)/coelacanth, see ENSLOC00000000036, that was named GHSR2; and a set of fishes (11, duplicates in cave fish), see ENSAMXP00000003803, also orthologous to human GHSR. Four goldfish receptors (GHS-R1a type 1/type 2, GHS-R2a type 1/type 2) have been previously suggested.

The thyrotropin-releasing hormone receptors 1–3 (TRHR) are resolved in Ensembl.R92 as one orthologue and two paralogues of the human TRHR receptor. Four receptors (THR1a, THR1b, THR2, THR3) have been previously characterized in medaka fish and three cloned in frog.

The neuromedin U receptors (NMUR) are divided into NMUR1 and NMUR2, both with lamprey orthologues (ENSPMAP00000009319, ENSPMAG00000001504). Two sets of fish genes were assigned to NMUR1 (based on Ensembl.R92). A set of sequences found in lamprey (ENSPMAP00000007962) and gar/fishes(10)/coelacanth, see ENSLOC00000004052, was named NMUR3.

Figure 3. Overview of vertebrate GPCR clades mapped onto neighbor-joining trees of the β-branch. PEP receptors. Caption otherwise similar to Fig. 1.
NPFF; QFRPR; HCTR; NPY4,8,6,1; NPY5; NPY7; NPY2R; PRLHR. Two groups of genes are likely ophnologies of NPFFR; a gar/fishes(11) set, named ENSLCP00000019807, and gar/fishes(3), named ENSLCP00000018373, named NPPFR12,5, and gar/fishes(3). Two ree genes branch with either of these receptors.

Two groups of two novel prolactin releasing hormone receptors (PRLHR) are found: the first group is rooted by lamprey (ENSPMAP0000011235) and comprised of one set comprising gar/fishes(16, duplicated)/coelacanth/amphibian/birds(3), see ENSLCP00000018414, named here PRLHR3; and another set with gar/amphibian/sauropsids(7)/marsupials(2), see ENSLCP00000022432, named PRLHR4. The other group is also rooted by lamprey (ENSPMAP00000069808) and comprises a gar/fishes(7)/coelacanth/amphibian/sauropsids(6) set, named PRLHR5; and another gar/fishes(9)/coelacanth set, see ENSLCP0000014694 (removed from Ensembl subsequent releases). Three homologues of the mammalian receptor have been cloned in chicken68, presumably PRLHR2–4.

In the neuropeptide Y receptor family, three unannotated receptors NPY5, NPY7, and NPY8 have been described previously65–70.

AVPR; OXTR; NPSR; GNHR. Three clusters of vasopressin (AVPR) receptor genes are found: one with gar/amphibian/sauropsids(6), see ENSLCP0000001209, named AVPR12; one comprised of fishes(4) genes, see ENSDARP00000119491, named AVPR2; and one with gar/fishes(14)/coelacanth, see ENSLCP0000013628, named AVPR3C. Presumably AVPR3 and AVPR4 have been previously reported and named respectively V2-like and V2B71.

We found several interesting gonadotropin-releasing hormone receptors (GNRHR) groups, most including lamprey sequences at their root, whose annotation will require further study since Ensembl.R92 has clarified some of the branchings, but not all. The first contains gar/fishes(27)/coelacanth/amphibian, see ENSLCP0000017682. The second includes fishes(16)/coelacanth/amphibian, see ENSLCP0000010658. The remaining groups are coelacanth/amphibian/reptile(1), reptiles(5), and placental(31)/marsupial(3) mammals (see ENSMUEP00000014678). The evolution of GNRHR has been previously studied72 and four zebrafish GNRHR previously cloned73.

GRPR; BR53; NM8R; EDNRA,B; GPR37,37L. A group constituted of gar/fishes(8)/coelacanth/amphibian/sauropsids(7)/platypus, see ENSLCP0000018439, likely corresponds to a previously reported endothelin receptor, EDNRA274.

TACR1,2,3; PROKR1,2; GPR83; GPR165. Ensembl.R92 resolves Pgr131 as a new orphan present in many species: gar/fishes(8)/coelacanth/amphibian/sauropsids(6)/mammals(14 genes from rodents/rabbit), see ENSLCP0000018091. Ensembl.R95 reveals a new subtype of GPR83, annotated GPR83L, and Pgr131 to be a mammalian duplicate of GPR165.

Chemokine (CHEM) receptors. In CHEM, we report eight novel receptors, of which four are likely orphans, one divided into two subtypes (Fig. 4a). The assignment of receptors in CHEM is difficult due to a fast evolution rate and numerous internal duplications, and additional subtypes will certainly be defined when more data becomes available. Evolution of the chemokine system has been comprehensively studied75.

LTB4R, FPR1,2,3; GPR32, GPR33, CMKLR1, CSAR1, C3AR1, PTGDR2, GPR1, GPR152. Near the leukotriene-4 receptors (LTB4Rs) we suggest a new orphan receptor GPR190a, composed of two (or three) subtypes. A set of gar/(duplicated)/fishes(16)/coelacanth/amphibian/sauropsids(7) genes, see ENSLCP0000021707, was named GPR190a. The second subtype is composed of gar/fishes(32, internal duplications)/marsupials(2), see ENSLCP00000021705, and named GPR190b. A lonely platypus sequence (ENOANA0000021820), complete with respect to its GPCR signature, may represent a third subtype. Two sets of genes near LTB4R1 (in Ensembl. R92) may be orthologues of existing human receptors (sets removed in E.95). Three LTB4R have been identified in zebrafish76.

The branching near the formyl peptide receptors FRP1,2,3, and GPR132 is especially blurred, and Ensembl.R92 helps understand the recent history. FRP1,2,3 have arose through local duplications, and marsupial(3) sequences, see ENSHAP0000001972, are likely orthologous to the FRP1,2,3 set. GPR132 is likely rooted by seven monotreme species: gar/fishes(8)/coelacanth/amphibian/sauropsids(6)/mammals(14 genes from rodents/rabbit), see ENSLCP0000018091. Ensembl.R95 reveals a new subtype of GPR83, annotated GPR83L, and Pgr131 to be a mammalian duplicate of GPR165.

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ENSLOCP00000000990, named CCR8B. A third group comprising gar(fish)/coelacanth, see ENSLOCP00000022422, is located at the root of the mammalian CCR1,2,3,4,5 and CX3CR1 and probably correspond to orthologues of CCR2 and CCR5. We also identified already reported fish-specific receptors, CXCR3B characterized in common carp77 (ENSLACP00000016977) and CCR12A,B identified in orange-spotted grouper (see ENSLACP00000022265)78.

Figure 4. Overview of vertebrate GPCR clades mapped onto neighbor-joining trees of the γ-branch. (a) CHEM (b,c) SOG and MCH receptors. Caption otherwise similar to Fig. 1.

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AGTR1,2; BDKRB1,2; GPR15,25,182; APLNR; ACKR3; RXFP3,4. The bradykinin receptor B2 (BDKRB2) has an additional set gar(fish)/coelacanth (also a fragmental wallaby sequence), see ENSLACP00000020198, may be paralogous to the apelin receptor (APLNR). A second group of fishes(11) nearby, see ENSAMXP00000021714, was assigned as APLNR2.

Human relaxin/insulin-like family peptide receptor 3 (RXFP3) has orthologues in fish (ENSLOCP00000021654); a paralogous group comprising gar(fish)/coelacanth, see ENSLACP00000009554. Near RXFP4 (that has fish orthologues too) there is evidence of a new subtype comprised of gar(fish)/coelacanth/
mammal (2, including one marsupial) sequences, see ENSLOCP00000022049), as well as a fish-specific set of duplicates (9, annotated rxfp3.2h). These new receptors have been named here RXFP3,2 pending further studies and probably have been previously described as RXFP3,2 and RXFP3,3,79,80.

**Somatostatin-opioid-galanin (SOG) and melanin-concentrating hormone (MCH) receptors.** In the SOG and MCH families we suggest five previously unreported receptor types (Fig. 4b,c), including three orphans. For a review of neuropeptide signaling systems see81.

OPRD1, -M1, -K1, -L1; NPWR1,2; SSTR1,2,3,4,5; MCHR1,2. In the somatostatin (SSTR) receptor group, near SSTR3, a gar/fishes(5) group named SSTR3b, probable result of an internal duplication, see ENSLACP00000021860, has been described previously as SSTR.85 Nearby, a group of fishes and coelacanth represent a new SSTR subtype, SSTRb, since SSTR3 has lost its fishes with the exception of coelacanth.82,83

Branching close to MCHR1, there is a gar/fish(10)/coelacanth(duplicated) set, see ENSLACP00000004413. Near MCHR2, two new subtypes are suggested: a coelacanth/amphibian/reptile(1) set, see ENSLACP00000017963; and a large set comprising gar/fishes(14, duplicated)/coelacanth/sauropsids(5), see ENSLACP0000009737. Mammals have five melanocortin receptors and one or two MCHR receptors, zebrafish six melanocortin (extra MC5RB) and three MCHR receptors; fugu four MCR (MC3R missing) and two MCHR; Two MCHR receptors have been cloned in goldfish and four MCHR receptors have been cloned in *Xenopus tropicalis*. No annotations are proposed due to the complexity of the Ensembl trees for this subfamily.

CCKAR; CCKBR; GPR19; KISS1R; GALR1,2,3. Near the Kisspeptin receptor 1 (KISS1), three unassigned sets of genes were detected. The first contains gar/amphibian, see ENSLACP00000002856, and was named KISS1a. The second set contains gar/fishes(10)/coelacanth/amphibian, see ENSLACP0000003036, and was named KISS1b; this receptor has likely been studied in ray-finned fishes.85 A related set contains gar/coelacanth/reptiles(2), see ENSLACP00000008391, and the tree furthermore includes fragmental sequences at the root of KISS1 (not discussed here).

In Ensembl.R92, the human galanin receptor 1 (GALR1, having its own fishes), rooted by a lamprey sequence (ENSPMAP00000000353). A set of gar/fishes(7)/coelacanth(2)/sauropsids(7) genes, see ENSLACP00000010695, was named GALR1b. Near GALR1, a paralogous branch with gar/fishes(10)/coelacanth/amphibian/sauropsids(4), see ENSLACP0000001326, was named GALR1b. Avians have been reported to have two new types in addition to Galr1 and Galr2; Galr1, Galr1-like, Galr2-like; four galanin receptors have been isolated from sauropsids.86 The other is fish-specific and in Ensembl.R92 occupies its own tree, see ENSLACP00000007402. It contains lamprey, 3R-duplicated ray-finned fishes(16), and coelacanth, and was named as GPR196.

UTS2R. Previously four urotensin 2 receptor (UTS2) subtypes have been named as UTS2a,b,c. A set of gar/fishes(6)/coelacanth/amphibian/sauropsids(6), see ENSLACP0000012826, is orthologous to human UTS2a,b,c. A set from gar/fishes(9)/coelacanth/amphibian, see ENSLACP00000030313 is a probable new UTS2 subtype. The Ensembl.R92 gene tree suggest two subtypes of a new orphan receptor, GPR197a, gar/fishes(2)/sauropsids/marsupials(2)/monotreme, see ENSLACP00000022204, named GPR197a,b; and gar/fishes(10)/amphibian/birds(3), see ENSLACP00000021948, named GPR197b.

**Leucine-rich repeat (LGR) and Mas-related (MRG) receptors.** In the MRG and LGR families, no new vertebrate receptors were found (Fig. 5a,b).

LGR4,5,6; LHCCR; FSHR; TSHR; RXFP1,2. The LGRs are rooted by a set of fish genes (6, see ENSTRUP00000003361) likely orthologous to human LGR. A set of gar/fishes(2)/coelacanth/amphibian/sauropsids(5)/marsupials(2) genes, see ENSLACP0000018098, corresponds to the previously described RXFP1,2,84,86,87 annotated here RXFP1.88 (marsupial and bird genes not found in R.95). A fish-specific luteinizing hormone receptor (LH), see ENSLACP00000020888, has also been reported89.

MRGPRD; MRGPRF; MRGPRG; MRGPRE; MRGPRX1,2,3,4; MRGPRa,b; MAS1;1L. The evolution of the MS1, MAS1 oncogene-like, and MAS-related GPR (MRG) families is complexified by numerous internal duplications. A large group of sauropsid sequences, locally duplicated, could be orthologous to the mammalian MRGPR, see ENSGALP00000047269, named here MRGPR1; A group of placental and marsupial mammals, see ENSXETP00000063951, named MRGPRH. Nearby is a cluster of reptile genes, see ENSACAP0000019338, which may be orthologous or may represent a new subtype. MAS receptors have been reported to arise in amphibians88, and the MAS family tree is rooted by a group of amphibians, MAS1, see ENSHAP00000063951. Three clusters of fish genes were positioned in Ensembl.R91 with MAS/MRGPRD but in the newer Ensembl.R92 are aggregated with GPR1b (see ENSLACP0000001563, described above in the CHEM/CMKRLI tree).

**Purine (PUR) receptors.** The PUR family is the most uncharted and we identified 27 new receptors, including 12 orphans (Fig. 5c).

LPAR4,5,6; PTAFR; F2L1R1/2/3; P2RY8; P2YR10; F2R; GPR4,18,35,55,65,68,132,174: Nearby LPAR4, a set of gar(duplicated)/fishes(2)/coelacanth(3, internally duplicated) was assigned to a new orphan probably with two
subtypes, see ENSLOCP00000021495, named LPAR5A/B. Near PTAFR, a set of genes comprising gar/coelacan-
th(duplicated)/sauropsids(2)/mammalian (2, one marsupial), see ENSLOCP00000002041, was named GPR199.
Near LPAR4/6, a set with gar/fishes(4)/coelacanth/reptile(1), see ENSLOCP00000021661, was named GPR202.
Another orphan is composed two amphibian and two lamprey sequences, see ENSXETP00000036876, named
GPR200. Near GPR55, a full set of genes from gar/fishes(2)/coelacanth(duplicated)/sauropsids(5)/monotreme(1),
see ENSLOCP00000021497, was named GPR55B.
The receptors GPR4, GPR68, GPR65 and GPR132 branch together with a new orphan, named GPR201, divided
into two subtypes. One of these subtypes contains gar(7 duplicates)/fishes(2)/reptile(1)/marsupials(2), see
ENSLACP00000013584; the other contains gar/fishes(10)/coelacanth, see ENSLACP000000021497, was assigned to

Figure 5. Overview of vertebrate GPCR clades mapped onto neighbor-joining trees of the δ-branch. (a) LGR
(b) MGR (c) PUR. Caption similar to that of Fig. 1.
GPR20A and GPR17A. A group of gar/fish(3)/coelacanth, genes, as ENSLACP00000004667, was named GPR20A.

The coagulation factor II receptor F_2R_L3, is prone to local duplications, for example in spotted gar (see ENSLOCP00000014196) or coelacanth (see ENSLACP00000003039). Near F_2R_L3, a group of amphibian/birds(5), see ENSXETP0000005770, was named F_2R_L4. A set of fishes(18) paralogous to human F_2R, see ENSAMXP00000020201, need further consideration for annotation.

Near P_2RY_{10}, a group constituted of gar/fish/coelacanth/amphibian/reptile(2), see ENSLACP00000021876, was named P_2RY_{10}. Nearby a coelacanth duplicate (ENSLACP00000004454) could not be assigned. In P_2RY_{10}, a set of receptors resulting from a mammalian-specific internal duplication, see ENSMUSP00000133122, were assigned to P_2RY_{10B}.

GPR20B, 31; HCAR1,2,3; OXER1. Near P_2RY_{30}, we suggest a new orphan, divided into two subtypes: a group of constituted of gar/fishes(6)/reptiles(3)/monotreme(1), see ENSLOCP00000001967, named here GPR30A; and gar/coelacanth (duplicated), see ENSLOCP00000021450, named GPR30B. A set of fishes(21), see ENSLOCP00000021469, is probably orthologous to the mammalian oxoeicosanoid receptor 1 (OXER1); in such case a set of fish/amphibian(2) genes, see ENSLACP00000020331, is paralogous. The subtypes 1–3 of the hydroxycarboxylic acid receptors HCA, found only in mammals, are closely related and thus the likely result of recent duplications. Two sets of fishes are branched to the mammalian subtypes, of which gar/fishes(8)/coelacanth, see ENSLOCP00000022252, was named HCAR1L.

GPR34B, 82, 87, 171; P2RY12, 13, 14. P_2RY_{13} has an extra set of fishes(3), see ENSLOCP00000022722, indicating an internal duplication. Near P_2RY_{14}, there are two groups of sequences: one gar/fishes(41, many duplicates), see ENSLOCP00000021478, named here GPR210A; and gar/coelacanth, see ENSLACP00000019189 (not in R.95); gar/fish/amphibian/sauropsids(5), see ENSLOCP00000016952, was named GPR210B.

SUCNR1; OXGR1; P2RY1, 2, 4, 6; GPR17; GPR183; CYSTLR1, 2. Branched distantly from others is a new orphan divided into three sets: two with genes from gar(2)/coelacanth/reptile, see ENSACAP00000013831, named GPR208; and P_2RY_{1} (NPSR, in R.95) there is a cluster of genes from gar/fishes(23)/coelacanth/amphibian (triplicates)/sauropsids(7), see ENSLOCP00000022457, that we named NPSR_{1L}. Near P_2RY_{6}, there are two sets of unannotated genes: a complete group with gar/fishes(3)/coelacanth/reptile(1)/mammals(21), see ENSLOCP00000021492, named here P_2RY_{16} and, distant and rooted by duplicated lampreys (see ENSPMAP00000011278), a cluster comprised of gar/fishes(18, duplicates)/coelacanth/amphibian, see ENSLOCP00000021662, that we named GPR214A. A set of genes from ray-finned fishes(10)/coelacanth (duplicated)/sauropsids(5)/mammals(5), including one monotreme and one marsupial, see ENSLOCP00000021877, was assigned as GPR214A. A gar/fishes(8) specific group of genes, see ENSLACP00000014291, is branched near P_2RY_{7}, and we named it GPR214B. Another group rooted by lamprey (triplicate, ENSPMAP00000011192) comprised of gar/fishes(4)/coelacanth, see ENSMUSP0000011192, was named CYSLTR_{1L}. Two other fishes (gar, cave fish) nearby open the possibility of another subtype CYSLTR_{1B}.

FFAR_{1, 2, 3} Free fatty acid receptors (FFAR) have generally undergone many internal duplications. A small gene cluster from coelacanth/amphibian/reptile, see ENSLACP00000002775, is likely a new subtype FFAR_{1L}(annotation in process). A set of 32 genes from fishes, see ENSLOCP00000006360, is likely belonging to FFAR_{1L} (Ensembl.R92).

GPR141; GPR101, 161, 176. These two clusters of orphan receptors were initially aggregated with PURIN receptors but form independent groups in Ensembl.R92. A set containing gar/fishes(10)/coelacanth, see ENSLOCP00000014127, was named GPR141C. A set with coelacanth/sauropsids(4)/mammals(15, including two monotremes), see ENSLACP00000014290, was named GPR141B. A set of fishes(10)/amphibian, see ENSXETP00000035434, was named GPR141B.

Other families. We grouped together receptors that we could not link to the main families, and in this group we identify seven novel receptors (Fig. 6). Interestingly, nine of the orphan groups are closely associated with a lamprey or tunicate sequence, showing an ancient origin; and there are clear grouping for some of the orphans into subtype groups.

GPR22. A set from gar/fishes(17, duplicated)/coelacanth/amphibian/reptile/marsupial, see ENSLOCP00000017943, is a new subtype GPR22A, which may divide further into different subtypes.

GPR139; GPR142. Three sets of genes indicate an orphan divided into three subtypes: coelacanth/fishes(4), see ENSLACP00000014196, named here GPR210A; gar/coelacanth, see ENSLACP00000019189 (not in R.95); gar/fishes(2)/coelacanth, see ENSLACP00000015589, named GPR210B.

GPER1. A new subtype in ray-fin fishes (8) has been previously reported, which may be a fish-specific duplicate (3R) 90.

GPR20. An independent group of fishes(8), see ENSAMXP00000025847, was named GPR20A; a set of fishes(10)/coelacanth/amphibian/sauropsids(7)/marsupial/monotreme genes, see ENSAMXP00000019172, is distant...
from GPR20’s placental mammals and may represent a new subtype. A group of gar/fishes (15) is also nearby in Ensembl.R92 (annotated lpar5b), see ENSLOC00000021496.

**GPR151.** A set of fishes (9)/coelacanth/amphibian, see ENSLACP00000009338, was named GPR151B.

**GPR148.** A set of fish (2)/sauropsids (5)/marsupial (1), see ENSLOC00000021506, was named GPR209. A second spotted gar, see ENSLOC00000021505, may indicate another subtype.

**GPR61.** A set coelacanth/amphibian, see ENSLACP00000015922, is likely paralogous to GPR62.

**GPR21,52.** A set of gar/fishes (10)/coelacanth/amphibian/lizard, see ENSLACP00000013616, named GPR211.

**Conservation across families.** We used the set of sequences aligned across families to study the relative conservation of the subtypes across four representatives species (human, mouse, bird, amphibian and fish) (see Supporting information; 189 aligned positions). Generally, as expected the more “ancient” branches such as α -branch and β -branch are relatively conserved compared to more recent branches (γ -branch and δ -branch).

In the α -branch (Fig. 7A,B), the most conserved are LPAR 1 and CNR 1 in MECA that share sequence identity above 90% towards their human orthologue (human-zebrafish LPAR 1, 96%; human-mouse LPAR 1, 99%; human-zebrafish CNR 1 92% and human-mouse CNR 1 99%). Conservation is also high in the AMIN family (Fig. 7A) where the DRD 1/2, ADRA 2C and CHRM 3/4/5 have percent sequence identities above 90% towards the human receptors. In the β -branch, in PEP GPR 22 and HCRTR 2 maintain sequence identity above 90% from amphibian to mouse (Fig. 7C). Few receptors of the γ -branch maintain sequence identity above 80% through species (Fig. 7D), but this is the case of OPRD 1, OPRK 1, GALR 1, and SSTR 1 (most conserved OPRM 1, human-zebrafish 93%; human-mouse 99%). In the δ -branch conservation is less (Fig. 7E), and the most conserved are TSHR (80–93%) and P2RY 1 (81–98%). In the unassigned receptors (Fig. 7F), GPER 1 is the most conserved (human-zebrafish 87%; human-mouse 95%). Not surprisingly, GPR 85 (human-zebrafish, 94%; human-mouse, 100%), an orphan receptor closely related to the amine family and named “Super Conserved Receptor” shows a very high conservation too (initially grouped with AMIN, Fig. 7A).
Concluding remarks. The novel receptors are identified based on sequence data alone. The complete integrity of the sequences, validated by the conserved GPCR motifs, suggest that these are not pseudogenic. The groupings have been highly stable during the last five years, especially inside monophyletic groups, and most likely to remain so. Generally, the branchings in the fast-evolving PUR and CHEM families (see eg especially near FRP1,2,3 and GPR32,33), and for distant sequences such as lamprey, have been the most rearranged over the different Ensembl releases. The automated rearrangements have been nonetheless parsimonous, ie groups have been reassigned as orthologues rather than paralogues of known receptors.

This study raise numerous nomenclature issues, but in the same time sets a framework on the extent of yet-to-be studied vertebrate receptors. A difficult issue is that without establishing ligand binding preferences new sequences cannot be defined as "subtypes" or "orphan" receptors; we thus chose to keep the "subtype" denomination only for the likely cases. Importantly, the ligand binding preferences are not fully indicative of close evolutionary relationships; some receptors have acquired the same ligand specificity several times, for example α₁-, α₂-, and β-adrenoceptors31,32.

This study maps the extent of orthologues of human GPCRs to vertebrate species. Furthermore, it explores the pool of yet-to-be-studied GPCRs, suggesting at minima 69 sets of novel receptors in vertebrates not orthologous to human. The clustering data allows to group orphan receptors and thus probably brings down the number of endogenous ligands yet to be identified. Nineteen of these groups are maintained in Ensembl.R92 and thus worthy of consideration: In MECA, GPR3/6/12; in PEP, GPR83/165, EDNR/GPR37/37L1, GPR39/NTSR; in CHEM, GPR15/25, GPR182/ACK3; in PUR, GPR20/35/55, GPR101/161, P2RY10/GPR174, GPR17/183, GPR87/P2RY14, GPR31/HCAR; and in other families GPR85/27/173, GPR45/63/135, GPR153/162, GPR139/142, GPR21/52, GPR61/62, GPR26/78; for a few of these receptors an endogenous ligand has been suggested91. The data at the basis of this study are available online (see the Experimental section) and will continue to be improved following especially the sequencing of novel genomes and the increased coverage of existing genomes.

Experimental Section
Ensembl data. The Ensembl.R90 includes 89 vertebrate genomes: eleven ray-finned fishes (e.g. zebrafish, spotted gar), one lobe-finned fish, one amphibian, five birds, two reptilians, three marsupial, one monotreme and 65 eutherian mammals, as well as early vertebrates (lamprey, two tunicates) and invertebrates (one insect and one nematode). The most recent release, Ensembl.R92, includes 94 genomes.
New receptors were identified based on the branching presented on the Ensembl-generated trees. Three genomes are at the cornerstone for assessing a new receptors: coelacanth, frog, and spotted gar, and we separate them (‘spotted gar’ referred to simply as “gar”) from the other fishes. Spotted gar diverged from the teleost lineage before the 3R[38]. New orphan receptors were named in numerical order starting from 181, using the numbers left empty (for human genes) by the HUGO Gene Nomenclature Committee. New subtypes were identified either numerically or alphabetically.

Guide trees. The data used to build the guide trees are based on an automatically extracted and curated set of 14,000 sequences is described as Supporting Information. This earlier data are used in this manuscript only to build guide trees and compute sequence identities in transmembrane regions.

Data Accessibility
All genomic data from the Ensembl releases is freely accessible online in few simple steps: (1) log to http://www.ensembl.org or to the Ensembl archives https://www.ensembl.org/info/website/archives/index.html (2) query the name of the receptor of interest or the provided codes; (3) select (usually) the first Gene hit on the list; (4) click on ensembl.org or to the Ensembl archives https://www.ensembl.org/info/website/archives/index.html (2) query the All genomic data from the Ensembl releases is freely accessible online in few simple steps: (1) log to http://www.ensembl.org or to the Ensembl archives https://www.ensembl.org/info/website/archives/index.html (2) query the name of the receptor of interest or the provided codes; (3) select (usually) the first Gene hit on the list; (4) click on ensembl.org or to the Ensembl archives https://www.ensembl.org/info/website/archives/index.html (2) query the

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