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Ornithorhynchus anatinus (Platypus) Links the Evolution of Immunoglobulin Genes in Eutherian Mammals and Nonmammalian Tetrapods\textsuperscript{1,2}

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The evolutionary origins of mammalian immunoglobulin H chain isotypes (IgM, IgD, IgG, IgE, and IgA) are still incompletely understood as these isotypes differ considerably in structure and number from their counterparts in nonmammalian tetrapods. We report in this study that the platypus (Ornithorhynchus anatinus) Ig H chain constant region gene locus contains eight Ig encoding genes, which are arranged in an μ-δ-ο-γ2-γ1-α1-α2 order, spanning a total of ~200 kb DNA, encoding six distinct isotypes. The o (ο for Ornithorhynchus) gene encodes a novel Ig H chain isotype that consists of four constant region domains and a hinge, and is structurally different from any of the five known mammalian Ig classes. This gene is phylogenetically related to θ (θ) and γ, and thus appears to be a structural intermediate between these two genes. The platypus δ gene encodes ten heavy chain constant region domains, lacks a hinge region and is similar to IgD in amphibians and fish, but strikingly different from that in eutherian mammals. The platypus Ig H chain isotype repertoire thus shows a unique combination of genes that share similarity both to those of nonmammalian tetrapods and eutherian animals and demonstrates how phylogenetically informative species can be used to reconstruct the evolutionary history of functionally important genes. The Journal of Immunology, 2009, 183: 3285–3293.

Immunoglobulins are the key components of the adaptive immune system in jawed vertebrates including mammals, birds, reptiles, amphibians, bony fish, and cartilaginous fish (1, 2). Eutherian (placental) mammals are known to express five classes of IgS including IgM, IgD, IgG, IgE, and IgA, among which only IgM, IgD, and IgA (or its functional equivalent, like amphibian IgX) are found in nonmammalian tetrapods such as birds, reptiles, and amphibians (1–7). Although IgM is structurally conserved in all tetrapods thus far investigated, both mammalian IgD and IgA differ from their respective counterparts in nonmammalian tetrapods by having a structurally flexible hinge and fewer numbers of constant (C) region domains.

IgD is an enigmatic Ig class, primarily because its function remains elusive. Until an ortholog was discovered in the teleost catfish (Ictalurus punctatus) a decade ago (8), IgD had been thought to be present only in primates and rodents (9). Recently, the gene encoding IgD was identified in various classes of animals including bony fish, amphibians, reptiles, and additional eutherian mammals such as pigs, cow, sheep, panda, and dogs (3, 4, 6, 10–12), thus showing a distribution across a wide phylogenetic spectrum. Based on analysis of the δ gene in Xenopus tropicalis and bony fish (3, 13), investigators uncovered a phylogenetic relationship of IgD with IgW (found in lungfish and cartilaginous fish; Ref. 14, 15), suggesting that the IgD/W lineage is as evolutionarily ancient as IgM. Therefore, the absence of the δ gene in birds (16, 17) and rabbits (18) is probably an outcome of evolutionary loss. Unlike IgM which is structurally conserved across all jawed vertebrates, IgD exhibits extensive variation, particularly in size. The length of IgD can range from two heavy chain constant region domains (C\textsubscript{H}\textsubscript{2})\textsuperscript{5} to more than ten C\textsubscript{H}\textsubscript{4} domains in different species (8–10, 13, 19–21). In addition to the length variations, in some species, such as catfish and pigs, IgD can also be expressed as a chimeric Ig H chain with an inclusion of the IgM C\textsubscript{H}1 (8, 22). Compared with their counterparts in nonmammalian vertebrates, IgD molecules

\textsuperscript{5} Abbreviations used in this paper: C\textsubscript{H}1, heavy chain constant region domain; IGHC, immunoglobulin heavy chain constant region gene; PFGE, pulsed field gel electrophoresis; TM, transmembrane; BAC, bacterial artificial chromosome.

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\textsuperscript{2} The IgD and IgD\textsubscript{o}DNA sequences reported in this study have been deposited in the National Center for Biotechnology Information GenBank under the accession numbers: EU503149-EU503150.

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from eutherian mammals contain fewer C_H domains and a structurally disordered, flexible hinge region which is not seen in non-mammalian vertebrates. However, the evolutionary process that led to shortening of the IgD molecule and creation of the hinge region remains unclear.

The mammalian IgG and IgE molecules are believed to have evolved through gene duplication and subsequent evolution of IgY (23), an ancient, four-CH-domain, hinge-lacking Ig class found in birds, reptiles and amphibians. Evolution of IgY to IgE seems to be straightforward, as both Igs have four CH domains and no hinge, and essentially share sequence homology and similar functions. However, no evidence is available to explain how the hinge of mammalian IgG evolved from IgY, although two hypotheses have been proposed. The first suggests that the IgG hinge region was evolutionarily condensed from the pre-existing IgY CH2 (23), while the second argues that the hinge originated from the 3 sequence of an intron (24).

Mammals (class Mammalia) are taxonomically classified into Prototheria (monotremes) and Theria (Metatheria: marsupials, and Eutheria: eutherians). Monotremes contain only five living species, the platypus and four species of echidna. The platypus is indigenous to Australia and displays an unusual mixture of mammalian and reptilian features, both at the genome level (25) and anatomically: females lay eggs but also provide colostral milk for their pups, and males are venomous. Monotremes last shared a common ancestor with therian mammals 166 million years ago (26), placing them in a prime position for reconstructing the evolutionary history of biologically important molecules.

The duck-billed platypus (*Ornithorhynchus anatinus*) has previously been shown to express four Ig classes: IgM, IgG1/IgG2, IgA1/IgA2, and IgE, that all structurally resemble their respective counterparts in eutherian mammals (27, 28). To explore the evolutionary origins of the mammalian Igs, we analyzed the platypus Ig heavy (H) chain constant region gene locus and identified a novel Ig type encoding gene (IgO, O, or IgO for *Ornithorhynchus*) and a ten-CH domain encoding O gene.

**Materials and Methods**

**Platypus spleen cDNA library, cDNA, and bacterial artificial chromosome (BAC) clones**

The platypus spleen cDNA library was previously constructed by Vernerson et al. (28). BAC clones (CH236-821N20, CH236-99D18, and CH236-647E09) were purchased from BACPAC Resource Center (BPRC) at Children’s Hospital Oakland Research Institute in Oakland, California. All platypus tissues were collected opportunistically from animals found dead in the wild. Tissues were frozen at −80°C before DNA/RNA extraction. RNA was extracted using TriReagent according to the manufacturer’s instructions (Molecular Research Center). RNA samples were subjected to DNase digestion using standard protocols (Promega). The Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR was then used to make cDNA using the Oligo(dT)20 primer as per the manufacturer’s instructions (Invitrogen).

**Database searches**

BLAST searches were performed against the platypus genome sequence deposited in the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/genome/guide/platypus/). Genomic contigs were retrieved for further analysis. To search for putative Ig domains, the
FIGURE 2. Phylogenetic analysis using the terminal C14 domain. Note: C14δ for the gecko IgD, C14γ for both the *X. tropicalis* and platypus IgD were used for the phylogenetic analysis. The scale shown as a bar represents the genetic distance. The credibility value for each node is shown. Except the sequences of the platypus IgD and IgG (obtained in this study), and *Xenopus tropicalis* IgF, IgM, and IgX (4), the remaining Ig sequences were obtained from the NCBI GenBank with the following accession numbers: α or χ genes: chicken, S40610; duck, AJ314754; echidna, AF416951; human, J00476; mouse, J00085; opossum: AF108225; platypus, AU055778, AU055779; *X. laevis*, BC072981. β genes: cow, AF515672; dog, DQ297185; gecko, EU312156; human, BC021276; horse, AE631942; mouse, J00449; pig, AF515674; rat, AJ148499; sheep, AF515673; *X. tropicalis*, DQ350806. γ genes: echidna, AF416949; human, J000228; mouse, J00453; opossum, AF035195; platypus, AU055781, AU055782. ε genes: cow, U63640; echidna, AE631942; human, J00222; mouse, X01857; opossum, AF035194; platypus, AU055780. μ gene: chicken, X01613; duck, AJ314754; echidna, AF416952; gecko, EU287911; human, X14940; mouse, VO0018; opossum, AF108226; platypus, AE168639; turtle, U53567; *X. laevis*, BC084123. ν genes: chicken, X07175; duck, X78273; gecko, EU827594; *X. laevis*, X15114.

**PCR detection of the platypus Ig gene expression in different tissues**

cDNA samples derived from seven tissues including gastrointestinal tract, kidney, liver, lung, reproductive tract, spleen, and testsis, were used in PCR detection using normal *Taq*DNA polymerase (Tiangen Biotech). PCR primers were: IgM-detections: 5′ ACA AGC GTA TTC CAC CAT CAT CTC 3′; IgM-detectionAs: 5′ GTT CAA GTG CTG CTC CAC ACA 3′; IgE-detections: 5′ GCG CTA GAA CTC TGG AGA CTA GGT CAC 3′; IgE-detectionAs: 5′ CTC CAC GAC TAT CTC CAT CTT CGT 3′; IgD-detections: 5′ CTC CCT TGG CAC TGT GGT AGA AGG GGT 3′; IgD-detectionAs: 5′ GGT GGA CAG AGA GGG GGA 3′; IgA1-detections: 5′ GCA AGC ACA ACC ACC ACC 3′; IgA1-detectionAs: 5′ GGT GCA TCT TCT TCT CGT 3′; IgA2-detections: 5′ GCA GCC AGC ACA ACC ACC ACC 3′; IgA2-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgE-detections: 5′ GGA GGC CAA ACA ACC ACC ACC 3′; IgE-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgG1-detections: 5′ GGT GGA CAA ACA ACC ACC ACC 3′; IgG1-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgG2-detections: 5′ GGT AAT CGA GAA ACC ACC ACC ACC 3′; IgG2-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgG3-detections: 5′ GGT GGA CAA ACA ACC ACC ACC 3′; IgG3-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgH2-detections: 5′ GGT AAT CGA GAA ACC ACC ACC ACC 3′; IgH2-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgH3-detections: 5′ GGT AAT CGA GAA ACC ACC ACC ACC 3′; IgH3-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgX-detections: 5′ GGT AAT CGA GAA ACC ACC ACC ACC 3′; IgX-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′; IgO-detections: 5′ GGT AAT CGA GAA ACC ACC ACC ACC 3′; IgO-detectionAs: 5′ GCT GGA AGA GGA CGG CAG GAC AGC AGC 3′;

**DNA computations, structural prediction, and construction of phylogenetic trees**

DNA and protein sequence editing, alignments and comparisons were performed using the DNASTAR program (DNASTAR). Phylogenetic trees were
made using MrBayes 3.1.2 (29) and viewed in TREEVIEW (30). Multiple sequence alignments were performed using Clustalw. Protein disordered regions were predicted using a series of online programs (http://www.disprot.org/predictors.php).

Results
Identification of the genomic sequences encoding IgM (μ), IgG1/G2 (γ1/γ2), IgA1/A2 (α1/α2), and IgE (ε) in the platypus

The platypus has previously been shown to express four Ig classes, encoded by six genes: IgM (μ), IgG1/G2 (γ1/γ2), IgA1/A2 (α1/α2), and IgE (ε) at the cDNA level (27, 28). Using these cDNA sequences as templates, we performed BLAST searches against the platypus genomic sequences deposited in the NCBI GenBank. This allowed us to identify a 680-kb genomic contig (NW_001794226) containing those previously known μ, γ1, γ2, α1 genes. The γ2 and α1 genes were found on two other short contigs (NW_001705893.1, NW_001777603.1, the latter contig contains only a part of the α1 genomic sequence), which are not assembled into the 680-kb contig in the current version of the platypus genome assembly. Alignments of these identified genomic sequences with their respective cDNA revealed that all genes had a similar genomic organization as their corresponding genes in eutherian mammals (Fig. 1).

Identification of the platypus IgD encoding gene

There is an ~90-kb of DNA sequence (including sequence gaps with unknown sizes) between the μ and γ1 genes in the 680-kb contig, which is of sufficient size to accommodate additional Ig isotype encoding genes. Approximately 4.3-kb downstream of the μ TM2 exon, an Ig Cε domain encoding exon was identified. Further downstream, nine additional Ig Cε domain-encoding exons and two typical IgM/D transmembrane exons were also observed, indicating the presence of a 10 CH domain-encoding Ig gene (Fig. 1). Blast searches using the deduced amino acid sequence revealed that it shared a relatively high degree of homology with IgD from fish, Xenopus, leopard gecko, and some mammals. Together with phylogenetic analyses (Fig. 2, supplementary Fig. 1), these data strongly suggest that the identified gene is the platypus δ.

We failed to amplify the δ-encoding cDNA from a platypus cDNA library (28), probably due to its low rate of expression. We thus resorted to a nested RT-PCR directly using total RNA as a template and primers derived from JH and the predicted TM1. An ~3.3 kb PCR product was successfully amplified and directly sequenced, and was shown to contain all the ten predicted CH exons (supplementary Fig. 2). This clearly suggests that the platypus IgD can be expressed as a membrane-bound form consisting of multiple CH domains. However, we did not succeed in cloning a secreted form encoding cDNA. Like in most other mammals, the platypus IgD gene is most likely expressed through alternative splicing of a long primary transcript which consists of both the μ and γ1 genes, as no putative switch region sequence was identified in the γ1-μ intron (data not shown) (31).

Despite its apparent absence in birds (chickens and ducks), IgD has recently been identified in some reptiles (Ref. 6, and our unpublished data) showing a roughly similar size (11 CH domains) as the platypus IgD. We subsequently performed a domain to domain sequence comparison of IgD between the platypus and two reptiles (leopard gecko and the green anole lizard), that revealed that nearly all the 10 platypus IgD CH domains have a corresponding homologous CH domain in both reptile IgD molecules (supplementary Table 1), supporting the view that these molecules are related. A domain-to-domain sequence comparison revealed that the CH1, CH6, and CH7 domains of the platypus IgD are homologous to the CH1, CH2, and CH3 of IgD of eutherian mammals, respectively (supplementary Table 2 and Fig. 3), indicating that this molecule was shortened by a selective loss of CH domains during evolution.

6 The online version of this article contains supplemental material.
IgD in eutherian mammals usually contains a structurally flexible hinge in the H chain constant region (9, 12, 22). However, a detailed examination of the platypus IgD sequence reveals no hinge region (Fig. 3), indicating that the IgD hinge developed after divergence of therian mammals from monotremes.

Identification of a novel Ig gene in the platypus

In addition to the above mentioned five genes including the $\mu$, $\delta$, $\gamma$, $\epsilon$, and $\alpha$, a sixth H chain isotype encoding gene was unexpectedly found downstream of the $\delta$. This gene consists of four $\text{CH}_4$ and two TM encoding exons and is structurally similar to $\mu$ and $\epsilon$. However, it has only 29.4 and 45.1% amino acid identity to the platypus IgM and IgE, respectively. We termed this gene $\omega$ (for *Ornithorhynchus anatinus*, encoding IgO) (Fig. 1). RT-PCR shows that IgO is exclusively expressed in the spleen (Fig. 4). We cloned the IgO C region encoding cDNA using a nested PCR-amplification of a duck-billed platypus spleen cDNA library (Fig. 5). Surprisingly, alignment of the IgO C region with that of the platypus IgE revealed an extended $\text{CH}_4$ domain at the N-terminal (Fig. 6). This extended sequence is abundant in prolines and very similar, in amino acid composition, to hinges of mammalian IgA and IgG (supplementary Fig. 3). Proline rich regions usually play a structural role in proteins as spacers and are normally devoid of a secondary structure, i.e., being disordered or forming a random coil. Definite Ig hinges usually display an extended (at least partially), disordered protein structure, conferring structural flexibility (32). A series of prediction programs suggested that the extended peptide in the IgO $\text{CH}_4$ region is structurally disordered, as exemplified in supplementary Fig. 4. Therefore, it is likely to form a hinge unit together with the first cysteine residue in the $\text{CH}_4$ domain. An Ig class with four $\text{CH}_4$ domains plus a hinge has thus far not been
observed in any species, suggesting that IgO represents a distinct Ig class in mammals.

IgO, a structural intermediate between IgY(E) and IgG?

To determine the phylogenetic position of IgO, we performed thorough phylogenetic analyses using its separate CH domains or the entire constant region sequence. All analyses showed that IgO is phylogenetically related to IgG, IgE, and IgY (relatively closer to the evolutionary route from a four CH domain Ig to a three CH domain). Two other lines of evidence support the notion that likely, IgO represents a structural intermediate between IgY(E) and IgG.

**Localization of the χ2 and α1 genes in the platypus immunoglobulin heavy chain constant region (IGHC) gene locus**

Although the genomic sequences of the platypus χ2 and α1 could be found in the genome database, they were not assembled in the main Ig gene locus (680-kb contig). This could be due to the presence of sequence gaps within the genomic contig, as in nearly all tetrapods so far investigated, all the IGH genes are arranged in a main Ig gene locus (680-kb contig). This could be due to the presence of sequence gaps within the genomic contig. Nevertheless, it provides data to support the above-mentioned model, explaining how a genetic hinge (encoded by a separate exon) could be developed.
CH236-647E9, the most plausible location for \(\alpha_1\) is in the gap between CH236-821N20 and CH236-647E9 (Fig. 1). This was confirmed by sequencing of PCR products amplified using primers derived from the \(\gamma_2\) gene and the sequences flanking the gap. The genomic organization of the \(\gamma_2\) was deduced by PCR and sequencing.

According to our PCR results, CH236-821N20 was shown to contain the \(\delta\) gene. To determine the location of the \(\gamma_2\) gene, a couple of primers were designed covering all sequence gaps from the \(\delta\) to the \(\gamma_1\) gene, and used together with the primers derived from \(\gamma_2\) in PCR amplifications using CH236-821N20 BAC DNA as a template. By sequencing of the obtained PCR products, it was deduced that the \(\gamma_2\) gene was located in the first gap downstream of the \(\delta\) gene (Fig. 1). These data allowed us to conclude that the platypus IGHC genes are arranged in a \(\mu-\delta-\alpha-\gamma_2-\gamma_1-\alpha_1-\gamma_1\) order (Fig. 1).

Supporting functionality of the identified platypus IGHC locus, a \(J_H\) (Ig H chain joining gene segment) locus was identified \(\sim 10\)-kb upstream of the \(\mu\) gene. The \(J_H\) locus spans \(\sim 4.2\)-kb DNA and contains 10 structurally functional \(J_H\) gene segments and 1 pseudo \(J_H\) (Fig. 8). The \(D_H\) and \(V_H\) gene loci could not be analyzed as there are too many sequence gaps upstream of the \(J_H\) locus.

**Discussion**

In the present study, we identified two Ig H chain isotypes in the duckbilled platypus, IgD and IgO, in addition to the previously known IgM, IgG1/G2, IgE, and IgA1/A2 classes, showing that the platypus expresses eight Ig H chain constant region genes, arranged in an \(\mu-\delta-\alpha-\gamma_2-\gamma_1-\alpha_1-\gamma_1\) order (Fig. 1). This also demonstrates that all Ig H chain isotypes expressed by eutherian mammals are present in monotremes even though these two mammalian lineages diverged \(\sim 166\) million years ago (26).

Although it has been known for some years that monotremes express typical mammalian IgM, IgG, IgA, and IgE (27, 28, 35–38), the presence of an ortholog of IgD has remained elusive. We have previously tried, but failed, to clone the \(\delta\) gene in the platypus using a PCR-based approach and degenerate primers covering the conserved \(\delta\) gene sequence of eutherian mammals, suggesting that if it was present, it should be distinct from that of its counterparts in eutherian mammals. In this study, we show that the platypus contains a \(\delta\) gene strikingly different from the \(\delta\) gene in eutherian mammals. It does, however, share great similarity to the \(\delta\) gene in nonmammalian vertebrates such as reptiles, amphibians, and fish. In eutherian mammals, IgD typically contains three \(C_H\) domains and a hinge segment (9), which is much shorter than its counterparts in nonmammalian vertebrates, which usually have eight or more CH domains but no hinge (3, 4, 8). The platypus \(\delta\) gene encodes ten \(C_H\) domains, representing the first long form of IgD molecule identified in mammals. The discovery of such an IgD gene has evolutionary implications as: 1) it establishes a clear relationship between the short IgD molecules in eutherian mammals and the long IgD molecules in nonmammalian vertebrates and 2)
it shows that the platypus has a unique combination of Ig genes which share similarity both to those of nonmammalian vertebrates and eutherian animals.

Sequence comparisons suggest that the platypus δ gene shares an immediate ancestor with the recently identified δ gene in a reptile, leopard gecko (6), as all its 10 Cδ exons are homologous to exons found in the reptile (gecko) δ gene (containing 11 Cδ encoding exons). We also recently identified the δ gene in another reptile, the green anole lizard (our unpublished data), displaying a similar structure to that of the gecko gene by having 11 Cδ encoding exons. Presence of these closely related δ genes in phylogeny in both mammals and reptiles, strongly suggests that the absence of the δ gene in birds (7, 16) is due to a recent genetic modification that occurred after their divergence from reptiles.

The platypus Igδ does not contain a hinge region. Sequence comparisons suggest that the 5′CH1, δCH2, and δCH3 of eutherian mammals share homology with the 6′CH1, 6′CH6, and 6′CH7 of the platypus, suggesting a selective loss of other Cδ exons during the evolution. To provide insights into the timing of the loss of these genes, we searched for a δ gene in a marsupial, the gray short-tailed opossum, by using its genome sequence. Typical mammalian μ, γ, ε, and α genes are found in tandem in a single locus (data not shown). In the 200-kb of DNA between the opossum μ and γ genes (where the δ usually located), we did not find any evidence of a δ gene (or even any putative Ig encoding sequences). A δ gene was also not found anywhere in the entire opossum genome, suggesting an absence of the gene in this species. A future search for the IgD encoding gene in additional marsupials might be expected to provide more clues to this evolutionary issue.

The o gene represents another interesting finding in platypus. It encodes four Ig CH domains and a hinge attached to the N-terminal of Cδ2, giving rise to an Ig H chain constant region that has thus far not been observed in vertebrates. Our data suggest that it might be an immediate evolutionary precursor of IgG. This hypothesis is based on several facts: 1) the IgO encoding gene, o, is physically located in the position where a γ gene is usually found in the mammalian IgH locus; 2) the o gene is phylogenetically related to the mammalian γ gene; and 3) the o gene encodes a hinge segment. IgG is thought to have originated from IgY, an ancient, hinge-lacking, four CH domain Ig isotype. Compared with IgY, mammalian IgG usually has a hinge segment and one Cγ domain less (a loss of CH2 of IgY). The structure of IgO suggests that the hinge was formed before the loss of a CH domain. This hypothesis agrees with a model that explains the origin of the murine IgA hinges (24). The pivotal point in that model is that the Ig hinge region was originally derived from the noncoding 3′ end sequence of an intron that was rich in pyrimidine (especially cytidine), potentially encoding the prolines that are often seen in Ig hinge regions. This originally noncoding stretch of nucleotides was subsequently attached to the 5′ end of a Cγ exon and inserted in frame with the reading frame of the Cγ exons. A final loss of the Cγ exon (or a gain of a separate genetic hinge exon) involved could be achieved through mutations of hinge-Cγ junctional sequence generating a 5′ RNA splice site, which would result in a removal of the Cγ exon in mature message RNA. Nonfunctionality of the Cγ exon would finally cause it to be gradually lost, forming the genomic structure of the mammalian γ genes.

In summary, the discovery of IgO and Igδ in the platypus highlights the important position this species holds in mammalian phylogeny and provides unique insights into the evolution of the mammalian Ig repertoire. It also provides support for the continuous evolution of immunoglobulins in vertebrates and the importance of comparative studies for reconstructing the evolutionary history of the immune system.

**Disclosures**

The authors have no financial conflict of interest.

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