Analysis of C3 Suggests Three Periods of Positive Selection Events and Different Evolutionary Patterns between Fish and Mammals

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Abstract

Background: The third complement component (C3) is a central protein of the complement system conserved from fish to mammals. It also showed distinct characteristics in different animal groups. Striking features of the fish complement system were unveiled, including prominent levels of extrahepatic expression and isotypic diversity of the complement components. The evidences of the involvement of complement system in the enhancement of B and T cell responses found in mammals indicated that the complement system also serves as a bridge between the innate and adaptive responses. For the reasons mentioned above, it is interesting to explore the evolutionary process of C3 genes and to investigate whether the huge differences between aquatic and terrestrial environments affected the C3 evolution between fish and mammals.

Methodology/Principal Findings: Analysis revealed that these two groups of animals had experienced different evolution patterns. The mammalian C3 genes were under purifying selection pressure while the positive selection pressure was detected in fish C3 genes. Three periods of positive selection events of C3 genes were also detected. Two happened on the ancestral lineages to all vertebrates and mammals, respectively, one happened on early period of fish evolutionary history.

Conclusions/Significance: Three periods of positive selection events had happened on C3 genes during history and the fish and mammals C3 genes experience different evolutionary patterns for their distinct living environments.

Introduction

The complement system was first identified as a heat-sensitive factor in fresh serum that ‘complemented’ the effects of specific antibody in the lysis of bacteria and red blood cells. It is a group of humoral and cell surface proteins which play an essential role in innate immune defense against invading microorganisms [1]. In vertebrates, the complement system not only mediates functions contributing to pathogen killing and elimination but also serves as a bridge between the innate and adaptive responses (reviewed in [1,2,3,4]). The vertebrate complement system can be activated through three overlapping pathways: the classical, alternative and lectin pathways [5,6]. The classical pathway is induced by antigen-antibody interactions, whereas the other two pathways function only in innate immune system. These pathways converge in the formation of the third complement component (C3) convertases, which cleave C3 into the small anaphylatoxin C3a and the large, reactive C3b that may covalently couple to target surfaces [7,8]. Afterward, the lytic pathway is activated and the membrane-attack complex (MAC) is formed on target cells resulting in cell lysis. And host cells can express both serum and cell surface regulatory proteins to protect against attacking on self cells [9].

C3 is a central protein of the complement system, this versatile and flexible molecule interacts with various proteins to perform its functions. It emerged over 700 million years ago [10] and belongs to the α2-macroglobulin (α2M) family. Members of this family, such as the complement components C3, C4 and C5, the proteinase inhibitor α2M and the insect and nematode thioester-containing proteins (TEPs) [11], are characterized by homologous sequences features, including a unique thioester motif enabling covalent attachment to target particles and a central, highly variable part likely involved in recognition; and, the propensity to undergo conformational changes for distinct protein binding interactions [11,12,13]. Researches of crystal structures of human C3 and its derived products revealed thirteen domains. The core of the protein is formed by eight homologous domains, which were named macroglobulin (MG) domains referring to the related immunoglobulin fold and to the family of α2M proteins. The other five domains are crafted onto this core of eight MG domains in two large insertions and one extension [14,15,16]. The first insert is located in MG6 and includes the linker region (LNK), the tetra-arginine pro-C3 processing site, the anaphylatoxin (ANA) domain and a linker (αNT) that connects the ANA domain back to MG6. The second insert is between domains MG7 and MG8 and consists of the CUB (for ‘complement C1r/C1s, Uegf, Bmp1’)
domain and the thioester domain (TED) that carries the reactive
thioester. The TED domain itself is inserted in loop of the CUB
domain [15]. The C345C (for ‘the C-terminal parts of the
complement components C3, C4 and C5’) domain at the C-
terminal end forms an extension and is connected to MG8 via a
short anchor region.

The conformational changes during the conversion of C5 to
C3b make many proposed ligands binding sites more accessible.
Large conformational changes in several domains are pivotal to
the conversion of inactive C3 to active C3b which make C3b more
elongated and open than C3. As a direct consequence, putative
binding sites for large ligands, such as factor B and properdin,
are spatially dispersed, reducing potential steric collisions between
them [14]. For the reason of versatility of ligands to C3 and its
derived products and lack of structural data, up till now there is
not a comprehensive and integrated view of these binding sites.

Since their discovery, complement molecules have been found
and studied in a variety of organisms, principally vertebrates.
Then the homologs of complement C3 have been identified from
invertebrates including sea squid, sea urchin, horseshoe crab,
coral, and sea anemone [17,18,19,20,21,22]. These findings
demonstrated that the origin and evolution of the complement
system is traced to the earliest radiations of the animal kingdom
(reviewed in [23,24,25,26]). These animals differed in immune
weapons and lived in distinct environments with huge differences.
The innate immune system is the only defense weapon of
invertebrates while vertebrates evolved and developed acquired
immune system furthermore to against pathogen invasion. As
vertebrates, fish faced more intense selection pressures imposed by
the aquatic environment in which is filled with countless types and
numerous amounts of bacteria and viruses than the terrestrial
organism. Moreover, the copy numbers and functions of C3 also
varied in different animals. Mammalian C3 is encoded by a single
gene, while many teleost fish studied thus far were found to possess
multiple forms of C3 which are the products of different genes
[27]. Functional studies in trout, carp and seahream showed different binding efficiencies of these C3 isoforms to several
complement activating surfaces, suggesting that teleost fish may
have evolved a novel strategy to enlarge the innate recognition and
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Figure 1. The putative gene tree for C3 reconstructed by Bayesian approach with no constraints on the topology. The Darwin selection pressures were detected by the branch-site models in the ancestral lineages to vertebrates (in green), mammals (in red) and ostariophysian together with protacanthopterygian (Ost+Pro) fishes (in blue). The positive selected sites with posterior probabilities larger than 0.95 (PP > 0.99 in bold) were showed on the corresponding lineages. The synonymous substitution (dN), non-synonymous substitution (dS) of nucleotides and the ratio of \( dN/dS \) of these ancestral lineages were showed. The sequences of mammalian (red) and fish C3 (blue) were then tested by site-model tests in next analysis, respectively.

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Table 1. Likelihood ratio tests of branch-site models on C3 genes.

| Model        | np | lnL   | Model comparison | \( 2\Delta (\ln L) \) | P-value | positive selected sites |
|--------------|----|-------|------------------|------------------------|---------|------------------------|
| 1: Null-vert | 69 | -79225.31 |                   |                        |         |                        |
| 2: Vert      | 70 | -79210.21 | 1 and 2          | 30.20                  | 1.0E-6  | 140L, 310G, 318P, 510S, 594R, 660S, 708D, 758Q, 957E, 976E, 982G, 1106N |
| 3: Null-Mam  | 69 | -79231.38 |                   |                        |         |                        |
| 4: Mam       | 70 | -79211.96 | 3 and 4          | 38.84                  | 0.0     | 220H, 242K, 591E, 855T, 974Q |
| 5: Null-OP   | 69 | -79236.76 |                   |                        |         |                        |
| 6: OP        | 70 | -79209.22 | 5 and 6          | 55.08                  | 0.0     | 797E, 826Q, 964Y, 1218E, |
| 7: Null-Acan | 69 | -79241.15 |                   |                        |         |                        |
| 8: Acan      | 70 | -79216.70 | 7 and 8          | 48.90                  | 0.0     | 451N, 1026H, 1106W, 1110E, 1147V, 1653T |
| 9: Null-Seabass2 | 69 | -79241.51 |                   |                        |         |                        |
| 10: Seabass2 | 70 | -79230.22 | 9 and 10         | 64.58                  | 0.0     | n/a                    |
| 11: Null-Stickle5 | 69 | -79234.35 |                   |                        |         |                        |
| 12: Stickle5 | 70 | -79230.63 | 11 and 12        | 7.44                   | 0.0063  | n/a                    |

Note: np number of parameters, lnL ln(likelihood) value, \( 2\Delta (\ln L) \) twice the difference of ln(likelihood) between the two models compared, vert, mam, OP, and Acan the ancestor branches of the vertebrates, mammals, ostariophysian together with protacanthopterygian fishes, and acanthopterygians fishes, respectively, examined in present study, seabass2 the seabass C3-2, stickleback5 the three-spined stickleback C3-5. The P-values < 0.01 are shown in boldface. The human C3 sequence was used as reference to mark the positions of the positive sites in all cases. Sites with the P-values < 0.05 are shown and those with P-values < 0.01 are in boldface.

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and mammals, respectively. The site models treat the \( \omega \) ratio for any site (codon) in the gene as a random variable from a statistical distribution, thus allowing \( \omega \) to vary among codons \([32,33]\). Positive selection is defined as presence of some codons at which \( \omega > 1 \) (in model M2a or M8). An LRT is constructed to compare a null model that does not allow for any codon with \( \omega > 1 \) (M1 or M7) against a more general model that does. Site models were conducted on subset of mammal or fish \( C3 \) sequences, respectively.

For mammals, no positive selected sites were detected by either M2a model or M8 model (Table 2, mammal subset). However the fish \( C3 \) showed different evolutionary pattern. The LRT test statistic \((2\Delta \ln L)\) of M7-M8 comparison of fish subset was 150.34 \((P < 0.01, \) Table 2, fish subset), indicating that positive selected sites probably existed in fish. Then the BEB approach detected seven sites under positive selection on fish \( C3 \) based on model M8, of which six had PP values >0.95 (293L, 294T, 432G, 433P, 737T, 802D, and 1360S, see Table 2, model M8 of fish subset). These results provide evidence of different selection pressures on fish and mammal \( C3 \), indicating the strong Darwin selection pressures on fish \( C3 \) genes.

**Discussion**

In this study, we surveyed the thirty-four \( C3 \) sequences of twenty-five different species to explore the evolutionary process of \( C3 \) genes and to examine whether the different environments had caused different selection pressures between aquatic and terrestrial organisms. Because the aquatic environments contain countless kinds of bacteria and virus and fishes are armed with less developed adaptive immune system comparing with mammals, one may expect the innate immunity including the complement system of fish plays much more important roles in defense against pathogen invading. The site-models tested on fish and mammalian \( C3 \) genes revealed that these two groups of vertebrates which are flourishing in the aquatic and terrestrial environments, respectively, experienced different evolutionary patterns. No evidence of positive selection was detected in mammalian \( C3 \) while seven sites were found to be under positive selection in fish \( C3 \) (Table 2), indicating the different evolutionary pressure on these two groups whose living environments differed hugely.

Molecular evolution analyses were also conducted to explore the possible evolutionary process of \( C3 \). Many positively selected sites were detected among the common ancestral lineages to the vertebrates, mammals and protacanthopterygian and ostariophysian fishes, indicating that episodic positive selection events had happened during the \( C3 \) evolution along these lineages. The first period of positive selection happened with the emergence of vertebrates. From the evolutionary standpoint, the complement system is present in both of vertebrates and a wide range of invertebrates. Unlike the vertebrates, the complement system of invertebrate was more primitive although they showed some complexity and diversity \([34,35]\). Those invertebrate complement systems lack the antibody and thus the classical pathway, which is based on the antibody-recognizing activation cascade, and seem to represent a prototypic opsonin system composed of \( C3 \) and its activation cascades that seem to correspond to mammalian lectin and/or alternative pathways \([36,37]\). The ancient origin of \( C3 \) gene can be traced back to cnidarians, one of the most primitive metazoan members \([10,30]\) and it has been evolutionarily retained.
Table 2. Site model tests on subset of fish or mammalian C3 genes.

| Model                  | np | lnL        | parameter                              | Model comparison      | Δ(lnL) | P-value | positive selected sites \(^1\) |
|------------------------|----|------------|----------------------------------------|-----------------------|--------|---------|-------------------------------|
| **Data set: mammals**  |    |            |                                        |                       |        |         |                               |
| M0 (one ratio)         | 27 | -41738.40  | \(\omega = 0.178\)                    |                       |        |         |                               |
| M1a (nearly neutral)   | 28 | -41035.20  | \(\omega = 0.794, (\rho_1 = 0.206)\)   |                       |        |         |                               |
| M2a (positive selection)| 30 | -41035.20  | \(\rho_0 = 0.794, \rho_1 = 0.000, (\rho_2 = 0.206, \omega_2 = 1.00\) | M2a and M1a          | 0.0    | 1.0     | Not allowed \(^2\)            |
| M3 (discrete)          | 31 | -40858.91  | \(\rho_0 = 0.266, \rho_1 = 0.530, (\rho_2 = 0.204, \omega_0 = 0.027, \omega_1 = 0.132, \omega_2 = 0.706\) | M3 and M0            | 1758.98| 0.0     |                               |
| M7 (beta)              | 28 | -40929.27  | \(\omega = 0.706, p = 2.565\)         |                       |        |         |                               |
| M8 (beta & w > 1)      | 30 | -40851.91  | \(\rho_0 = 0.890, p = 1.182, q = 7.291, (\rho_1 = 0.110, \omega_1 = 1.000\) | M7 and M8            | 154.72 | 0.0     | None                          |
| **Data set: fish**     |    |            |                                        |                       |        |         |                               |
| M0 (one ratio)         | 41 | -54906.39  | \(\omega = 0.309\)                    |                       |        |         |                               |
| M1a (nearly neutral)   | 42 | -53795.95  | \(\omega = 0.683, (\rho_1 = 0.317)\)    |                       |        |         |                               |
| M2a (positive selection)| 44 | -53795.95  | \(\rho_0 = 0.683, \rho_1 = 0.265, (\rho_2 = 0.052, \omega_2 = 1.00\) | M2a and M1a          | 0.0    | 1.0     | Not allowed \(^2\)            |
| M3 (discrete)          | 45 | -53584.70  | \(\rho_0 = 0.320, \rho_1 = 0.455, (\rho_2 = 0.225, \omega_0 = 0.058, \omega_1 = 0.273, \omega_2 = 0.987\) | M3 and M0            | 2643.38| 0.0     |                               |
| M7 (beta)              | 42 | -53637.19  | \(\omega = 0.694, q = 1.342\)         |                       |        |         |                               |
| M8 (beta & w > 1)      | 44 | -53562.02  | \(\rho_0 = 0.864, p = 1.045, q = 3.305, (\rho_2 = 0.136, \omega_1 = 1.197\) | M7 and M8            | 150.34 | 0.0     | 293L, 294T, 432G, 433P, 737T, 802D, 13605 |

Note: the site models were conducted on mammalian or fish C3 gene sequences with two invertebrate C3 as out-group, respectively.

\(^1\): only the sites with PP \(> 0.95\) were shown and those with PP \(> 0.99\) are in bold.

\(^2\): this means that the models which allowed the positively selected sites exist in sequences did not pass the likelihood ratio test and thus the positively selected sites were not allowed to exist.

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in both deuterostomes and some lineages of protostome, such as arthropods (horseshoe crab) [22] and mollusks [39,40]. The antimicrobial activities of the invertebrate C3, through a complement-mediated phagocytosis, have been proven only in the sea urchin (echinoderm) [36] and ascidians (urochordate) [20]. And no evidence of direct cytolytic activity has been proven in invertebrate primitive complement system. Thus, we speculate that with the evolution of antibody in the vertebrate, the complement system had experienced the first period of positive selection on the ancestral vertebrates to evolve the classical pathway of C3-activation and the cytotytic pathway. These huge advances of immunity, emergence of antibody (adaptive immunity) and the classical pathway of activating complement system (innate immunity), promoted the flourish of the ancestral vertebrates.

The second period of positive selection happened on the early period of fish evolutionary history. The ancestral lineage leading to ostariophysian and protacanthopterygian fish also showed positively selected sites, indicating one more period of positive selection event on C3. Besides that, six positive selection sites were also detected among the ancestral lineage leading to all actinopterygian fish (Table 1). The positive selection sites detected among the ancestral lineages to fish reflected the second period of positive selection on the early period of fish C3 evolutionary history. Fish are most primitive groups of jawed vertebrates, their complement system had evolved all three C3-activation pathways and the cytolytic pathway, showing many of the effecter activities recognized in the mammalian complements, such as target cell killing, opsonization, and anaphylatoxic leukocyte stimulation [41]. Although fish complement system has showed a high degree of structural and functional conservation of the complement pathways and their components comparing with mammals, striking features of the fish complement system were also unveiled, including prominent levels of extrahapletic expression and isotypic diversity of the complement components. Whole-genome duplication (WGD) is to be one of the major evolutionary events that shaped the genome organization of vertebrates. Three WGD events have been proposed in ancient vertebrate history; two at the origin of the group and a third specific to fish [42]. The distinctiveness of fish complement system probably was the long evolution results of the ancient fish-specific genome duplication (FSGD) under the aquatic environment. Studies of the genomes of zebrafish and two close relative Tetraodontiformes (Tetraodon and Takifugu) [43,44] have confirmed that ray-finned fish underwent a FSGD some 320-400 million years ago which might explain their evolutionary success.

The third period of positive selection of C3 happened on ancestral mammalian lineage. After its discovery, intensive and detailed researches have focused on human complement system, more than 30 plasma and cell-surface complement proteins have been found. The complement system is known to be a highly sophisticated host-defense system that is engaged in both the innate and adaptive immunities [45,46]. It involves in a range of functions from direct cell lysis to the enhancement of B and T cell responses [47,48]. Given the multiple pathways of activation and the versatile functions of derived products of complement members, regulation of the complement system is complex and necessary. Activation of complement is critical for protection against pathogen infection; however, inappropriate activation of complement contributes to the pathogenesis of immunological and inflammatory diseases [49]. To limit host destruction, the system makes use of both serum and cell surface regulatory proteins. The cell-expressed members of this family are membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55) and complement receptors one (CR1; CD35) and two (CR2; CD21) while the plasma members are C4-binding protein (C4BP) and factor H (FH). Complement regulators at the C3 cleavage step possess cofactor activity (CA) or decay accelerating activity (DAA). Almost all mammalian cells express regulators of complement to protect against attacking on self [9]. Nearly half of the complement proteins participate in regulation. And five positive selection sites were detected in ancestral mammalian lineage (Fig. 1). The presumed positively selected residue 1193 is on the surface of TED domain and located in the proposed binding site (residues 1187–1249) for factor H [50]. Other four positive selection sites were located in the internal of MG4, MG7 and CUB domains, respectively. Factor H, decay-accelerating factor (DAF, CD55) and complement receptor 1 (CR1, CD35) are three important members of regulators of complement activity which inhibit the C3 convertase activity [51]. Different from the diversity of C3 and quantitatively evolution manner in fish, mammals took more elaborately regulation on the existing members of complement system.

In conclusion, although C3 gene is conserved from invertebrate to vertebrate, it had happened three periods of positive selection events during animal evolutionary history. Two happened on the ancestral lineages to all vertebrates and mammals, respectively, one happened on early period of fish evolutionary history. For the reason of huge differences between aquatic and terrestrial environments, the C3 genes of fish and mammals had experienced different evolution patterns.

Materials and Methods

Ethics statement

All work was conducted with the approval of the Animal Ethics Committee.

Taxonomic Coverage

We screened and obtained partial length of C3 from the spleen cDNA library of miuui croaker, Miichthys miuui [52]. The 5' and 3' RACE-PCR was performed following the manufacturer’s instructions to obtain the full length of miuui croaker C3 (with accession number JQ033711). Furthermore, we retrieved eighteen C3 sequences from nine fish species and twelve sequences from twelve mammals, together with three C3 from two invertebrates and one bird, from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) or Ensembl (http://wwwensembl.org) database for evolutionary analyses (see Table 3).

Evolutionary Analysis

The miuui croaker C3 sequence with those of other species retrieved from public database was aligned under codon model with MUSCLE software for its high accuracy and speed [53,54]. With the alignment, the phylogenetic tree was reconstructed by using MrBayes3.1 [55]. For Bayesian inference, we evaluated the best-fit model as GTR+I+Γ by Bayesian information criterion (BIC) using jModeltest [56,57]. The MrBayes3.1 program was run with 5,000,000 generations with a burn-in of 25%. Ancestral sequences were reconstructed using the Bayesian method [58] implemented in the BASEML program in PAML 4.1 [59]. To investigate the evolutionary process of C3 and whether the different environments had caused different selection pressures between aquatic and terrestrial organisms, such as teleost and mammals, we employed the codon-based method to estimate the ratio of nonsynonymous and synonymous substitutions (ω) using PAML4 [59]. The subset of mammalian and fish C3 sequences, both of which used the two invertebrates C3 as out-group, were analyzed by site-models to detect the possible selective pressures.
on these two groups of animals with huge difference in living environments. Basically, a free-ratio model was first employed to allow the $\sigma$ ratios to vary for each branch. Then, the likelihood ratio test (LRT) was used to evaluate whether this model fits the data significantly better than the one-ratio model which assumes all branches have only one ratio. Then the branch-site model was used to detect positive selection that affects the interesting foreground lineages (for example, the ancestor lineages to teleost or mammals or the duplicated C3 lineages of some fish). Finally, six site models were applied to subset of teleost and mammalian C3 sequences, respectively, to examine the possible positively selected sites among those lineages. In all cases, twice the difference of log-likelihood values ($2\Delta \ln L$) between the two models was calculated following a chi-squared distribution with degrees of freedom.

Table 3. Taxonomy of species and accession numbers of C3 sequences used in this study.

| Taxonomy               | Common name | Species name         | Accession Number |
|------------------------|-------------|----------------------|------------------|
| Class Echinoidea       |             |                      |                  |
| Order Echinoidea       | purple sea urs | Strongylocentrotus purpuratus | NM_214521.1      |
| Class Asciidiacea      |             |                      |                  |
| Order Enterogona       | ascidian    | Ciona intestinalis   | NM_001032512.1   |
| Class Aves             |             |                      |                  |
| Order Galliformes      | chicken     | Gallus gallus        | NM_205405.1      |
| Class Mammalia         |             |                      |                  |
| Order Rodentia         | mouse       | Mus musculus         | ENSMUST00000024988 |
|                       | rat         | Rattus norvegicus    | NM_016994.2      |
|                       | guinea pig  | Cavia porcellus      | NM_001172903.1   |
| Order Didelphimorphia  | opossum     | Monodelphis domestica| ENSMODT00000034216 |
| Order Perissodactyla   | horse       | Equus caballus       | ENSECAT00000007684 |
| Order Cetartiodactyla  | cattle      | Bos taurus           | ENSBATAT00000022979 |
|                       | pig         | Sus scrofa           | ENSSCT000000014800 |
| Order Carnivora        | giant panda | Ailuropoda melanoleuca| ENSAMET00000007996 |
| Order Probosidea       | elephant    | Loxodonta africana   | ENSLAFT00000010468 |
| Order Monotremata      | platypus    | Ornithorhynchus anatinus | ENSOANT00000009742 |
| Order Primates         | human       | Homo sapiens         | ENST000000245907 |
|                       | orangutan   | Pongo pygmaeus       | ENSPPYT000000011025 |
| Class Actinopterygii   |             |                      |                  |
| Superorder Acanthopterygii |        |                      |                  |
| Order Tetraodontiformes| spotted green pufferfish | Tetraodon nigroviridis | ENSTNIT000000017333 |
|                       | tiger puffer | Takifugu rubripes    | ENSTRUT000000027127 |
|                       |              |                      | ENSTRUT00000004988 |
|                       |              |                      | ENSTRUT000000045315 |
| Order Pleuronectiformes| Japanese flounder | Paralichthys olivaceus | AB021653.1        |
| Order Perciformes      | miuuy croaker | Milichthys miuuy     | JQ033711          |
|                       | spotted wolffish | Anarchias minor      | AJ30957.1         |
|                       | European seabass |Dicentrarchus labrax | HM563078.1        |
|                       |              |                      | HM563079.1        |
| Order Gasterosteiformes| three-spined stickleback | Gasterosteus aculeatus | ENSGACT00000024968 |
|                       |              |                      | ENSGACT00000024978 |
| Order Beloniformes     | medaka       | Oryzias latipes      | NM_001105082.1    |
|                       |              |                      | NM_001105083.1    |
| Superorder Ostariophysi|            |                      |                  |
| Order Cypriniformes    | zebrafish    | Danio rerio          | NM_001037236.1    |
| Superorder Protacanthopterygii |      |                      |                  |
| Order Salmoniformes    | rainbow trout | Onchorhynchus mykiss | AF271080.1        |
|                       |              |                      | U61753.2          |

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equalizing the difference in parameter numbers estimated in the nested models.

Sliding windows analysis
To explore further the heterogeneous selection pressure across codons of $C3$ genes between fish and mammals, a sliding window analysis of $\omega$ values was conducted using the Nei and Gojobori method [60]. Sliding windows were implemented in the program SWAAP 1.0.2 [61] with window and step sizes of 90 and 36 nucleotides, respectively.

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