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Identification of *Cytochrome P*450 (*CYP*) Genes in Zhikong Scallop (*Chlamys farreri*)

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Abstract Cytochrome P450 (*CYP*) superfamily is one of the membership largest and function most diverse protein superfamily recogniozed among living beings. Members of this superfamily were further assigned to different families and subfamilies based on their amino acid similarities. According to their phylogenetic relationships, the *CYP* genes which likely diverged from common ancestor gene and may share common functions were grouped into one clan. Widely distributing scallops are a group of the most conspicuous bivalve; however the studies on their *CYP* is acarce. In this study, we searched the genome and expressed sequence tags of Zhikong scallop (*Chlamys farreri*) for *CYP* genes. In total, 88 non-redundant *CYP* were identified, which were homed in 13 *CYP*s gene families. Phylogenetic analysis divided these genes into 4 *CYP* clans. As in deuterostomes, Clan 2 was the largest, which contained 33 genes belonging to *CYP*1, *CYP*2, *CYP*17 and *CYP*356 families. Clan 3 contgained 19 genes belonging to *CYP*3, *CYP*5 and *CYP*30 families. Clan 4 contained 23 genes, all belonging to *CYP*4 family. The mitochondrial *CYP* clan contained 9 genes belonging to *CYP*10 and *CYP*24 families. In comparison, protostomes (*C. farreri*, *D. pluex*, *D. melanogaster*) contained more *CYP* genes than deuterostomes (*S. purpuratus* and vertebrates) in Clan 2 but less genes in Clan 3 and Clan 4. Our findings will aid to deciphering *CYP* function and evolution in scallops and bivalves.

Key words cytochrome P450; *CYP*; Zhikong scallop; *Chlamys farreri*; phylogenetic analysis

1 Introduction

Cytochrome P450 (*CYP*), a group of hemeproteins, is the membership largest and function most diverse protein superfamily found in nature (Estabrook, 2003). To date, more than 12000 members of *CYP* belonging to over 1000 families have been identified in living beings ranging from virus to human beings (Nelson, 2011), especially in those with genome sequences. The number of *CYP* genes identified is highly variable among species. For example, 57 *CYP* genes were found in *Homo sapiens*, 54 in *Takifugu rubripes*, 94 in *Danio rerio*, 120 in *Strongylocentrotus purpuratus*, 85 in *Drosophila melanogaster* and 75 in *Daphnia pulex* (Nelson, 2009). All the members of *CYP* identified in animals cluster into 10 clans (clusters of phylogeneticly related *CYP* families). For example, in vertebrate, Clan 2 includes *CYP* family 1, 2, 17, 18 and 21; Clan 3 includes *CYP* family 3, 5, 6 and 9; Clan 4 includes *CYP* family 4; and the mitochondrial *CYP* clan includes family 11, 24 and 27 (Nelson, 2011). *CYP* superfamily is believed to be extremely ancient and originate from a common ancestral gene, and consecutive duplications and subsequent divergence of the gene diversified this multigene family (Nebert and Gonzalez, 1987; Nelson *et al.*, 1993). *CYP* members are found in all living beings ranging from prokaryotes to eukaryotes (Nebert and Gonzalez, 1987).

Bivalve is one of the oldest and evolutionary most successful classes of invertebrates. From expressed sequence tags, *CYP* genes have been identified in two bivalve species, mussels and oysters, which included 58 *CYP* genes in *Mytilus californianus*, 12 in *Mytilus galloprovincialis*, 39 in *Crassostrea gigas* and 14 in *C. virginica* (Zanette *et al.*, 2010). Phylogenetic analysis indicated that *CYP* genes identified in *M. californianus* mainly falled into Clan 2, Clan 3, Clan 4 and Mitochondrial *CYP* clan. Clan 2 was the membership largest in deuterostomes. It is clear that identification of *CYP* genes in different bivalve species will aid to revealing the diversity of *CYP* and deciphoring *CYP* function in this old invertebrate class.

As one of the most conspicuous groups of bivalve, scallops are widely distributed on the world with about over 360 species (Shumway and Parsons, 2006). At present, little is known about scallop *CYP* (Miao *et al.*, 2011). Recently, transcriptome sequencing using 454 GS FLX

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platform and ~50-fold genome sequencing using Solexa platform have been carried out for Zhikong scallop (*Chlamys farreri*) (unpublished), which provided comprehensive transcriptomic and genomic resources of identifying *CYP* genes. A total of 88 *CYP* genes belonging to 13 families were identified in available *C. farreri* sequence data with their phylogenetic relationships to human and other mollusks *CYP* examined in this study.

2 Methods

2.1 Gene Identification

Significant homology to *CYP* was identified by Hidden Markov Model searches (HMMER v2.3.2) (Eddy, 1998) using the global PFAM model for *CYP* (PF00067) (http://pfam.sanger.ac.uk/) from *C. farreri* transcriptome data (accession number SRA030509). The detected sequences were compared with the genomic sequence of *C. farreri* (~50 fold coverage; unpublished) using BlastN with an E-value of 1e-5 as significant matching and then assembled to consensus genes. Gene identities were examined by reciprocal BLAST of the predicted genes against the NCBI non-redundant (Nr) protein database and Swiss-Prot database (http://www.ncbi.nlm.nih.gov/).

The identified sequences were translated and named based on the homology of *C. farreri CYP* to those of other species using the standardized nomenclature previously assigned by Nelson *et al.* (2004). Values of 40% and 55% amino acid identity were cut-offs for *C. farreri CYP* to be assigned to families and subfamilies, respectively. *C. farreri CYP* of different families were divided into clans as those in vertebrates (Nelson, 2011).

2.2 Sequence Alignments and Phylogenic Analysis

All sequence alignments were performed using Muscle v3.6b (Edgar, 2004), and adjusted by hand based on the MUSCLE (multiple sequence comparison by log expectation) alignment scoring function. Maximum likelihood phylogenetic trees were constructed by analyzing *CYP* amino acid sequences of *C. farreri*, human (Nelson, 2003) and other mollusks (Teunissen *et al.*, 1992; Brown *et al.*, 1998; Toledo-Silva *et al.*, 2008; Whalen *et al.*, 2010; Miao *et al.*, 2011; Pan *et al.*, 2011) using MEGA (v 5.05) (Tamura *et al.*, 2011). The Whelan and Goldman model (WAG) of amino acid substitution with a gamma distribution of substitution rates was used (Whelan and Goldman, 2001).

3 Results and Discussion

3.1 Annotation of *C. farreri CYPs*

Two hundred and twenty *CYP*-containing fragments including 85 contigs and 135 singletons were identified among *C. farreri* EST. Finally, 88 consensus *CYP* genes were assembled manually based on BlastN results from genome sequence of *C. farreri*. Forty-one of them could be translated into full-length or nearly full-length *CYP* proteins, and the others were partial. Based on the previously described rules for naming *CYP* (40% identity for family designation, 55% identity for subfamily designation) (Nelson *et al.*, 2004), in this study all *C. farreri CYP* genes could only be provisionally classified at level of family, so *C. farreri CYP* genes assigned to one *CYP* family were further numbered consecutively, with a 'like' between the family number and a subordinate number in the gene name. The 88 *CYP*s fall into 13 families based on the similarity of inferred amino acid sequences with previously named *CYP*, and were mainly from Clan 2, Clan 3, Clan 4 and Mitochondrial *CYP* clan. Four of the 88 *CYP* fall in other clans, including 1 in *CYP*20 (Clan 20), 2 in *CYP*46 (Clan 46) and 1 in *CYP*39 (clan not assigned) (Table 1) (Nelson, 2011).

3.2 Phylogenetic Analysis of *C. farreri CYP*s

Maximum likelihood phylogenetic tree was constructed with the 41 full-length and nearly full-length *C. farreri CYP*, 57 *CYP* from human, and 10 *CYP* from other mollusks, revealing the relationship among *CYP* families in these species (Fig.1). In general, the tree showed that 4 major clans were found in *C. farreri*, including Clan 2, Clan 3, Clan 4 and mitochondrial *CYP*. A majority of *CYP* in insects, mammals and fishes also fall into these clans (Nelson, 2009).

3.2.1 *C. farreri CYP*s in Clan 2

The *CYP* members in Clan 2 were mainly involved in metabolism of drugs and steroids in human. Some of them could be induced through pregnane X receptor (PXR) or aryl hydrocarbon receptor (AHR) (Dogra *et al.*, 1998; Waxman, 1999). As in deuterostomes and other mollusks, Clan 2 was the largest in *C. farreri* in which 33 *CYP* in family *CYP*1, *CYP*2, *CYP*17 and *CYP*356 were found.

So far, 4 *CYP*1 subfamilies (*CYP*1A-D) have been identified in vertebrates. A number of potential *CYP*1 homologs have also been identified in deuterostomes, such as *S. purpuratus* and *Ciona intestinalis*, but *CYP*1 have not been reported so far in protostomes (Goldstone *et al.*, 2007). In this study, 3 *CYP*1 genes were found in *C. farreri*, and 2 of them were full-length genes (member 1 and 3). The amino acid sequences of the two genes were used for phylogenetic analysis. The 2 *CYP*1 members from *C. farreri* clustered together and then clustered with those of human *CYP*1 (Fig.1). In human, members of *CYP*1C were not found and genes of *CYP*1D members were pseudogene, and only *CYP*1A and *CYP*1B could be used for the construction of the phylogenetic tree (Goldstone *et al.*, 2009; Kawai *et al.*, 2010).

*CYP*2 is the largest *CYP* family in mammals. The members in this family were responsible for a tremendous variety of substrate oxidation (Danielson, 2002). Twenty-three genes in *CYP*2 were identified in *C. farreri*, accounting for 26% of the total, about 30%–49% in vertebrates and 60% in sea urchin (Goldstone *et al.*, 2006). All the 10 full-length *CYP*2 members of *C. farreri* were used for phylogenetic analysis, 9 of them were clustered into one clade with human *CYP*2 except protein 17 (Fig.1).

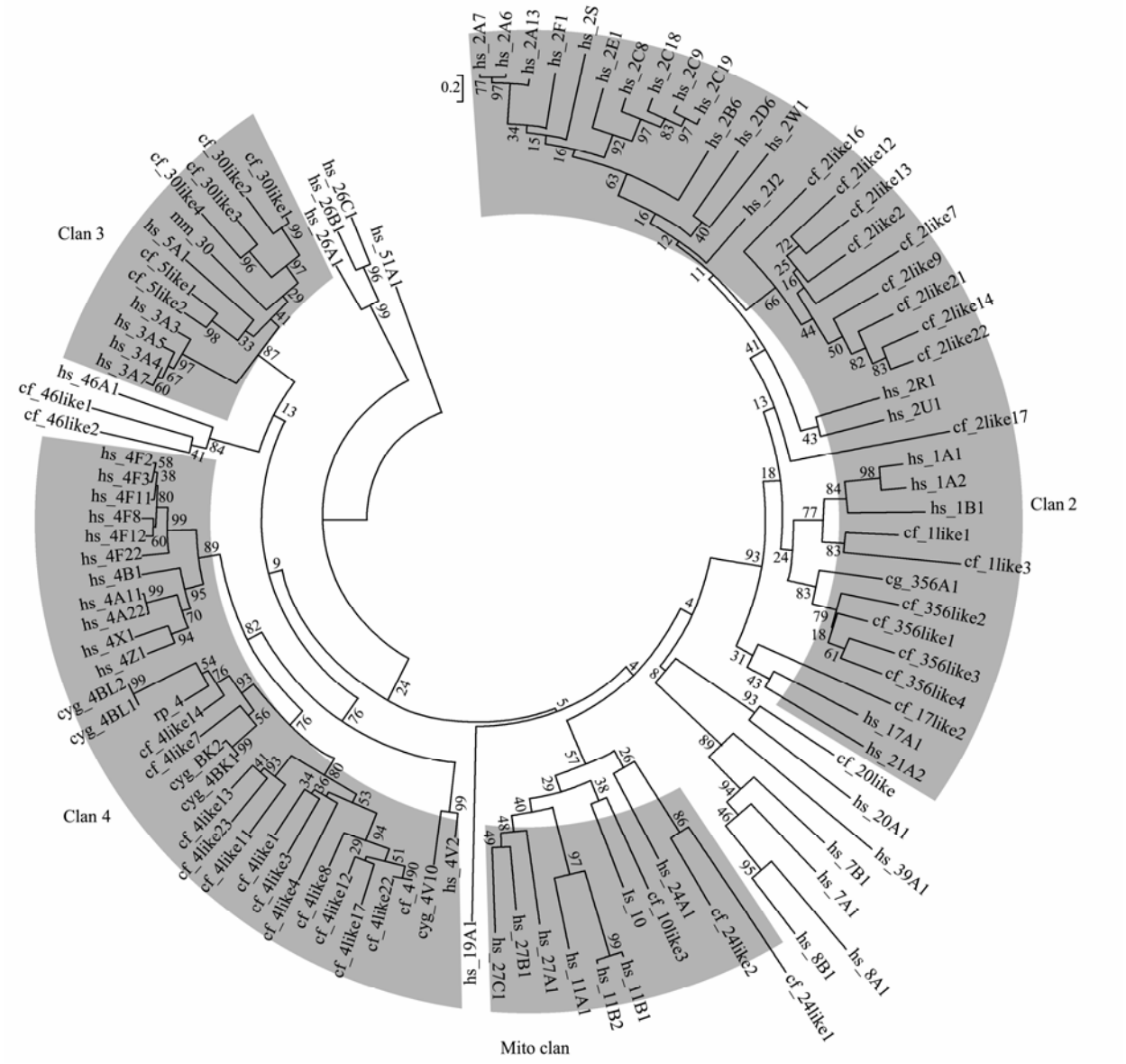

Fig.1 Maximum likelihood phylogenetic tree of 41 full-length and nearly full-length members of *C. farreri CYP*, 57 members of human *CYP* and 10 members of other mollusks *CYP*. Support values above branches are derived from 100 bootstrap replicates. cf, *C. farreri*; cg, *C. gigas*; mm, *Mercenaria mercenaria*; cyg, *Cyphoma gibbosum*; rp, *Ruditapes philippinarum*; ls, *Lymnaea stagnalis*; hs, *H. sapiens*.

This number 17 protein might be a member of a new *CYP* family closely relating with *CYP*2, which would be further classified by *CYP* Nomenclature Committee (Nelson, 2009).

Members in *CYP*17 played key roles in gonadal and adrenal steroids biosynthesis in vertebrates (Gilep *et al.*, 2011). Only one member in *CYP*17 has been found in mammals, and 2 in fishes (Zhou *et al.*, 2007). In invertebrates, 4 members in *CYP*17 were found in *Hydra vulgaris* (Nelson, 2009) and 6 in *S. purpuratus* (Goldstone *et al.*, 2006). Three members in *CYP*17 were found in *C. farreri*, of them only member 2 was nearly full-length and could be used for phylogenetic analysis. It clustered with those in human *CYP*17 and *CYP*21 (Fig.1). Members in *CYP*21 were also involved in steroid metallization, which were known to be the closest relatives to those in *CYP*17 (Lewis *et al.*, 1998). Members in *CYP*17 and *CYP*21 were thought to be monophyletic in molecular evolutionary researches (Lewis *et al.*, 1998; Nelson, 1999). Newly added scallop members into *CYP*17 will aid to analyzing their relationships with the vertebrate homologs.

*CYP*356 was a family newly identified from *C. gigas*. It is closely related to *CYP*1 and *CYP*17 of vertebrates (Toledo-Silva *et al.*, 2008). Herein, 4 members of *CYP*-356 were identified in *C. farreri*, which all clustered with *CYP*356A1 of *C. gigas* (Fig.1). Since no members of this family had been identified in other species, *CYP*356 might be a bivalve specific *CYP* family that participates in special biological processes of species in this class.

3.2.2 *C. farreri CYP*s in Clan 3

Clan 3 consists of various members of *CYP* associated

with detoxification of xenobiotics and endobiotics (Danielson, 2002). Nineteen members of *CYP* from *C. farreri* were assigned to *CYP*3, *CYP*5 and *CYP*30 of Clan 3 (Table 1).

Table 1 Distribution of *C. farreri CYP*s in *CYP* clans

CYP clan	*CYP* family	Number of *CYP* members
Clan 2	1	3
	2	23
	17	3
	356	4
Clan 3	3	13
	5	2
	30	4
Clan 4	4	23
Mitochondrial *CYP*	10	3
	24	6
Other clans	20	1
	39	1
	46	2

As one of the most important *CYP* families in human, members in *CYP*3 catalyze the metabolism of more than 50% of all clinically used drugs. Members of this family also metabolized a diverse range of other substrates such as bile acids, endogenous hormones, fungal and plant products and environmental pollutants (Danielson, 2002). Genes of *CYP*3 members were believed to arise from a common ancestor gene and diverged before deuterostome-protostome splicing (Nelson, 1998; Williams *et al.*, 2004). An early deuterostome *CYP*3 closely resembling the vertebrate *CYP*3 was reported in tunicate species, but no members of *CYP*3 has been described in protostomes (Verslycke *et al.*, 2006). Thirteen members of *CYP*3 were found in *C. farreri*, and all of them were partial, being not long enough for merging into phylogenetic tree. More genomic sequences are needed for phylogenetic analysis of members of *CYP*3 in deuterostomes and protostomes.

Two members of *CYP*5 were identified in *C. farreri*, and both clustered with human *CYP*5A1 (Fig.1). Members of *CYP*5 function as thromboxane synthase in vertebrates, and may derive from an ancestral member of *CYP*3 (Verslycke *et al.*, 2006). So far, no members of *CYP*5 have been reported in invertebrates. The identification of members of *CYP*5 in *C. farreri* might provide important information for revealing the evolutionary relationship of *CYP*5 and *CYP*3.

Four members of *CYP*30 were identified in *C. farreri*. *CYP*30 was a new cytochrome P450 family originally identified in gonadal tissue of the clam *Mercenaria mercenaria*, which showed high sequence identity to mammalian *CYP*3 and insect *CYP*6, and was considered to be involved in the metabolism of steroid hormones (Brown *et al.*, 1998). No member of this family has been reported in other species, suggesting that *CYP*30 might be mollusck specific.

3.2.3 *C. farreri* CYPs in Clan 4

Twenty-three members of *CYP* fall into Clan 4 in *C. farreri*, and as in vertebrates, all of them were members of *CYP*4, one of the most ancient *CYP* families in animal (Lewis *et al.*, 1998; Nelson *et al.*, 2004). In vertebrates, members of *CYP*4 catalyze the metabolism of fatty acids, eicosanoids and vitamin D and played important roles in chemical defense (Kirischian and Wilson, 2011). Many members of *CYP*4 have been identified in mollusks, some of them may be involved in fatty acid metabolism (Whalen *et al.*, 2010). The only *CYP* member reported in scallop was a member of *CYP*4 of *C. farreri*. Its expression was significantly decreased in gill and digestive gland of scallops exposing to Benzo(α)pyrene (BaP). Member 22 of *CYP*4 identified in this study shared 86% amino acid similarity with it (data not shown) (Miao *et al.*, 2011). As shown in Fig.1, 12 members documented earlier, 1 of *C. farreri* and 6 of other mollusks in *CYP*4 merged into member of human *CYP*4. *CYP*4V10 of *Cyphoma gibbosum* clustered with members of human *CYP*4V, the only subfamily shared by vertebrates and invertebrates (Nelson, 2009). The other 18 members of mollusk *CYP*4 analyzed in this study clustered together in a distinct clade, in which 10 members of *CYP*4 identified in this study and one member of *C. farreri CYP*4 reported before (Miao *et al.*, 2011) clustered into a subclade without members of other mollusk *CYP*4, suggesting that this subclade may be scallop specific. Two members of *C. farreri CYP*4 (7 and 14) clustered with the homologues of *C. gibbosum* and *Ruditapes philippinarum CYP*4 in another subclade, indicating these members of mollusk *CYP*4 may function similarly.

3.2.4 Mitochondrial *CYP*s in *C. farreri*

Mitochondrial *CYP* was named by their subcellular locations (Nebert *et al.*, 1991). The mitochondrial *CYP*s contain 3 *CYP* families in vertebrates, *CYP*11, *CYP*24 and *CYP*27. The members of these families involve in essential physiological functions such as metabolism of sterols, steroids and secosteroids (Danielson, 2002). At least 10 families of mitochondrial *CYP* have been identified in arthropods, and some members of them can metabolize xenobiotics, which is a clear difference from those of vertebrates (Danielson, 2002; Feyereisen, 2006; Feyereisen, 2011). *CYP*10 from the dorsal bodies of *Lymnaea stagnalis* was the only mitochondrial *CYP* reported in mollusks (Teunissen *et al.*, 1992). In this study, 3 members of *CYP*10 and 6 of *CYP*24 were found in *C. farreri*, and 1 member of *CYP*10 and 2 of *CYP*24 were assigned to phylogenetic tree. No homologue of arthropod mitochondrial *CYP* was identified in *C. farreri*. The members of molluscan *CYP*10 clustered with members of human *CYP*11 and *CYP*27, while the 2 memers of *C. farreri CYP*24 clustered with human *CYP*24A1 of another clade, implying that molluscan *CYP*10 is evolutionarily more close to CYP11 and CYP27 than CYP24 in human.

3.3 Expansion and Reduction of *CYP* Genes

A comparison of gene number in each *CYP* clan was made among *C. farreri*, *D. pluex*, *D. melanogaster*, *S.*

purpuratus, *D. rerio*, *T. rubripes* and *H. sapiens*, and difference was found between deuterostomes and protostomes (Fig.2). Clan 2 *CYP* contained 40% to 68% members of deuterostomes (*S. purpuratus*, *D. rerio*, *T. rubripes* and *H. sapiens*) *CYP*, but only 8% to 38% members of protostomes (*C. farreri*, *D. pluex* and *D. melanogaster*) *CYP*, indicating that an expansion in the number of members of this clan exists in deuterostomes. Of the protostomes investigated, *C. farreri* had the greatest percentage (38%) of clan 2 *CYP*s.

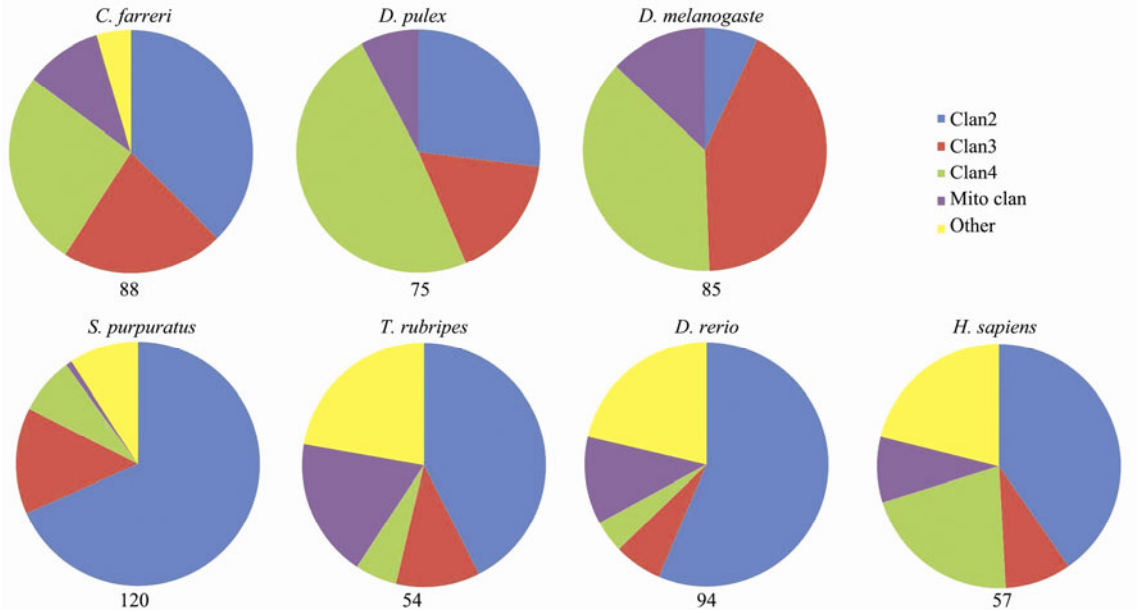

Fig.2 Distribution of gene numbers in *CYP* clans of *C. farreri*, *D. pluex*, *D. melanogaster*, *S. purpuratus*, *D. rerio*, *T. rubripes* and *H. sapiens*.

Number of Clan 3 members varied from 6% to 14% of the total in deuterostomes, while from 17% to 42% in protostomes, indicating a reduction in the number of members existed in this clan in deuterostomes. There was also a relative reduction in the number of clan 4 members in deuterostomes in comparison with protostomes. The members of *CYP* 4 account for 8%-21% of the total in deuterostomes, while those account for 26%-49% of the total in protostomes. Further screening in the genomes of more species will aid to our understanding of the species distribution and evolution of CYP.

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