

In Silico Sequence Analysis, Structure Prediction and Function Annotation of Melanocortin 1 Receptor Gene (MC1R) from the Guppy *Poecilia reticulata*

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Abstract: The common guppy, *Poecilia reticulata* is one of the most popular aquarium fish used as a model organism in toxicology, embryology and genetical studies. Vibrant colors in males are a secondary sexual character which attracts the females for mating. Pigmentation in guppy is found to be linked with melanin synthesis. Melanocortin genes are involved in the synthesis of melanin pigments. Alteration in Melanocortin 1 Receptor (MC1R) genes results in polymorphism. Analysis of the MC1R gene of *Poecilia reticulata* revealed a 966 bp open reading frame encoding 322 amino acids. The deduced MC1R protein sequence was predicted to possess three domains: 7TM GPCR (7 transmembrane G-protein-coupled receptor) chemoreceptor Srsx at the N-terminal, 7 TM receptor and the G-protein-coupled chemokine receptor like protein domain at the C-terminal. Predicted mutations in the MC1R protein sequence shows Indel (Insertion and deletion) and block mutations. Phylogenetic analysis revealed that MC1R of *Poecilia reticulata* and other fish species were separated from other vertebrates. The 3D structure of the 54-105 region of MC1R protein predicted for the first time comprised of two helices namely $\alpha 1$ and $\alpha 2$ from amino acid residues 59-69 and 86-97 respectively. The extracellular region is composed of two loops from amino acid residues 54-58 at the N-terminal and 88-105 at C-terminal. The intracellular region of the receptor contains a beta turn from the amino acid residues 71-73 which connects the $\alpha 1$ and $\alpha 2$ helices by means of a loop. Docking of α -MSH, ligand (Ca^{2+} ions and Cholesterol) interactions and the presence of CRAC domain (Cholesterol Recognition Amino acid Consensus sequence) within the TM region of receptor further reiterates the predicted structure has a definite functional role in pigmentation.

Keywords: *Poecilia reticulata*, MC1R, GPCR, CRAC, Function Annotation

Introduction

The common guppy, *Poecilia reticulata* is one of the most popular aquarium fish used as a model organism in toxicology, embryology and genetic studies. Male guppies are brightly colored and show tremendous pigmentation on their bodies when compared to females. Vibrant color in male is a secondary sexual trait, which is complex, conspicuous and manifested as spots, speckles and lines of various pigmented colors, including black, white, red-orange, yellow and green. Female guppies show preferences for a specific type of male

pigmentation, including orange and black and various color spots (Houde, 1997). Pigmentation exhibits various roles in the behavior of several animal species such as mate choice, camouflage, deterrence (Maderspacher and Nusslein-Volhard, 2003), warning, thermoregulation, protection from ultraviolet radiation and courtship display (Slominski *et al.*, 2004). Animal color patterns often evolve as adaptations to environmental surroundings, in which it frequently involves the evolution of cryptic coloration. Specifically in cichlid fish, color probably represents an important cue for assortative mating and is related to the establishment

and maintenance of reproductive isolation and, hence, speciation (Henning *et al.*, 2010). Studies on the teleost *Amphilophus* which includes the Midas cichlid species from the crater lakes in Nicaragua show color polymorphism, with dark and gold morphs. In this group, dark individuals have a barred pattern with vertical black bars that are intensified during social interactions, such as during mating behavior and territorial defense and has diverged sympatrically at least twice (Barluenga *et al.*, 2006). Various color spots are maintained as polymorphic traits within populations. Therefore, individual variations due to mutations can modify color patterns on the fish and hence seem to undergo rapid evolutionary change.

The identification of genes responsible for pigmentation polymorphism has gained considerable attention in the evolutionary biology of vertebrates (Hoekstra, 2006). The genes that can cause darkening of coat color have been studied most thoroughly in the laboratory mouse (Bult *et al.*, 2008) but spontaneous coat-darkening mutations have been reported in only four genes: The *Agouti* signaling protein (*Agouti*), attractin (*Atrn*), Melanocortin-1 Receptor (*MC1R*) and mahogunin (*Mgrn*) (Robbins *et al.*, 1993; Nagle *et al.*, 1999; Phan *et al.*, 2002; Bult *et al.*, 2008). The protein products of three of these genes, *Agouti*, *Atrn* and *MC1R*, interact at the surface of pigment-producing cells (melanocytes) and constitute the machinery responsible for “pigment type switching,” the ability of melanocytes to switch between the production of dark brown/black (eumelanin) and light yellow/red pigment (pheomelanin). Black pigmentation is produced by melanin synthesis, which involves many genes. The coding sequence of one of these genes, Melanocortin 1 Receptor (*MC1R*) is reported to contain variations that are associated with melanin pigmentation polymorphism in natural populations in many animals

including fish (Hoekstra, 2006). *MC1R* is a membrane bound-receptor, when it is active it signals the melanocyte to produce eumelanin, whereas low activity of *MC1R* leads to production of pheomelanin or an absence of melanin synthesis (Jackson, 1997).

Ornamental fish are the most valuable fisheries commodity in the world today in terms of returns per unit area. Hence, they could prove beneficial in disease study, bioassay, toxicology studies, proteomics, genomics and phylogenetics: There by in multidisciplinary applied research (Swain *et al.*, 2010). However, there are issues and concerns that need to be addressed such as survival of existing species, maintaining the potential of species for continual evolution, collection of base line data on ecosystems, socio-economic and diversity of the population in order to analyze the impact of ornamental fish trade on social and natural environments, expanding their biodiversity and genetic characterization studies. The increasing world demand for ornamental fish has opened the market for new varieties of fish with novel shapes or colors through the use of transgenics, the availability of the genes encoding fluorescent proteins for the production of green, red, blue, yellow fish in an almost endless variety of combinations (Melamed *et al.*, 2002).

The purpose of this study is to characterize the *MC1R* gene from the guppy *Poecilia reticulata* using *In Silico* methods and to deduce its phylogenetic relationship with other teleosts and vertebrates. The present study would contribute to an understanding of the molecular characterization of *MC1R*, its possible function in melanin synthesis and to predict mutational sites and their possible alterations in protein functions (Fig. 1).

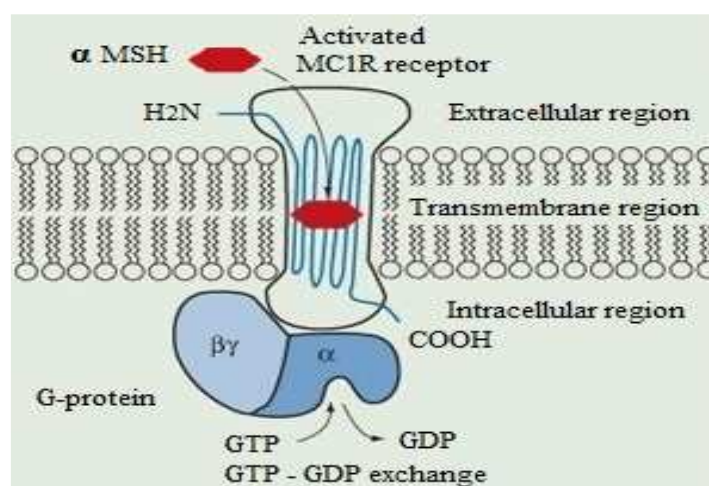


Fig. 1. Schematic diagram of the *MC1R* structure

Materials and Methods

Sequence Retrieval

The melanocortin receptor gene in ornamental fish was searched using GenBank at the NCBI. MC1R of *Poecilia reticulata* was selected with GenBank ID: AB563501.1 (Tezuka *et al.*, 2011) and the sequences were retrieved in FASTA format (Pearson and Lipman, 1988).

Sequence Analysis

Bioinformatic analyses of the MC1R gene of *P. reticulata* was carried out at the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The Open Reading Frame (ORF) and deduced amino acid sequence was predicted using ORF finder (www.ncbi.nlm.nih.gov/gorf). The conserved domains were predicted with CD search program (www.ncbi.nlm.nih.gov/Structural/cdd/).

Mutations in the protein sequence were predicted using MSBAR from EMBOSS (<http://bioinfo.nhri.org/tw/gui/>). The phylogenetic analysis of *P. reticulata* MC1R and MC1Rs from other species was aligned using BLAST pair wise alignment tool (Altschul *et al.*, 1990). The phylogenetic tree was constructed using a Neighbour-Joining method (NJ) (Saitou and Nei, 1987) using the BLAST Tree program (<http://www.ncbi.nlm.nih.gov/blast/treeview>).

Primary Structure Analysis

Physical and chemical parameters of the entire Melanocortin 1 receptor protein of *Poecilia reticulata* was computed by ProtParam tool (<http://web.expasy.org/protparam/>) which analyzed for molecular weight and theoretical iso-electric point (pI).

The Hum-mPLoc v2.0 server (Shen and Chou, 2009) was used to predict the subcellular localization of MC1R protein. The TMPred server (Hofmann and Stoffel, 1993) was used to analyze the presence of the transmembrane domains within the MC1R protein.

Secondary Structure Prediction

The SOPMA server (Geourjon and Deleage, 1995) was used for the secondary structure prediction of the MC1R protein. It was used to assess the conformational information about positional possibilities of the α -helices, β -strands, turns, random and coils within the protein structure.

Tertiary Structure Prediction

A BLASTP search of MC1R protein of *P. reticulata* with default parameter was performed against the Brook Haven Protein Data Bank (PDB) to find the suitable template for comparative or homology modeling (<http://blast.ncbi.nlm.nih.gov/>). The PDB structures based on sequence identity/similarity were retrieved

in PDB format and stored for further analysis. Structural alignment between the MC1R protein and the selected templates were carried out to predict the tertiary structure of the MC1R protein using ModSim server (Rodriguez *et al.*, 2012). The model with low Discrete Optimized Protein Energy (DOPE) score value and Ramachandran plot (stereochemistry quality) were taken into consideration for model refinement and validation. The PyMol (www.pymol.org) interface was utilized for viewing the predicted model.

Model Refinement and Validation

The predicted model was subjected to ModSim server for Molecular Dynamics study for the optimization of hydrogen bonding network and energy minimization. The optimized 3-D structure of the model was assessed by Structural Analysis and Verification Server (SAVES) (<http://nihserver.mbi.ucla.edu/SAVES>) and Rampage (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) which determines the stereo chemical aspects along with main chain and side chain parameters with comprehensive analysis (Lovell *et al.*, 2003). Finally, the model quality assessment program ProQ (Wallner and Elofsson, 2003) has been used to evaluate the 3D model.

Functional Characterization of the Predicted Structure

Functional assessment of the predicted model was done by searching against a Pfam database and the active site residues of the modeled MC1R protein (54-105 region) of *P. reticulata* was assessed by CASTp server (Dundas *et al.*, 2006).

Docking and Ligand Prediction

Alpha-Melanocyte Stimulating Hormone (α -MSH) was searched at the Protein Data Bank (www.pdb.org). PDB file, 1BOQ (Giblin *et al.*, 1998) was retrieved to dock the MC1R protein (54-105 region) of *P. reticulata*. The SwarmDock server was used for docking (Torchala *et al.*, 2013). The modeled 3D structure was submitted to the coach server (Yang *et al.*, 2013) to predict probable ligands.

Results

Primary Structure Analysis

The MC1R gene of *P. reticulata* has 969 bp and contained a 966 bp ORF, which encodes 322 amino acids. PSI-BLAST showed that MC1R belonged to the G-Protein Coupled Receptor (GPCR) family. MC1R of *P. reticulata* shared 98, 91, 90, 89, 84 and 78% identities with MC1Rs from *Xiphophorus maculatus* (GenBank number ABI34468.1), *Nothobranchius furzeri* (AEJ87154.1), *Scophthalmus maximus* (ACN38801.1),

Acanthamblemaria exilispinus (AET22614.1), *Tetraodon nigroviridis* (AAQ55176.1) and *Carrasius auratus* (BAJ83471.1) respectively. Multiple alignments of MC1R with other fish MC1Rs indicated that the MC1R of *P. reticulata* and *X. maculatus* were the most similar (Fig. 1). A conserved domain search revealed that MC1R contains three domains. The first domain (residues 54-105) is a Serpentine type 7TM GPCR chemoreceptor Srsx domain of 52 amino acid residues at the N-terminal of MC1R of *P. reticulata*. Srsx is a solo family amongst the superfamilies of chemoreceptors. The second domain (residues 65-269) is a 7 transmembrane receptor of Rhodopsin family containing 204 amino acid residues and the third domain (residues 142-264) is the G-protein-coupled chemokine receptor like protein of 122 amino acid residues at the C-terminal of MC1R of *P. reticulata* (Fig. 2).

Predicted mutations in the MC1R protein sequence using MSBAR showed an insertion of lysine at the 51st position of the amino acid residue between leucine and alanine, deletion of asparagine at the 28th position between threonine and serine. Various block mutations were also predicted between 258 and 259 amino acid residues in the following sequence: Histidine, leucine, alanine, cysteine, tryptophan, glutamine, glycine and alanine and between 267 and 268 amino acid residues with isoleucine, leucine and isoleucine (Fig. 3a-c).

A phylogenetic tree was constructed based on the MC1R sequence retrieved from GenBank to investigate the evolutionary relationship among the MC1Rs of different species. The results indicated that MC1R of *P. reticulata* and other fish were separated from those of reptiles and mammals and were grouped into one large cluster (Fig. 4).

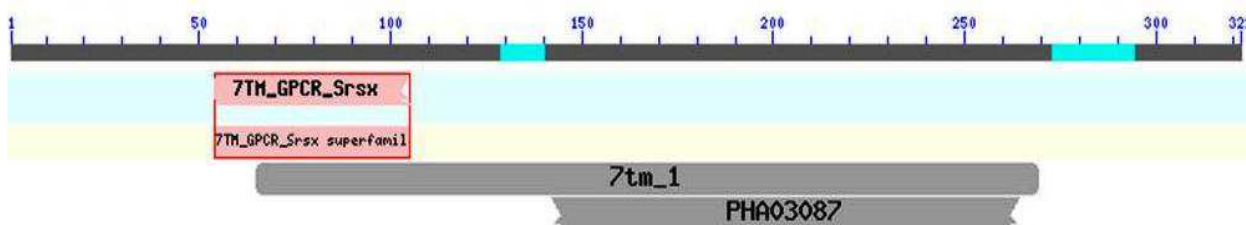


Fig. 2. *P. reticulata* showing three MC1R domains: 7TM_GPCR_Srsx domain, 7tm_1 and PHA03087 domain

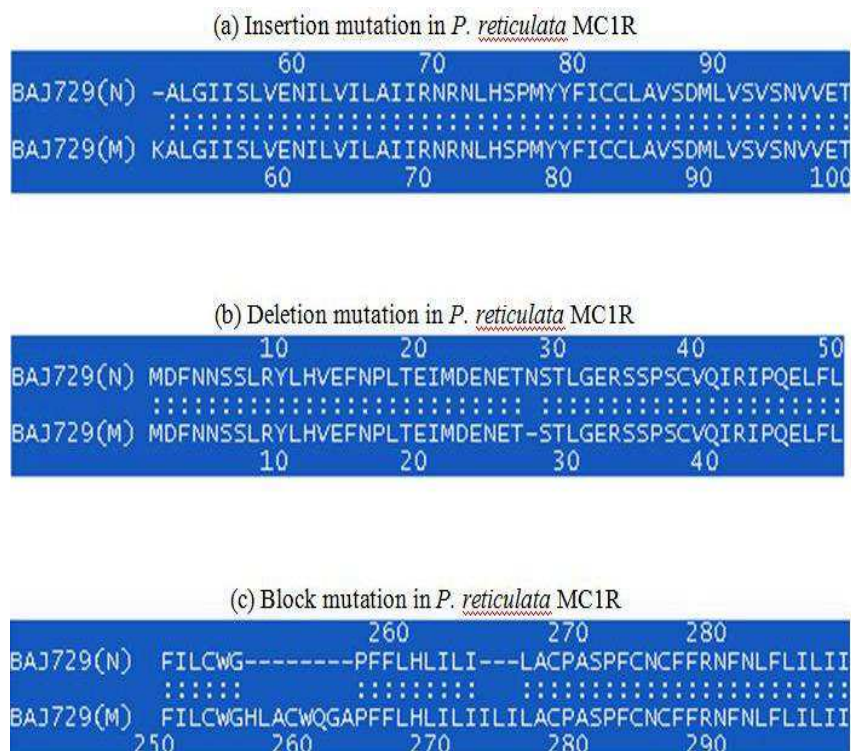


Fig. 3. Predicted mutations in *P. reticulata* MC1R using MSBAR (N-normal; M-mutated)

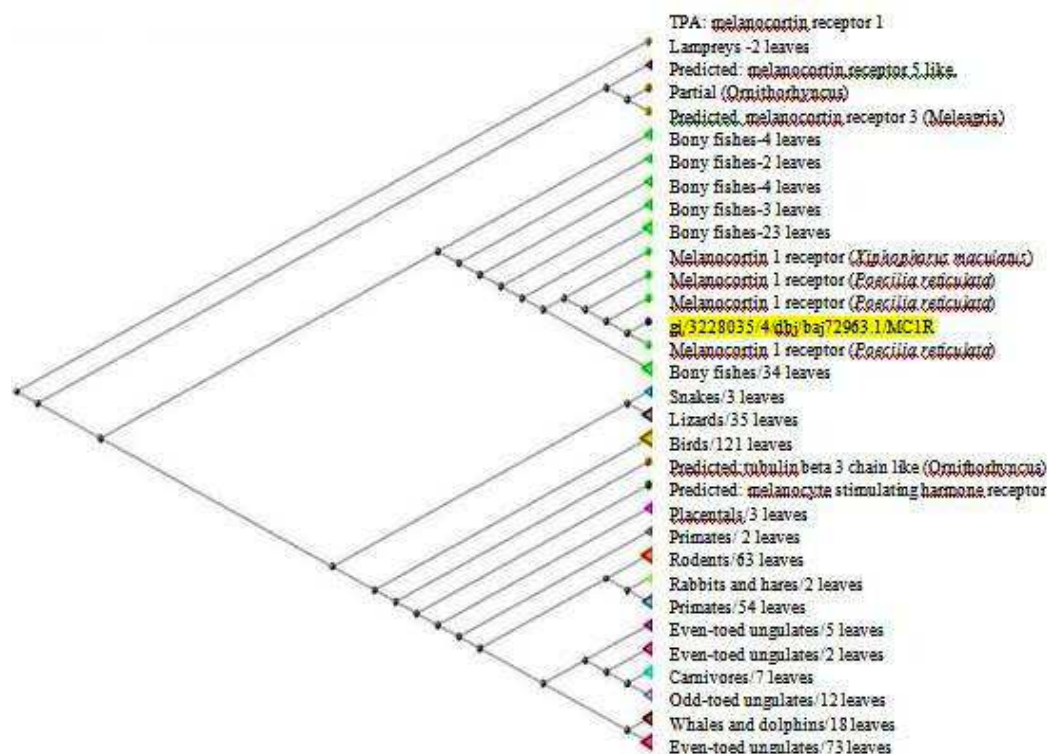


Fig. 4. Phylogenetic analysis of *P. reticulata* MC1R and other animal species from BLAST alignments. The neighbour-joining method was used to construct the tree

The MC1R protein was predicted to have a molecular weight of 36.58 kDa. The theoretical pI was found to be 6.84. The subcellular localization prediction using HumPLOC v 2.0 server predicted that the MC1R protein is a membrane protein and localized in the plasma membrane. Furthermore, TMPred server predicted that the sequence positions 55-70, 74-92, 94-114, 125-151, 172-190, 211-232, 237-258 and 286-305 are the probable transmembrane helix regions of the protein.

Secondary Structure Prediction

Secondary structure prediction of the entire sequence of MC1R protein using SOPMA (with default parameters) showed the protein having the composition of Helix = 47.24%, Strand = 23.76%, Beta turn = 8.29% and Coil = 20.72%. As evident from this secondary structure prediction, MC1R protein is mostly comprised of alpha helices, extended strands and coils with traces of beta turns (Table 1).

BLASTP analysis (<http://www.ncbi.nlm.nih.gov>) with the entire amino acid sequence showed good alignment to the MC1R protein (54-105 region) of *P. reticulata*. Hence, the similarity was restricted to amino acid residues ranging from 54-105 in the 7TM_GPCR domain of MC1R protein. Since, structures of the *P. reticulata* Melanocortin 1 receptor protein have not been determined, a Multiple Sequence Alignment (MSA)

was carried out between the residues 54-105 of MC1R protein sequence. Crystal structure of Adrenergic G-Protein coupled receptor, PDB: 3V2W showed good alignment to the MC1R protein (54-105 region) of *P. reticulata* with different homology percentages (Total 67.31% identity, TM 67.65% identity, loops 60.00% identity) are represented in the Table 2. 3V2W was predicted as the best template for comparative or homology modeling.

The 3D structure of the 54-105 region of MC1R protein of *P. reticulata* was generated using ModSim. Out of 10 models predicted, a PDB model with a low Discrete Optimized Protein Energy (DOPE) score (-0330) was selected for further studies. The predicted 3D structure of the 54-105 region of MC1R protein of *P. reticulata* mainly comprised of alpha helices, loops, extended strands and with traces of beta turns. The transmembrane regions comprised of two helices, namely $\alpha 1$ and $\alpha 2$, ranging from amino acid residues 59-69 and 86-97 respectively. The extracellular region is composed of two loops ranging from amino acid residues 54-58 at the NH₂ terminal (N-terminal) and 88-105 at COOH terminal (C-terminal). The intracellular region of the receptor contains a beta turn, comprised of amino acid residues 71-73 which connects the $\alpha 1$ and $\alpha 2$ helices by means of a loop, positioned at the amino acid sequence number 70 and from 74-75 of the receptor (Fig. 5).

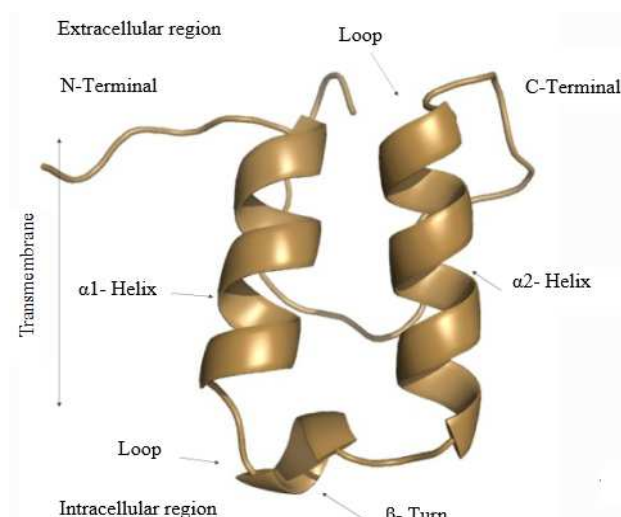


Fig. 5. Predicted 3D model of the MC1R protein (54-105 region) of *P. reticulata*. This model is based on ClustalW alignment and was generated using the ModSim server, viewed in PyMol

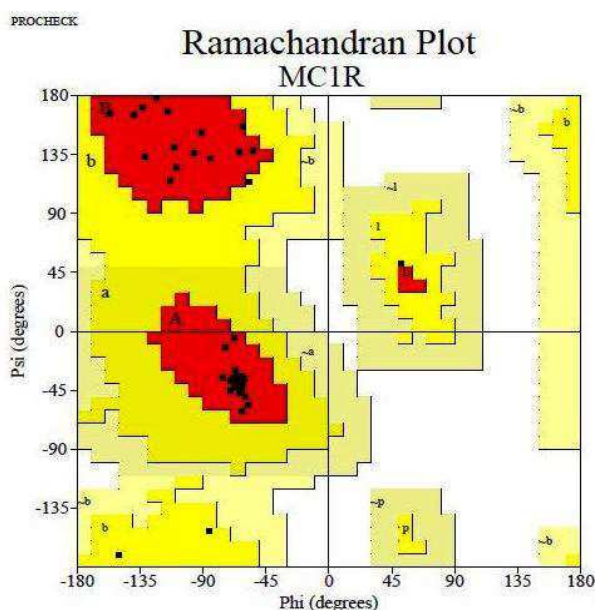


Fig. 6. Ramachandran plot of the predicted 3D structure of MC1R protein (54-105 region) of *P. reticulata* by saves server

Protein Model Validity

The geometrical and structural consistency of the predicted model was evaluated by different approaches. The Φ and Ψ distributions of Ramachandran plot analysis using PROCHECK revealed that 91.8 and 8.2% amino acid residues are present in the core regions, most favored (red in the Figure) and additionally allowed regions (yellow in the Figure) and no (0.0%) residues in the generously allowed and

disallowed regions (white in the Figure) (Fig. 6). This is in conformity to the rampage server, where 98.0 and 2.0% of the residues were observed in the favored and allowed regions and there were no (0.0%) residues in the disallowed regions (out of the core regions) of the Ramachandran plot (Fig. 7).

The predicted 3D model of MC1R protein (54-105 region) of *P. reticulata* showed the predicted LG score: 6.579 (>4: Extremely good model) and MaxSub score: 0.939 (>0.5: Very good model) were in acceptable range of a good model.

Functional Characterization of the Predicted Structure

Functional assessment of the ligand binding sites (pockets) of the predicted model was assessed by CASTp server. Out of 8 predicted structural pockets, the pockets with volume > 100 Å³ were reported. Pocket 1 (green) and Pocket 2 (blue) is located in cavity between $\alpha 1$ and $\alpha 2$ helices of the 54-105 region of MC1R protein (Fig. 8).

The best pocket site was selected on the basis of area and volume of the protein active site. The area and volume of the predicted model structure active site is given in Table 3, for a blue and green-colored domain.

Docking and Ligand Prediction

The Alpha-Melanocyte Stimulating Hormone (α -MSH) consists of 10 amino acids. The 3D structure contains the amino acid, cysteine at the N-terminal and Valine at the C-terminal regions of the peptide hormone. Extracellular access to the binding pocket is facilitated through the N-terminal region and extracellular loops of the receptor. Access is gained by the ligand, α -MSH entering laterally between α -1 and α -2 helices within the transmembrane region of the receptor (Fig. 9).

The ligand-receptor interaction was predicted between the amino residues 7-59 (Cysteine-Glutamic acid), 9-88 (Proline-Aspartic acid), 8-91 (Lysine-Valine) and 3-98 (Histidine-Glutamic acid) (Fig. 10).

The 3D structure predicted two more ligands namely CA (Ca²⁺ ions) and CLR (Cholesterol). Ca²⁺ ions was found to be complexed with amino acid residue serine 75 at the intracellular region of the receptor (Fig. 11).

The ligand CLR was bound to the $\alpha 1$ helix of MC1R protein (54-105 region) at amino acid alanine 66 of the C1R receptor. The binding of CLR to the transmembrane region has predicted the presence of CRAC domain (Cholesterol Recognition/interaction Amino acid Consensus sequence) ranging from the residues downstream, 63-69 (VILAIR) within the transmembrane region of the receptor. The amino acid, Valine at the 66th position is non polar followed by 5 types of amino acids residues and then the basic amino acid, Arginine at the 69th position (Fig. 12).

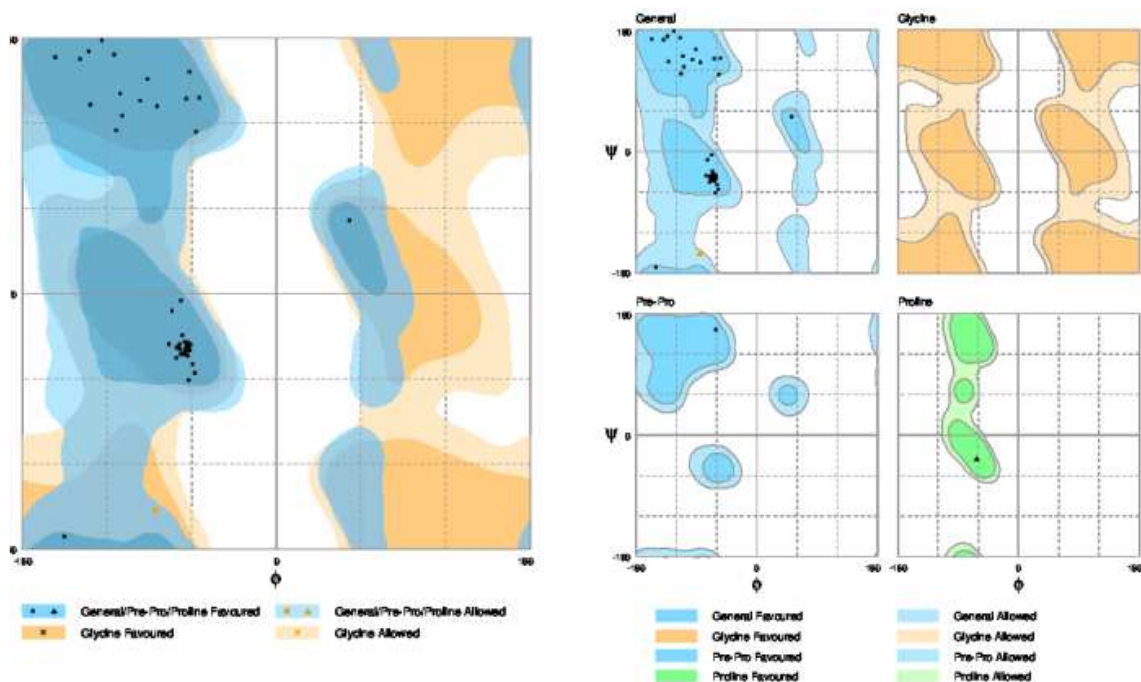


Fig. 7. Ramachandran plot of the predicted 3D structure of MC1R protein (54-105 region) of *P. reticulata* by rampage server

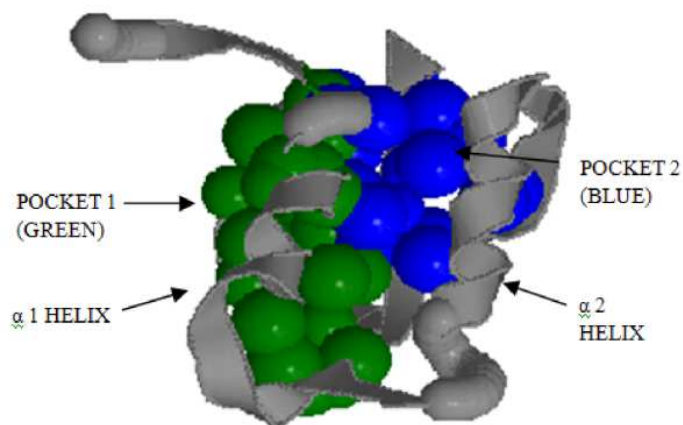


Fig. 8 Active site prediction of the predicted 3D structure of MC1R protein (54-105 region) of *P. reticulata* by CASTp server

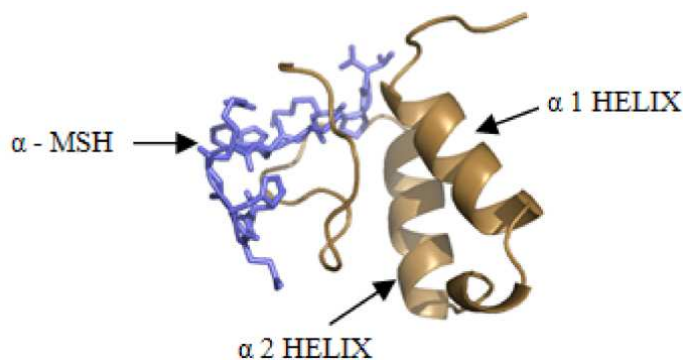


Fig. 9. Alpha Melanocyte Stimulating Hormone (PDB: 1BOQ) binding to the MC1R protein (54-105 region) of *P. reticulata*, viewed in PyMol (Predicted by Swarm Dock Server)

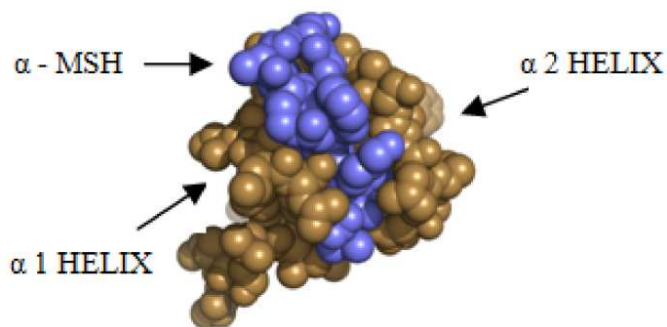


Fig. 10. α -MSH- MC1R protein (54-105 region) complex of *P. reticulata*

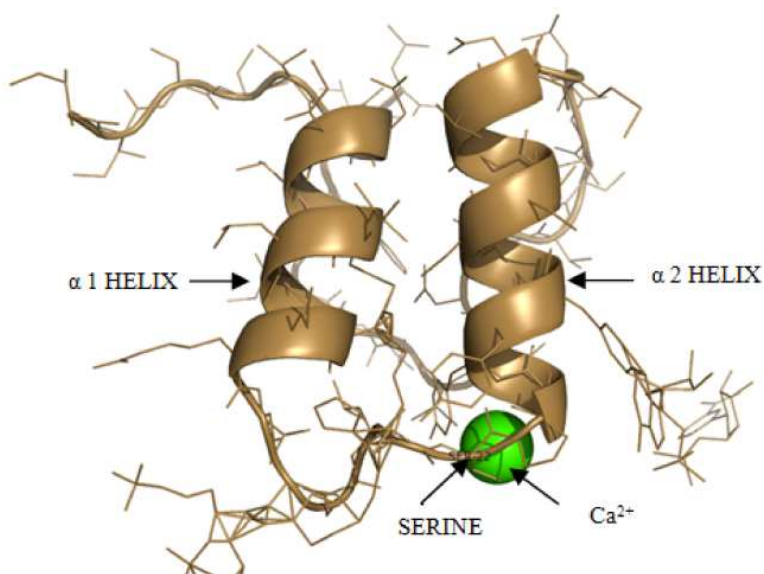


Fig. 11. Ca^{2+} ion binding to MC1R protein (54-105 region) of *P. reticulata* (Predicted by coach server)

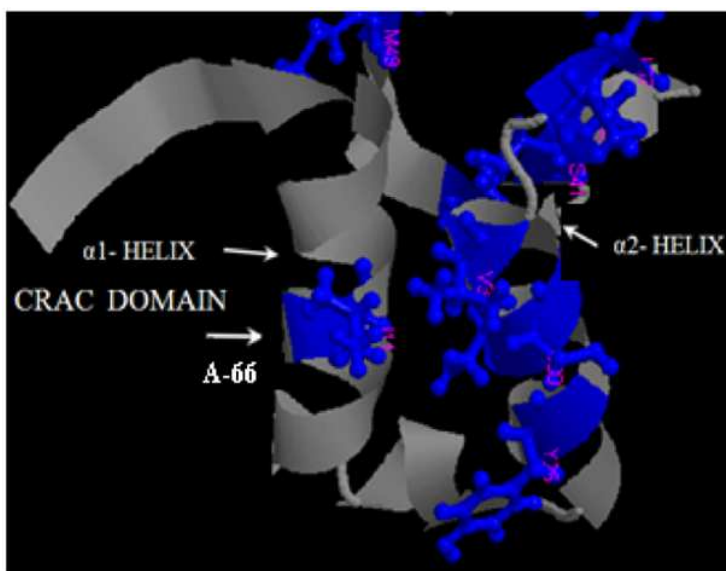


Fig. 12. Cholesterol (CLR) binding to the CRAC region of MC1R protein (54-105 region) of *P. reticulata* (predicted by coach server)

Table 1. Secondary structure prediction of *P. reticulata* using SOPMA

| Protein structure unit | No. of amino acids | Percentage of structural unit |
|------------------------|--------------------|-------------------------------|
| Alpha-helix (Hh) | 171 | 47.24 |
| 310-helix (Gg) | 0 | 0.00 |
| Pi-helix (Ii) | 0 | 0.00 |
| Beta-hridge (Bb) | 0 | 0.00 |
| Extended-strand (Ee) | 86 | 23.76 |
| Beta-turn (Tt) | 30 | 8.29 |
| Bend-region (Ss) | 0 | 0.00 |
| Random-coil (Cc) | 75 | 20.72 |
| Ambiguous states | 0 | 0.00 |
| Other states | 0 | 0.00 |

Table 2. ModSim report of MC1R protein of *Poecilia reticulata*

| | Total idents | Total % idents | TM % idents | Loops % idents |
|-------------------|--------------|----------------|-------------|----------------|
| 1u19-bRhoR | 12 | 23.08 | 29.41 | 13.33 |
| 2rh1-hB2R | 15 | 28.85 | 41.18 | 6.67 |
| 2vy4-tB1R | 14 | 26.92 | 38.24 | 6.67 |
| 2z73-sRhoR | 11 | 21.15 | 26.47 | 13.33 |
| 3eml-hA2aR | 16 | 30.77 | 35.29 | 26.67 |
| 3odu-Hcxcr4-IT1t | 14 | 26.92 | 35.29 | 13.33 |
| 3oe0-hCXCR4-CVX15 | 13 | 25.00 | 35.29 | 6.67 |
| 3pbl-hD3R | 16 | 30.77 | 41.18 | 13.33 |
| 3rze-hH1R | 15 | 28.85 | 35.29 | 20.00 |
| 3uon-hM2R | 16 | 30.77 | 38.24 | 20.00 |
| 3v2w-hSP1R | 35 | 67.31 | 67.65 | 60.00 |
| 4daj-Rm3r | 16 | 30.77 | 41.18 | 13.33 |
| 4djh-hKOpR | 16 | 30.77 | 44.12 | 6.67 |
| 4dkl-hMOpdR | 13 | 25.00 | 35.29 | 6.67 |

Table 3. Predicted structural pockets showing area and volume within the MC1R protein (54-105 region) of *P. reticulata*

| Pocket | Area, A ^{o2} | Volume, A ^{o3} | Residuse |
|-----------|-----------------------|-------------------------|---|
| 1 (Green) | 161.8 | 162.6 | 59, 62, 63, 66, 67, 73, 78, 95, 97, 98, 99, 100, 101, 102 |
| 2 (Blue) | 127.4 | 109.7 | 60, 81, 84, 85, 91, 94 |

Discussion

Coloration and pigmentation of ornamental fish is of primary importance in their culture and breeding. Fish skin coloration is mainly attributed to the presence of chromatophores that contain pigments like melanins, pteridines, purines and carotenoids (Chatzifotis *et al.*, 2005). MC1R is a membrane bound-receptor, when it is active signals the melanocyte to produce eumelanin, whereas low activity of MC1R leads to production of pheomelanin or an absence of melanin synthesis (Jackson, 1997). Since, MC1R structure of *P. reticulata* was not available in the protein data bank, an attempt was made to predict and elucidate its structure using bioinformatic tools. This is the first study of its kind to unravel the structural implications of MC1R with its biological function.

MC1R of *P. reticulata* belongs to the GPCR family and it contains three domains: 7TM GPCR chemoreceptor Srsx at the N-terminal, 7 transmembrane receptor and the G-protein-coupled chemokine receptor like protein at the C-terminal. The 7TM GPCR motif is a

structural motif found in *Caenorhabditis elegans* involved in chemoreception mediated by the members of the seven-transmembrane G-protein-coupled receptor class (7TM GPCRs) of proteins which are serpentine. Srsx is a solo family amongst the superfamilies of chemoreceptors. These genes are unusually clustered on chromosomes, both within and between families and are enigmatically concentrated on the large chromosome V (Robertson and Thomas, 2006). MC1R is involved in various physiological processes in vertebrates. Melanocortins are pituitary peptide hormones including adrenocorticotropin and melanocyte-stimulating hormones. In mammals and birds, MC1R is involved in pigmentation and expressed in melanocytes and melanoma. Activation of MC1R leads to eumelanin production as well as to proliferation and survival of melanocytes in the epidermis (Selz *et al.*, 2007).

Animals recognize a wide variety of chemicals using senses of taste and smell. The nematode *C. elegans* has only fourteen types of chemosensory neurons but it detects several stimuli. The G-protein-coupled receptor family contributes to this functional diversity. A single

type of chemosensory neuron can potentially express four different receptor genes. These genes might encode receptors for water-soluble attractants, repellents and pheromones (Troemel *et al.*, 1995). The 7 transmembrane receptor have been considered to be typical members of the rhodopsin superfamily. They share several motifs, mainly the seven transmembrane structure similar to that of the GPCRs, but are distinguished by a lysine residue that is a retinal binding site in the seventh helix. All opsins bind a chromophore, such as 11-cis-retinal. The function of most opsins other than photoisomerases are light absorption and G-protein activation whereas photoisomerases are not coupled to G-proteins but they are thought to generate and supply the chromophore that is used by visual opsins (Terakita, 2005).

The present study shows various Indel (insertion and deletion) and block mutations in the MC1R protein sequence of *P. reticulata* which could change pigmentation patterns and influence adaptive radiation as reported earlier in other teleosts (Joshua *et al.*, 2009; Tezuka *et al.*, 2011). Though, the consequences of the insertion, deletion and block mutations in the melanocortin 1 receptor of *P. reticulata* have not been reported in the present investigation, several studies have highlighted various alterations in melanin synthesis and pigmentation like depigmentation in cavern teleosts (Joshua *et al.*, 2009) and disturbances in black pigment production in *P. reticulata* (Tezuka *et al.*, 2011).

The phylogenetic tree analysis identified that MC1R of *P. reticulata* was clustered with MC1Rs of other fishes. MC1R of *P. reticulata* showed the highest identities with the MC1R of other teleosts like *Xiphophorus maculatus*, *Nothobranchius furzeri* and *Scopthalmus maximus*. The molecular and evolutionary analysis of MC1R from three major fish models, the zebrafish *Danio rerio*, the medaka *Oryzias latipes* and the platyfish *Xiphophorus maculatus* has been conserved as a single copy gene in divergent fish species. Protein sequence comparison between fish and mammalian MC1R revealed a remarkable concordance between evolutionary and functional analyses for the identification of residues and regions critical for receptor function (Selz *et al.*, 2007).

The MC1R protein was predicted to have a molecular weight of 36.58 kDa and the theoretical isoelectric point (pI) of 6.84, indicating that the protein is negatively charged. The MC1R protein is a membrane protein and localized in the plasma membrane. Furthermore, TMPred server predicted that the sequence positions 55-70, 74-92, 94-114, 125-151, 172-190, 211-232, 237-258 and 286-305 are the probable transmembrane helix regions of the protein. The secondary structure prediction of the entire sequence of MC1R protein is mostly comprised of alpha helices, extended strands and coils with traces of beta turns.

Melanocortin receptors belong to the seven-transmembrane (TM) domain proteins that are coupled to G-proteins and signaled through intracellular cyclic adenosine monophosphate. Many structural features conserved in other G-Protein Coupled Receptors (GPCRs) are found in the melanocortin receptors (Yingkui, 2011). The melanogenic actions of the melanocortins are mediated by the melanocortin-1 receptor (MC1R). MC1R is a member of the G-Protein-Coupled Receptors (GPCR) superfamily expressed in cutaneous and hair follicle melanocytes. Activation of MC1R by adrenocorticotrophin or alpha-melanocyte stimulating hormone is positively coupled to the cAMP signaling pathway and leads to a stimulation of melanogenesis and a switch from the synthesis of pheomelanins to the production of eumelanic pigments (Garcia-Borron *et al.*, 2005).

Interestingly, the predicted 3D model of MC1R protein (54-105 region) has the characteristic features of the GPCR family, namely Serpentine type 7TM GPCR chemoreceptor Srsx domain comprising of 52 amino acid residues at the N-terminal of MC1R of *P. reticulata*. Srsx is a solo family amongst the superfamilies of chemoreceptors. The extracellular region is mostly composed of loops and extended strand. The transmembrane regions contains two helices namely $\alpha 1$ and $\alpha 2$ helix followed by the intracellular region which has loops and a beta turn. However, a number of distinguishing characteristics are associated with the ligand binding pocket in the MC1R receptor. Extracellular access to the binding pocket is facilitated through the N-terminal region and extracellular loops of the receptor. Access is gained by the ligand, α -MSH entering laterally between α -1 and α -2 helices within the transmembrane region of the receptor. Similarly, T4-lysozyme gained access laterally between the helices I and VII of the transmembrane region of the sphingosine 1-phosphate receptor (Hanson *et al.*, 2012).

The structure-activity relationship is well demonstrated in the current investigation, where α -MSH acts as an agonist and reacts with the 54-105 region of MC1R protein of *P. reticulata*, which may result in a conformational change of the receptor due to various chemical reactions such as activation of G-protein coupled receptor within the intracellular regions of the receptor. The mechanism of melanogenesis which includes a series of chemical changes, Alpha-MSH molecules binds with the MC1R on melanocytes (pigment producing cell) to activate the production of melanin. Melanin granules are then deposited in packages called melanosomes and transported to the ends of the melanocyte projections, called dendrites. The tips of these dendrites are then covered by nearby keratinocytes into which the melanin granules are expelled. These spread out to

form a pigmented, protective barrier over the keratinocyte's nucleus (Hedley *et al.*, 1998). The melanin also protects the cells from UV damage by absorbing, reflecting and refracting light. However, α -MSH must be able to bind to the MC1R to achieve this function. In some fairer skin types and in individuals with mutations or damage to the MC1R is significantly impaired or absent (Bohm *et al.*, 2005).

The Ca^{2+} ion complexed to the intracellular loop region of receptor is indicative of its possible role in melanogenesis as a second messengers in the signaling pathway situated at the intracellular/cytoplasmic region. Calcium is a versatile signal that regulates many cellular functions (Berridge *et al.*, 2000), including cell survival and apoptosis (Orrenius *et al.*, 2003). The role of Ca^{2+} ions was well demonstrated by the application of calcium probes to elicit the aggregation of melanosomes in melanophores of the tilapia fish in culture (Oshima *et al.*, 1988). Similar kind of motile responses and intracellular changes of Ca^{2+} ions was also reported by Toyohara and Fujii (1992). They further concluded that an increase in the concentration of Ca^{2+} ions induces the aggregation of melanosomes in tilapia melanophores in culture. Thus, it is quite evident that in many teleost chromatophores the increase in Ca^{2+} ions is deeply involved in the aggregation of pigmentary organelles at the intracellular environment. Yamada and Fujii (2002) substantiated that in chromatophores, changes in the intracellular level of Ca^{2+} come at the expense of intracellularly deposited Ca^{2+} ions and it is independent of the extracellular concentration of the ions. Motile responses of the melanophores are mostly mediated by G-protein linked membrane receptors (Fujii *et al.*, 2000).

Cholesterol Recognition/interaction Amino acid Consensus sequences are generally referred as CRAC domains (Li and Papadopoulos, 1998). Previous molecular modeling studies have shown that the CRAC motif belonging to TM domains can have a good fit for cholesterol. In the present study, the ligand CLR was shown to be bound to the transmembrane region of the receptor and this indicates the significance of integral cholesterol in membrane proteins. The CLR bound to the receptor lies between the amino acid residues 63-69 (VILAIIR). This is apolar and basic and falls into the CRAC domain/motif of the cholesterol binding region of membrane proteins. The 5th TM domain of the human type 3 somatostatin receptor contains a CRAC domain which lies between the amino acid residues 221-231 and has the following sequence: Vlcyllylvvk which fulfilled the CRAC algorithm. Baier *et al.* (2011) further interpreted the interaction between cholesterol and CRAC domain complex, which involves essentially five residues, four of which belong to the CRAC motif (V-221, C-225, L-228 and I-229) and the fifth remaining outside (K-232). Pucadyil and

Chattopadhyay (2006) studied the effect of cholesterol depletion on ligand-binding characteristics of a few receptors in membranes. The thermal stability of both the oxytocin receptor and the beta-2AR is improved in the presence of cholesterol and CHS, respectively (Gimpl and Fahrenholz, 2002). The oxytocin receptor, cholesterol or cholesterol analogs that enhance thermal stability also shift the receptor to the high-affinity agonist binding state, implying allosteric modulation by cholesterol.

Cholesterol is a polycyclic amphipathic molecule derived from the sterane backbone (Fantini and Barrantes, 2009). Cholesterol plays a well established regulatory role in a number of membrane proteins, either indirectly through its ability to modulate the physical properties of lipid membranes, or directly through specific interactions with select proteins (Lee, 2004). Its polar section is restricted to a single hydroxyl (OH) group which can form two distinct types of hydrogen bond (acceptor and donor) with a polar group belonging to either a membrane lipid or a protein. Common molecular mechanisms deal with the interaction of transmembrane proteins with cholesterol. These mechanisms include the delineation of apolar area to accommodate the OH group of cholesterol at the membrane-water interface and the establishment of numerous vander Waals interactions in the apolar zone of the membrane (Epan, 2006; Baier *et al.*, 2011).

It is quite clear from the present study that, the binding sites of the 54-105 region of MC1R protein of *P. reticulata* for cholesterol may have distinct biological functions in bringing together the transmembrane domains, cholesterol could exert a condensing effect on the receptor which may help to acquire some of its functional characteristics like accommodation of ligands into the receptor pockets. It has also been reported that cholesterol may play an important role in GPCR function and pharmacology (Pucadyil and Chattopadhyay, 2006).

Conclusion

The predicted structure of the 54-105 region of MC1R protein of *P. reticulata* outlined for the first time in the present study provides a detailed view of structure-activity relationship between the receptor and ligands resulting in the modulation of various cell responses specifically pertaining to skin coloration in ornamental fish. Further, the scope and significance of GPCR is well established in human beings for drug targeting and consequent medical applications. This study opens up new avenues and horizons for exploring the GPCR domain of *P. reticulata* by developing new and innovative ligands for enhancing body coloration, drug delivery and high throughput in the ornamental fish industry.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all the other authors have read and approved the manuscript and no ethical issues involved.

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