Two dopamine D2-like receptor genes from the silkworm (Bombyx mori) and their evolutionary history in metazoans

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Dopamine is widely distributed in metazoans and is implicated in many physiological functions. Dopaminergic signaling is mediated through two classes of dopamine receptors, D1-like and D2-like. Phylogeny analysis reveals that the dopamine receptors probably appeared ahead of the cnidarian divergence, two distinct classes of dopamine receptors likely formed prior to the separation of deuterostomes and protostomes, and INDRs probably split from its ancestor before the emergence of nematodes. Two D2-like genes are closely linked on the same scaffold, and the chromosome region around D2-like gene loci show colinearity among different species within Lepidoptera. These indicate two D2-like and their adjunction genes are likely Lepidoptera-specific orthologs, and occur by gene duplication event taken place after Lepidoptera ancestor split from the common ancestor of Lepidoptera and Diptera. In silkworm, two D2-like genes were expressed in examined tissues, and encoded BmDop2R2 having all the features of D2-like receptors and BmDop2R1 being a truncated variant without the region of N-terminal to TM II. Only dopamine distinctly lowered cAMP levels in BmDop2R2-expressing cells, whereas all tested amines for BmDop2R1 had not markedly effect in pharmacological test. These suggest there is functional difference between the two genes, which are likely resulted from subfunctionalization of gene duplication.
D2-like receptors. Dop2 reduces intracellular cAMP levels in the presence of dopamine, and therefore, functions like D2-like receptors20–23.

Since the 1990s, a number of dopamine receptor genes have been identified and isolated from insects including Drosophila melanogaster14, 18, 21, Apis mellifera20, Periplaneta Americana19, Ctenocephalides felis25, Papilio xuthus17, Tribolium castaneum23, Chilo suppressalis24, 25 and Bombyx mori26. Interestingly, DmDopEcr, a GPCR receptor activated by steroid ecdysone, can be activated by dopamine to increase cAMP levels28. In honey bee, AmDop2 is widely expressed in the brain but differs from AmDop1R1 and AmDop1R2 in its expression pattern20. In red flour beetle, TcDop2 is highly expressed in both the central brain and the optic lobes, which is consistent with the role of dopamine as neurotransmitter23. In schistosomes (Schistosoma mansoni), SmDop2 is detected in the sub-integumental somatic musculature and acetabulum of larvae, and in the somatic muscles and the muscular lining of the adult caecum29. In flies, DmDop2 is expressed in the larval and adult nervous system, and is shown to regulate locomotion30. In Lepidoptera, CsDop2 gene in rice stem borer is identified and pharmacologically characterized in the recent report31, but the knowledge about D2-like receptors is still lacking. In this study, we cloned D2-like receptor genes from the silkworm, Bombyx mori, a Lepidopteran model, analyzed their evolutionary relationship to metazoan homologs, and identified their function in human embryonic kidney (HEK) 293 cells.

Results
Cloning BmDop2R1 and BmDop2R2. Comparing the amino acid homologous sequences of dopamine D2-like receptors from D. melanogaster, A. mellifera, S. mansoni and Caenorhabditis elegans, we searched the database and identified two genes in the silkworm genome. Abiding by the nomenclature of D1-like subclass of arthropod (Dop1R1 and Dop1R2), in this report, one gene having higher homology with the gene of D2-like receptor of other animal was designated as BmDop2R1, while the other was designated as BmDop2R2. Then, by RT-PCR and the RACE technique we cloned the 1406 bp long BmDop2R1 cDNA sequence containing a 1323 bp long ORF that encoded a protein with 440 amino acids and a predicted molecular mass of 46.29 kDa. The BmDop2R1 gene had seven exons and six introns (Fig. 1a, Supplementary Fig. S1). The cloned cDNA sequence of BmDop2R2 was 1636 bp long and contained a 1596 bp ORF that encoded a protein with 536 amino acids with a predicted molecular mass of 59.67 kDa. The BmDop2R2 gene contained eight exons and seven introns (Fig. 1a, Supplementary Fig. S1). Using the online domain prediction at EMBL, we found a G-protein coupled receptors a predicted molecular mass of 59.67 kDa. The BmDop2R2 gene contained eight exons and seven introns (Fig. 1a, Supplementary Fig. S1).

Analyses of amino acid sequences revealed that amino acid sequences were conserved mainly in the trans-membrane domains while most differences among species occurred in the amino termini and the non-transmembrane domains (Fig. 2). BmDop2R2 protein contained seven hydrophobic transmembrane

Figure 1. (a) The structures of BmDop2R1 and BmDop2R2 genes. Exons are represented by black boxes and the intron length is represented by lines. (b) Amino acid homology scores among insect Dopamine D2-like receptors. GenBank accession numbers of the proteins used are listed in Supplementary Table S1.

Sequence Analysis. Using the BLAST tool at NCBI we searched the homologs of D2-like receptor genes in the genome databases of insects, and found two D2-like receptor genes in Plutella xylostella (Lepidoptera, Plutellidae), Amyelois transitella (Lepidoptera, Pyralidae) and Papilio machaon (Lepidoptera, Papilionidae), but only one D2-like receptor gene in other species including A. mellifera (Hymenoptera), Tribolium castaneum (Coleoptera), D. melanogaster (Diptera, Drosophilidae), Culex quinquefasciatus (Diptera, Culicidae), Aedes aegypti (Diptera, Culicidae), Anopheles gambiae (Diptera, Culicidae), Musca domestica (Diptera, Muscidae), and Ceratitis capitata (Diptera, Tephritidae). Thus, Lepidoptera D2-like receptor had two subclass, Dop2R1 and Dop2R2, corresponding to BmDop2R1 and BmDop2R2, respectively. But the similarity of amino acid sequences within Lepidoptera was higher than that among species (detail in Fig. 1b). Unexpectedly, though the nucleotides of two D2-like genes of P. xylostella shared only 83% homology, the amino acid sequences coded by both genes were identical with BmDop2R1. Interestingly, the receptor genes identified in T. castaneum, C. quinquefasciatus, and A. aegypti genomes showed great divergence from BmDop2R1 and BmDop2R2. However, the receptor genes of A. mellifera and A. gambiae were similar to BmDop2R1 and BmDop2R2 (detail in Fig. 1b). For example, the A. aegypti D2-like receptor gene shared 45% amino acid similarity with BmDop2R1 and 29% with BmDop2R2, whereas the A. gambiae D2-like receptor gene was 46% and 48% similar to BmDop2R1 and BmDop2R2, respectively (Fig. 1b).

Analyses of amino acid sequences revealed that amino acid sequences were conserved mainly in the trans-membrane domains while most differences among species occurred in the amino termini and the non-transmembrane domains (Fig. 2).
domains (TM I-VII) with 3 intracellular loops and 3 extracellular loops (Fig. 2, Supplementary Fig. S2), indicating that BmDop2R2 belonged to the GPCRs family. BmDop2R1 protein had only five hydrophobic trans-membrane domains, and was a truncated variant lacking N-terminal region and the first to second trans-membrane domains (TM I-II) (Fig. 2, Supplementary Fig. S2). However, BmDop2R1 shared structural identity/similarity with other insect D2-like receptors and contained a relatively short cytoplasmic C-terminal tail and a relatively long third intracellular loop (Fig. 2), which were characteristics of invertebrate and mammalian D2-like receptors. In addition, the insect D2-like receptors along with BmDop2R1 and BmDop2R2 also contained several highly conserved sequence motifs and amino acid residues (Fig. 2). For example, the aspartate residue (D) in TM III was predicted to bind the amine group in catecholamines such as dopamine; the serine residues (S) in TM V may form hydrogen bonds with the hydroxyl groups in dopamine; the DRY sequence located at the end of TM III was involved

Figure 2. Comparison of amino acid sequences of silkworm dopamine D2-like receptors (BmDop2R1 and BmDop2R2) with Apis Dop2, Drosophila Dop2 and Tribolium Dop2. GenBank accession numbers of the proteins used are shown in Supplementary Table S1. Putative transmembrane domains (TM I-TM VII) are indicated by bold black lines. PKC phosphorylation sites (▼) and potential N-glycosylation sites (●) are indicated. IL represents intracellular loop and EL represents extracellular loop. Red box indicates the ligand-binding site.
in receptor activation; the characteristic CW-x-PFF in TM VI was critical for interaction with the aromatic ring of dopamine; and the conserved NPVIY in TM VII was crucial to stabilize the inactive conformation of the receptor\textsuperscript{33, 34}. Moreover, both BmDop2R1 and BmDop2R2 had a number of potential motifs for protein kinase C (PKC) phosphorylation sites and N-glycosylation sites (Fig. 2). These findings suggested that BmDop2R1 and BmDop2R2 encoded B. mori dopamine receptor and support the notion that BmDop2R1 and BmDop2R2 were within the biogenic amine receptors superfamily.

**Phylogenetic Analysis.** We constructed a phylogenetic tree of dopamine receptors based on homologous protein sequences from insects and humans (Fig. 3). The out-groups were human GPR54 protein and the silkworm FR protein belonging to the GPCRs family. All dopamine receptors were categorized into four clades. The first clade was D1-like clade, where the insect D1-like divided into two distinct subclades, Dop1R1 subclade and Dop1R2 subclade, and human D1-like (D1 and D5) appears to be closer to Dop1R1 than Dop1R2. Since Dop1R2 was unique to invertebrates it was named as invertebrate-type dopamine receptor (INDR) in the report by Mustard.J A\textsuperscript{12}. All insect D2-like clustered into the second clade containing two subclades, Lepidoptera D2-like subclade divided into two distinguishable branches (Dop2R1 branch and Dop2R2 branch) and other species D2-like subclade. Silkworm clustered with A. transitella not only in Dop2R1 branch but in Dop2R2 branch. Unexpectedly, the human D2 and D3 were incorporated into the third clade, and only the human D4 formed a independence clade.

A BLAST search (http://www.ncbi.nlm.nih.gov/Taxonomy) showed no homologous dopamine receptor gene in the genome of Amphinemnon queenslandica, an old multi-cellular animal. The genome of Hydra vulgaris contains seven sequences with homology to dopamine receptors and includes D(1 A)-, D(1C)-, D(2)A-, D(2) -, and 4-like receptors in annotation. In sea pansy, all three conventional transmitters dopamine, noradrenaline, and adrenaline were identified\textsuperscript{35, 36}. In S. mansoni, a dopamine D2-like receptor shared high homology with the dopamine receptor prototypes of mammals\textsuperscript{37}. It also contains two putative biogenic amine (dopamine, Dop-1 and Dop-2) receptors. Besides, the flatworms clearly have dopamine D1-like and D2-like receptors as evidenced by pharmacological analysis\textsuperscript{37, 38}. These suggest that biogenic amine (such as dopamine) receptors likely appeared before cnidarian divergence and that the D1-like or D2-like divergence from the ancestor took place before the separation of platyhelminthes. In C.elegans, there are six dopamine receptor genes including Dop-1 to -6 among which four were isolated and identified as D1-like or D2-like receptors\textsuperscript{39–41}. And there are four (three putative and one isolated genes) dopamine receptor sequences from its genome in the mollusk, Aplysia californica. Further, in Panulirus interruptus (Arthropoda, Malacostraca)\textsuperscript{42}, as well as in Ixodes scapularis (Arthropoda, Arachnida)\textsuperscript{43} and only three dopamine receptor genes (two D1-like and one D2-like) were identified in insect. Unexpectedly, we found two D2-like genes from the silkworm or other Lepidoptera genomes. This indicated that the numbers of dopamine receptor genes changed during the evolution of protostome. In parallel, these changes also likely occurred during the vertebrate evolution. For example, two were reported/identified in Lampetra fluviatilis (agnathans, jawless vertebrates), thirteen in Danio rerio (jawed vertebrates), seven in Alligator sinensis, six in Gallus
**gallus**, and five in mammals\(^6,^{44}\). Such increasing in the number of dopamine receptor genes likely resulted from gene duplication events followed by the selection of these duplicated genes (gene silencing).

To provide additional evidence for the evolution of dopamine receptors, we used 70 genes from 18 species across metazoa and constructed a second phylogenetic tree (Fig. 4). Since D1 (D1A) and D5 (D1B) exist in all vertebrates, they were used in this tree as a represent for vertebrate D1-like receptors. The *C. elegans* Dop5 was located at the root of the phylogenetic tree comprising two discrete clades, D1-like clade and D2-like clade (Fig. 4). The D1-like clade contained two distinct subclades. The vertebrate Dop1R2s were clustered in the first subclade with INDRs and *C. elegans* Dop4 (identified as D1-like receptor unique to invertebrates\(^{22}\)). Together with previous work\(^{12–16, 19, 20, 22, 42, 43}\), the results suggested that Dop1R2 probably as INDR splits from its ancestor before the emergence of nematodes during the protostome evolution. The *C. elegans* Dop1 (identified as D1-like receptor\(^{40}\)) as an outgroup was located at the second subclade containing the invertebrate Dop1R1 group (where invertebrate being consistent with the evolutionary relationship were incorporated) and the vertebrate D1-like group (where D1 and D5 were clustered respectively into two individual branches). The D2-like clade contained the vertebrate D2-like subclades and the invertebrate D2-like subclades. The *C. elegans* Dop2, -6 (identified as D2-like receptor\(^{22}\)), -3 (identified as D2-like receptor\(^{41}\)) and *S. mansoni* Dop2 (identified as D2-like receptor\(^{27}\)) were located at the outgroup of the invertebrate D2-like subclades. Lepidoptera D2-like were categorized into two evident branches, Dop2R1 branch and Dop2R2 branch, where the silkworm and *A. transitella* were clustered. This was alike to the manner of previous tree, implying that two subclasses of D2-like likely occurred before species divergence from their common ancestor in Lepidoptera. The vertebrate D2-like subclade comprised of two visible groups, the first group contained the vertebrate D2 branch and the vertebrate D3 branch, and the vertebrate D4 sequences appeared as an independent branches and formed a second group (Fig. 4), suggesting that vertebrate D2 and D3 could have a common ancestor who may not be the recent ancestor of vertebrate D4. Overall, the topologies of the phylogenetic tree were high similar to those in the previously constructed tree. This phylogenetic tree was rooted on putative biogenic amine (dopamine) receptor sequences from low animal as tree root are covered with pale white.

**Synten Analysis.** In this study, we found that the two D2-like genes (BmDop2R1 and BmDop2R2) were located on chromosome 18 even on the same scaffold (nscaf 2901), and there was a microRNA gene between...
them in the silkworm genome. Interestingly, the close linkage in two D2-like genes was conserved in all the Lepidopteran genomes mentioned above (Fig. 5a). Dop2R2 located at the downstream of Dop2R1 in the silkworm but at upstream of Dop2R1 in others (Fig. 5a). Observing the neighboring genes around two D2-like gene loci, there were XDH1 in upstream, APC1, CTR2 and ZNF492 in downstream in the silkworm. And there were CTR1, APC and ZNF708 in upstream, XDH1 and Krtap19-2 in downstream in *P. xylostella*; CTR1 in upstream, Krtap19-2, XDH and APC2 in downstream in *P. machaon* genome (Fig. 5a). These indicated that gene colinearity around D2-like loci existed in Lepidoptera. But such synteny did not emerge in non-Lepidopteran species.

Thus, the chromosome region including two D2-like and their adjunction genes were likely Lepidoptera-specific locus where the genes were likely Lepidoptera-specific orthologs. This view was also supported by the above phylogenetic analysis.

**Figure 5.** Synteny of D2-like receptor gene loci. (a) Synteny of D2-like receptor gene loci in Lepidoptera. The species tree (in the left) constructed by Cytochrome C is illustrated in white. The neighboring genes arounded D2-like receptor gene (in the right) are illustrated in gray. (b) Synteny of D2-like receptor gene loci in vertebrates and nematodes. Syntenies of vertebrate D2 are illustrated in red, D3 in blue-gray, D4 in yellow. The linkage of two D2-like genes in human and in a nematode are illustrated in gray.
In the different tissues. BmDop2R2 larvae, and adult thorax (Fig. 6a1,a2). These results indicated the functions of both product was detected in the ventral chain, hemolymph, testis, ovary, integument, malpighian tubule, midgut of larvae, and the thorax, abdomen, ovary and fat body of adults. A relatively weaker BmDop2R1 product was 753 bp was detected in the head, fat body, silk gland, tracheae, ventral chain, hemolymph, testis, ovary, integument, malpighian tubule, midgut of larvae, and the thorax, abdomen, ovary and fat body of adults. A relatively weaker BmDop2R1 product was detected in the larval fat body, and adult head and testis (Fig. 6a1,a2). Expression of the 660 bp BmDop2R2 product was robust in the head, fat body, silk gland, tracheae of larvae, and the head, abdomen, ovary, testis, and fat body of adults. A weaker BmDop2R2 product was detected in the ventral chain, hemolymph, testis, ovary, integument, malpighian tubule, midgut of larvae, and adult thorax (Fig. 6a1,a2). These results indicated the functions of both BmDop2R1 and BmDop2R2 in the different tissues.

Functional Analysis. In insects, five major amines, serotonin (5-hydroxytryptamine, 5-HT), dopamine, histamine, octopamine and tyramine, are involved in intercellular signalling. To determine the ligand specificity of the receptors, the HEK 293 cells expressing BmDop2R1 or BmDop2R2 were treated with five biogenic amines (1 μM or 10 μM) respectively. Control was empty pcDNA3.1 vector under the same conditions, and expression was confirmed by RT-PCR using total RNA extracted from transiently transfected cells (Supplementary Fig. S3). Since D2-like dopamine receptors could decrease cAMP levels which were low in normal cells by inhibiting adenyl cyclase activity, 10 μM forskolin was applied to stimulate adenyl cyclase to increase cAMP levels (Fig. 7) for observing clearly the effect of BmDop2R1/BmDop2R2 receptors.

No significant difference was observed in the cAMP levels among five biogenic amines in empty vector cells (Fig. 7a-d). In BmDop2R2-expressing cells, only dopamine distinctly lowered cAMP levels, which was down-regulated to 72.27% and 47.12% of the control group at 1 μM and 10 μM dopamine concentrations respectively (Fig. 7c,d). These displayed that dopamine was the most potent of the biogenic amines at activating BmDop2R2 receptors. However, all these amines did not induced a markedly change of the intracellular cAMP in BmDop2R1-expressing cells (Fig. 7a,b), or even high concentrations of dopamine (100 μM) (date not show).

For BmDop2R2 receptor, the dose-dependent effect of dopamine on cAMP levels was analyzed with concentrations ranging from 1 nM to 100 μM. The dopamine effect was concentration-dependent and saturable, resulting in a sigmoidal dose-response curve (Fig. 8). The intracellular cAMP levels was dramatically reduced at dopamine concentrations of ≥ 10 −8 M, and half-maximal activation (EC50) was achieved at a concentration of 6.57 × 10 −7 M (657 nM). When dopamine concentration was achieved at ≥ 10 −6 M, BmDop2R2 response was the
maximum (Fig. 8). These results showed that dopamine activation of BmDop2R2 receptors was responsible for down regulation of cAMP levels.

Discussion
Dopamine acts as a neurotransmitter, neuromodulator, and neurohormone in the nervous system, and depends on dopamine receptors to exert its effects in animals\(^4\),\(^5\). In sponges, several genes corresponding to postsynaptic scaffolding have been reported\(^4\),\(^5\), but there is a lack in defined neuronal cell types\(^4\),\(^5\). This is consistent with the
present study where no dopamine and other biogenic amine receptor genes are observed in the A. queenslandica genome. Homology search revealed that dopamine (biogenic amine) receptors appeared before the cnidarian divergence after the A. queenslandica split. Two distinct classes of dopamine receptors, D1-like and D2-like, are present in the phylogenetic tree and likely formed before the separation of deuterostomes and protostomes. The C. elegans Dop1 was identified as D1-like receptor unique to invertebrates is located at the out-group of invertebrate Dop1R2 branch, suggesting that Dop1R2 probably as INDR split from its ancestor before the emergence of nematodes. Previous study has been revealed that there was only one D2-like gene in the genome of arthropod including Malacostraca (as Pamulirus interruptus), Arachnida (as Ixodes scapularis) and Insecta (as D. melanogaster) and A. mellifera. However, we find two D2-like genes from the silkworm genome (Bombycidae) or other Lepidopteran genomes including platellidae (as P. xylostella), pyralidae (as A. transitella) and papilionidae (as P. machaon). Two subclasses of Lepidoptera D2-like are clustered into a branch in insect D2-like group, suggesting that the similarity in protein is higher between Lepidoptera than among other species. Further, we find that two D2-like genes are closely linked on the same scaffold, and the chromosome region around D2-like gene loci show collinearity in Lepidoptera, but not in other species including Hmenoptera (as A. mellifera), Cleoptera (as Tribolium castaneum) and Dptera (as D. melanogaster). These indicate that two D2-like genes probably emerged by a gene duplication event taking place after Lepidoptera ancestor split from the common ancestor of Lepidoptera and Dptera. Furthermore, unequal crossing over usually generates tandem duplicated genes, two D2-like genes loci in Lepidoptera are tandem arranged on one scaffold that possibly resulted from unequal crossing over.

Surprisingly, not only in Lepidoptera, there is also the vertebrates-specific collinearity related to D2-like receptor genes. Paralogous genes resulting from ancient duplications are often an indication of early syntenic regions, which was demonstrated for a large superfamily of homeobox genes. Shared gene synteny is a reliable criterion to explore orthologs derived from genomic evolution. Thus, the synteny surround D2-like genes in this study is a strong reference for comparative genomics research in Lepidoptera and/or vertebrates.

Sequence analysis shows that BmDop2R2 has the complete characteristic structure of a GPCR protein, while BmDop2R1 is a truncated variant deleting N-terminus and TM I-II of GPCR protein. Interestingly, this deletion in BmDop2R1 is shared identity/similarity with that of predicted D2-like receptors from P. xylostella genome. These imply that the variant without regions of N-terminus to TM II are widely occurred in Lepidoptera D2-like dopamine receptor. Currently, there are many reports on truncated GPCR having an absence of the sixth to seventh trans-membrane domains (TM VI-VII) and/or C-terminal region. For example, alternative splicing of the wild-type D2-like dopamine receptor DOP-3 resulted in the formation of DOP-3nf lacking TM VI-VII in C. elegans. Dop1R2B (a splice variant of INDR receptor Dop1R2A) has an alternative C-terminal domain in P. americana. Here, truncated GPCR variant lacking N-terminus and trans-membrane 1-2 domains (TM I-II) is the first report.

Truncated variants might associate with full-length receptors to modulate the signal transduction properties, which has been found in the D2-like receptor DOP-3 and its truncated splice variant Dop-3nf in C. elegans. Dop-3 attenuates forskolin-stimulated cAMP formation in response to dopamine, whereas Dop-3nf does not. When Dop-3 was co-expressed with Dop-3nf, the ability to inhibit forskolin-stimulated cAMP formation was reduced. Dop1R2B of P. americana is translated and transported to the plasma membrane but does not affect dopamine stimulation, and probably has a role to depress the capability of P. americana Dop1R2A. In this study, dopamine activating BmDop2R2-expressing cells decreases cAMP levels, whereas tyramine, octopamine, serotonin and histamine have no effect on intracellular cAMP. And the $EC_{50}$ value of 6.57 × 10$^{-7}$ M and the maximal response value of ≥10$^{-4}$ M are similar to that of the T. castaneum Dop2 (D2-like) receptor (640 nM and 100 μM) for dopamine lowering CAMP levels, respectively. Thus, BmDop2R2 is a functional D2-like dopamine receptor. In contrast, dopamine stimulation does not affect CAMP levels for BmDop2R1. However, BmDop2R1 as well as BmDop2R2 are almost ubiquitously expressed in all tissues examined by RT-PCR, and encode protein having conserved sequence motifs and amino acid residues in the third (DRY), sixth (CWLP), and seventh (NPXXY) TMs that establish affinity and/or determine efficacy for endogenous amines. Whether BmDop2R1 assemblies and impairs BmDop2R2 function will be investigated in a forthcoming investigation and is beyond the scope of this study.

Gene duplication generates functional redundancy. Two genes with identical functions are unlikely to be stably maintained in the genome, but can be retained when they differ in some aspects of their functions. Two D2-like receptors of Lepidoptera form two respectively independent branches, Dop2R1 branch and Dop2R2 branch, inside of Lepidoptera D2-like cluster, reflecting the proteins distinction which might be generated by the evolution of functional divergences for facilitating Lepidoptera-specific adaptation. Two duplicated genes via sub-functionalization interact to perform the original function that provides a mechanism for both duplicated gene copies being stably maintained in the genome. BmDop2R1 and BmDop2R2 have difference in spatial expression pattern for each other. The cAMP levels are clearly down-regulated in HEK293 cells expressing BmDop2R2 but not BmDop2R1 for dopamine stimulation. It is possible that two D2-like genes are sub-functionalized to cooperate to carry out the function of parental gene in silkworm or other Lepidoptera during evolving.

**Conclusion**

We identified and cloned two genes of D2-like receptors from the silkworm. This is different from a previous study in which only one D2-like receptor gene was found in insects and other arthropod. Our comparative genomics analyses suggested that the two genes are likely Lepidoptera-specific orthologs resulting from a gene duplication event in the common ancestor of Lepidoptera. We also found the truncated dopamine receptor deleting N-terminus to TM II region of GPCR structure for the first time, and demonstrated the function of
D2-like receptor by expression cells. Our results provide some new insights into to understanding gene function of D2-like receptors in Lepidoptera and their evolutionary history.

Methods

Silkworm strains.  Silkworm DaZao strains experimented were maintained at the Southwest University in china, and were reared under standard conditions.

mRNA isolation and cDNA synthesis.  Total RNA was purified using TRIZol reagent (Invitrogen) according to the manufacturer's instructions, and cDNA was synthesized using oligo (dT) primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega).

Cloning.  5’ RACE-ready cDNA was synthesized from the total RNA from the testis of the fifth silkworm using a GeneRacerTM Kit (TaKaRa, Dalian, China) according to the provided manual (The sequence of all primers in Supplemental Table S2). The cDNA from the heads of adults were used as templates in PCR to amplify BmDop2R1 and BmDop2R2 (Supplementary Table S2). PCR reaction conditions included 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 65 °C (BmDop2R1)/ 56 °C (BmDop2R2) for 1 min and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. PCR products were isolated and cloned into the pMD19-Tsimple vector (Takara) and sequenced (as described in ref. 51).

Semi-quantitative RT-PCR for expression analysis. Expression analysis by RT-PCR was performed using respective primers (Supplemental Table S2) for BmDop2R1 and BmDop2R2. Conditions of PCR consisted of 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 40 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. BmActin3 was used as an internal control. (as described in refs 51 and 52).

Construction of expression vectors.  The amplified products for the ORFs of BmDop2R1 and BmDop2R2 were ligated into the HindIII and NotI sites of the expression vector pcDNA3.1 (+) to produce the recombinant pcDNA3.1 (+)-BmDop2R1 and -BmDop2R2, respectively. Each insertion was confirmed by DNA sequencing.

Transfections and cAMP assays.  HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (D-MEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C with 5% CO2. Cells suspended in D-MEM containing 10% FBS were plated on 35-mm dishes 1 day before transfection. The attached cells were transfected with ~1.5 µg of the endotoxin-free plasmid in 350 µl of Opti-MEM I (Gibco) containing 3.5 µl Sinofection (Sino Biological). After incubation for 5 h at 37 °C with 5% CO2, the media was replaced with D-MEM containing 10% FBS (500 µl). And cell culture for 2 days, the culture media was removed and the drug diluted in Dulbecco’s modified Eagle’s medium was added into the stable transfected cells for incubation 30 min. The cells were washed two times with PBS (PH7.4) and lysed in 500 µl PBS by re-freezing before centrifugation at 2000 rpm for 20 min. The amount of intracellular cAMP extracted from harvested supernatant was determined using ELISA reagent (Huijia Biotech) according to the manufacturer's instructions. Each measurement was performed in duplicate and a minimum of three independent assays were carried out for each compound and concentration tested.

Multiple sequence alignment and phylogenetic analysis.  Protein sequences similar to dopamine receptors were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Multiple sequence alignments of the amino acid sequences were performed with DNAMAN and Clustalx. Transmembrane spanning domains were predicted by TMHMM (http://genome.cbs.dtu.dk/services/TMHMM/; http://smart.embl-heidelberg.de/). Phosphorylation sites and N-glycosylation sites were predicted by NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) respectively. Values for identity (ID) and similarity (S) were calculated by BioEdit. We utilized MEGA 6.0 to calculate the genetic distances among different species and to construct neighbor-joining (NJ) trees with 1000-fold bootstrap resampling.

Gene synten.  We examined the chromosomal localization of dopamine receptor genes and the neighboring genes in the available genomes of the NCBI (http://www.ncbi.nlm.nih.gov) Genome Browser.

Species trees.  Species trees in Fig. 5 was constructed using Cytochrome C protein whose accession numbers in GenBank were listed in Supplementary Table S1. Species divergence times, based on the assumption that R. mori and D. melanogaster diverged from 344.7 to 190.0 MYA 53,54, was estimated by the RelTime method.

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Conceived and designed the experiments: Ping Chen. Performed the experiments: Peng Chen, T.L., D.-F.Y., X.C., Y.L., W.Z. Analyzed the data: Ping Chen, Peng Chen, T.L., Q.S. Contributed reagents/materials/analysis tools: Peng Chen, T.L., L.Z., Q.S. Wrote the paper: Ping Chen, Peng Chen and Tian Li.

Additional Information
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