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## Abstract of Doctoral Dissertation

Degree requested Doctor of Life Science

Applicant's name: Hongdi Wang

### Title of Doctoral Dissertation

Transcriptional regulatory divergence underpinning species-specific learned vocalization in songbirds  
[鳴禽類(ソングバード)の種特異的な歌行動に関わる遺伝子発現制御の研究]

Species-specific behaviors are shaped by evolutionary forces to adapt to environment diversity. As an important factor for species co-occurrence, species-specific behavior contributes to a variety of inter- and intra-specific interactions including communication, sexual selection and predator avoidance. Species-specific behavior exists in most animal taxa, such as alarming gaits behavior between deer species, pair-banding behavior between vole species, and different courtship song behavior between *Drosophila* species. Figuring out the physiological and molecular difference in the neural systems that induced species-specific behavior between closely related animal species can help to understand how the neural mechanisms regulate behavior.

Evolutionary pressures shape organisms to produce species-specific behavior during speciation, which suggests neuronal changes exist among evolutionally closely related species. The nervous system incorporates the changes to generate novel species-specific behavior during speciation. The differences in structure and development of neural circuits among evolutionally-closely related species are the basis for species-specific behavior formation. Such structural and developmental differences can be caused by the genetic difference in closely related species. With development and application in the life science of genomics, finding out the genetic differences which induced species-specific behavior between closely related species is possible to explore the mechanisms of animal behavior on the molecular level. Genomic researches of species-specific behavior have been performed in some animal species. For example, the sin and pulse song, two features of species-specific courtship song of *Drosophila simulans* and *D. mauritiana*, were correlated with different genomic locus. In addition, quantitative trait locus (QTL) mapping found that species-specific nest digging behaviors of two closely related mice species, *Peromyscus polionotus* and *P. maniculatus*, were highly associated with a few genome regions. These two studies narrow down the candidate genomic regions by using backcross hybrid offspring between closely related species. F1 hybrids between closely related species are also useful to select candidate genomic regions related to species-specific behavior. For instance, genes related with parental nest building behavior were figured out by using biased allele expressed genes in F1 hybrid comparing with QTL mapping and species differentially expressed genes. Furthermore, the allelic expression ratio in the F1 hybrid and expression ratio between their parental species can be used to evaluate the transcription regulatory difference between closely related species. These studies suggested that genomic analysis utilizing hybrid offspring is powerful to explore genes/genome alleles which contributed to species-specific behavior formation.

The structure and development differences in the neural circuits between species are mainly caused by the expression difference of orthologous genes in conserved neural circuits. Previous research found that different expression levels of orthologous genes between closely related animal species induced species-specific behavior. For instance, the vasopressin 1a receptor (V1aR) gene show different expression levels in the ventral pallidum which induced species difference in pair-bonding behavior between monogamous (*Microtus ochrogaster*) and polygamous (*M. pennsylvanicus*) vole species. Further research found that the different length of microsatellites at the upstream of the V1aR changes the gene expression level, in turn, which generates different behavioral traits between the two vole species. These issues suggest that gene transcriptional regulatory difference which induced species different expression patterns of orthologous genes potentially contributes to the generation of species-specific behavior. Gene expression is driven by transcriptional regulatory elements which mainly contain two kinds, *cis*- and *trans*- regulatory elements. Transcriptional regulatory divergences of closely related species could induce the expression level difference of orthologous genes in the brain. Orthologous genes in closely related species are inherited from their ancestral species, while *cis*- and/or *trans*- elements may change during evolution among species. Such mutations can affect transcription

initiation, transcription rate, and transcript stability which induced species-specific expression patterns. Mutations such as single nucleotide polymorphism (SNP) and insert and deletion (indels) in the *cis*-sequence could induce expression level difference of orthologous genes. *Trans*- regulatory elements are genes that regulate the expression of distant genes through an intermolecular interaction. The different expression levels or amino acid substitutions of *trans*- regulators can drive different expression levels of their downstream genes between closely related species. However, little is known about how transcriptional regulatory divergence contributes to the generation of species-specific behavior, especially for learned behavior, by changing the expression level of orthologous genes.

There are more than 4,000 songbird species in the world and they produce species unique songs which play important roles for mating interaction and habitat declarin. Species-specific birdsongs are complex vocal signals acquired through vocal learning, which makes songbird species become an excellent model to explore the mechanism for vocal learning behavior. The species-specific songs as an important characteristic of speciation raised along with the evolution of songbird species. The species-specific songs are developed and produced by conserved neural circuits called the song pathway in the brain of songbirds. The song pathway consists of two major neural circuits: the anterior forebrain pathway (AFP) and the vocal motor pathway. There are two features of birdsong, the sound characteristics of song elements (syllables) featured as “syllable acoustic”, and the arrangement of each song syllable order featured as “syllable sequence”. The AFP contributes to song learning and vocal plasticity during song development, but it is not crucial for the production of the bird’s learned song. Species-specific songs of adult songbirds are produced by the vocal motor circuit of the song pathway. The robust nucleus of arcopallium (RA) and the song nuclei HVC (as a proper name) in the vocal motor circuit control the syllable acoustic and sequence features of birdsong, respectively. Both acoustic and sequence of the birdsong show species-specific differences, which suggests that the structure and physiological activity of RA and HVC are different among songbird species. That further meant these two nuclei could be important for determining species-specific song traits of adult songbirds. However, how the transcriptional regulatory differences of orthologous genes in the vocal motor pathway induce species differential expression which driven species-specific songs among songbird species is still unknown. Against this question, I hypothesized that gene expression difference induced by *cis*- and/or *trans*- changes in HVC and RA among songbird species is one of the potential reasons for the generation of species-specific birdsong. Identification of the transcriptional regulatory divergence which induced gene expression difference in the motor pathway can enhance our understanding of the molecular mechanisms for species-specific vocal learning behavior.

Based on the above information, I predicted that orthologous genes with different expression levels are regulated by *cis*- and/or *trans*- regulatory divergences among songbird species in HVC and RA. To verify this hypothesis, I used zebra finch (ZF; *Taeniopygia guttata*), owl finch (OF; *T. bichenovii*) and their reciprocal first-generation (F1) hybrids to analyse gene expression difference and regulatory divergence.

In Chapter I, I examined differences in song phenotypes between ZF and OF. To test whether the song difference between ZF and OF was genetically constrained, I analysed the vocal learning behavior of ZFs and OFs which were tutored by conspecific and cross-specific songs. To identify genes with potential contribution to the generation of species-specific songs, I analysed gene expression differences between ZF and OF in both HVC and RA.

In Chapter II, I analyzed the expression ratio between parental species (ZFs and OFs) of all detectable genes and the allelic expression ratio in the F1 hybrids. Depending on these two sets of values, I identified *cis*- and/or *trans*- regulatory difference between ZF and OF. To explore the biological function of genes with *cis*- and/or *trans*- regulatory divergences, I performed function analysis of genes belongs to all categories of regulatory divergence.

In Chapter III, depending on the candidate upstream regulators of *trans*-regulated different genes in RA, I performed a correlation analysis in F1 hybrids between parameters of acoustic and sequence features and ASE, or expression level of the most significant candidate *trans*-mediator, BDNF. To test the expression level of BDNF maintaining species-specific songs, I further performed pharmacological over-activation of BDNF receptor and RNA-seq (RNA sequencing) of the song nucleus RA of adult ZFs.

Based on the results obtained through a set of experiments, I will discuss the potential contribution of *cis*- and/or *trans*- regulatory differences for the generation of species-specific vocal learning behavior among songbird species.