Sexually dimorphic expression of the genes in the forebrain vocal-control nucleus RA of juvenile white-rumped munia *Lonchura striata* by mRNA differential display technique*

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Abstract Song behavior and its song-control nuclei (such as the robust nucleus of the arcopallium-RA) are highly sexually dimorphic in passerine songbirds. These dimorphisms are not present early in development, but arise through processes during sensitive periods in life. There are no sex differences in RA volume and neuron numbers of white-rumped munia at P35 (date after birth), but sexual dimorphism in RA volume became very markedly at P45. It is thought that the development of neural dimorphism is controlled by genes. In this experiment, the female and male RAs at developmental ages P35 (females only) and P45 (females and males) were studied by mRNA differential display (DD) technology. For the DDRT-PCR, combinations of the four T_{12}MN primers together with sixteen arbitrary primers, AP1-16, were used. This analysis yielded 1 158 bands in total, of which 273 bands were differentially generated. There were 161 differential bands which size was between 100 bp and 500 bp. The aim of this study was focused on the cDNAs co-expressed in P35 female and P45 male (sixty-one bands) or expressed only in female at P45 (nineteen bands), which might play a role in the dimorphic development of RA. After the second PCR amplification, sixteen differential bands were screened out, of which eleven were co-expressed in P35 female and P45 male, five were only expressed in female at P45, which might underlie the genetic mechanism of the RA dimorphic development in white-rumped munia. A differential segment named T3, which had 81% homology with Bcl complex in chichen, might be a member of Bcl-2 protein family or relate to the retaining of RA volume, was chosen to sequence and analyse. However, the information about T3 segment in detail needed further research. These results show that mRNA DD methodology can represent a potential tool for investigation of the gene expression in the brain of songbird development [*Acta Zoologica Sinica* 51 (1): 149－155, 2005].

Key words White-rumped munia, *Lonchura striata*, Dimorphism, RA, mRNA differential display, Apoptosis

应用 mRNA 差异显示技术寻找调控白腰纹鸟前脑发声控制神经核 RA 性双态性发育的基因*

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摘 要 鸣禽的发声行为及其前脑发声控制核团存在显著的性双态性，这种性双态性是在发育过程中逐步建立起来的。鸣禽白腰纹鸟 35 日龄（P35）前，雌、雄鸟前脑发声控制神经核 RA（Robust nucleus of arcopallium）不存在性别差异；到 45 日龄（P45）时，雌、雄鸟 RA 体积已经出现了显著的差异。推测这种神经结构的性双态性的形成与各种基因的不同时空表达有关。本实验以发育不同阶段（P35 时的雌性，P45 时的雌性和雄性）的雌、雄白腰纹鸟 RA 核团为研究对象，用 mRNA 差异显示银染技术来寻找前脑上述三组材料间的差异表达基因，试图发现调控 RA 核团性双态性发育的关键基因。本实验共选用了 4 种锚定引物与 16 种随机引物所组成的 64 组引物进行 DDRT-PCR（mRNA differential display and reverse transcription-polymerase chain reaction）反应。第一次扩增后，在测序胶上显示出1 158条 cDNA 扩增产物，其中 273 个为差异表达。长度分布于 100－500 bp 之间的差异

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条带共 161 条。本实验重点研究了 P35 雌性和 P45 雄性共表达或 P45 雌性单独表达的条带（共 19 条），推测这些基因片段可能与引起 RA 核团中的细胞凋亡和性双态性的形成有关。二次 PCR 扩增后，进一步筛选出 16 个差异条带，其中 11 个为 P35 雌性和 P45 雄性共同表达的，5 个是 P45 雌性特异表达的。本实验对其中一个差异片段 T3 进行了测序和分析，发现该片断对应的氨基酸序列与鸡（*Gallus gallus*）Bcl 复合体有 81% 的同源性，由此推测 Bcl-2 基因家族有可能在促进 RA 核团的细胞凋亡中发挥作用，但有关 T3 片段的具体功能还需进一步深入研究［动物学报 51（1）：149－155，2005］。

关键词 白腰纹鸟 性双态性 RA mRNA 差异显示 凋亡

The plasticity of vocal behaviour and song control system in songbirds is considered as the most outstanding model to study animal behavior, the plasticity of central neural system and the neural substrate of speech learning in humans (Nottebohm and Arnold, 1976; Nordeen and Nordeen, 1990; Bottjer, 1997; Zeng at al., 2004). In adult male passerines, singing is a prominent behavior. However, normal adult females sing rarely or not at all. Song learning and production are controlled by a system of discrete nuclei within the brainstem, midbrain, and telencephalon (Nottebohm and Arnold, 1976; Bottjer et al., 1989). Many researchers (Smith et al., 1997; Jiang et al., 1992; Li et al., 1992; Zuo et al., 1998) pointed out that there is dramatic sexual dimorphism in song behavior and its neural nuclei, that is, the volume of song nuclei, the size and number of neurons are 3 to 5 times larger in males than in females. Most of these sex differences arise through processes during sensitive periods in life, but not inherent (Johnson and Bottjer, 1992; Nixdorf-Bergweiler et al., 1995).

Some studies indicate that sex hormones play an important role in the development of song control system and vocal behavior in oscine (Arnold, 1975; Nottebohm et al., 1987; Nordeen and Nordeen, 1990; Stein, 2001). The female canary *Serinus canarius* and zebra finch *Poephila guttata* treated with testosterone during the adolescent period could produce male-typical song. Moreover, both the volume of song nuclei and the number of neurons increase markedly, almost approaching the level of males (Arnold, 1975). Similar results were obtained when administering estradiol, and the effect of estradiol on the development of song nuclei and song behavior was greater than that of androgens. These results implied that the genes controlling sex hormones exert important influence on the appearance of sexual dimorphism of song control system in songbirds. Recently, Arnold and his collaborators (Agate et al., 2003) found a rare gynandromorphic zebra finch in which the right half of the brain was genetically male and the left half genetically female. Because both halves of the brain were exposed to a common gonadal hormone environment, the hemispheric differences indicated that the genetic sex of brain cells (or genes), but not the gonadal factors, contributed to the process of sexual differentiation. Some researchers suggested that some sexual dimorphisms in the nervous system might develop under primary genetic control (Reisert and Pilgrim, 1991; Gahr and Metzdorf, 1999). At least 60% − 70% of the behavioral dimorphism is controlled by genes and 30% − 40% by hormones (Gahr and Metzdorf, 1999).

Sex difference in the morphology of the RA (Reiner et al., 2004) emerges during vocal development. In both male and female white-rumped munia *Lonchura striata*, the RA volume keeps growing from P5 (postnatal day 5, the birth day is P0) to P35. Moreover, there is not sex difference in RA volume before P35. After P35, the size of RA diverges as it increases in males and regresses in females. Female RA undergoes a significant decline in volume and neuron number while male RA substantially increases in volume with no change in neuron number. Sexual dimorphism in RA volume became very markedly at P45 (Zeng et al., 2001; Zuo et al., 2002). Some researches demonstrated that some afferent trophic supports from other song-control nuclei (such as HVC and MAN) play an important role in the formation of sex difference of RA volume (Gurney, 1982; Nordeen and Nordeen, 1990; Bottjer, 1997). However, the roughest process is not known. It is affirmative that the sex difference in the structure of the song control system results in the sex difference of vocal behavior. During the development of sexual dimorphism, the change of structure may relate to the control of genes. However, still now there is a complete blank about this kind of research.

Given the dramatic loss of RA neurons between fledgling and adult stages of female development, the female and male RAs of various developmental ages (the female at P35, the female and male at P45) were studied in this experiment. Our previous results showed that P35 and P45 were the key plastic period for the differentiation of RA (Zuo et al., 2002). Our aim in this experiment is to search for some differentially expressed genes that were the key genes controlling the RA development of white-rumped munia based on the developmental characteristic of RA by mRNA differential display (DD) silver-stained technology (Liang and Pardee, 1992).

1 Materials and methods

1.1 Animals

All white-rumped munias used in this study were bred and raised in animal center of College of Life Sciences, Beijing Normal University. The birds were divided into three groups by sex and age: the female at P35, the male and female at P45.

1.2 Tissue collection and RNA isolation for differential display

RA tissue in forebrain of white-rumped munias at P35 and P45 was isolated following rapid decapitation to prepare total RNA. Briefly, samples were homogenized in TRIZOL reagent (GIBCO) and chloroform was added to separate phases; then RNA was precipitated with alcohol and sodium acetate at low temperature. Washed RNA with ethanol was solubilized with DEPC-treated water. The integrity of RNA was estimated by 0.8% agarose gel electrophoresis with ethidium bromide (EB) stained.

To remove chromosomal DNA contamination, total RNA was incubated at 37℃ with RNase inhibitor (Promega) and RNase free DNase Ⅰ (Promega). RNA concentration was estimated by spectrophotometer reading at 260 nm.

To confirm the differentially expressed segments, the first strand cDNA synthesis was initiated for two times and every DD reaction was repeated for two times. That is to say, we did four repetitions for every primer pair (only three repetitions for few primer pairs). Only if three or more than three repetitions were the same, we considered the results positive; otherwise we considered them false positives and discarded them.

1.3 mRNA differential display

RNA reverse transcription was carried out as previously reported with some minor modifications (Rindi et al., 1999). Briefly, a sample of 0.5 μg total RNA was heated at 65℃ for 15 min and then cooled on ice before cDNA synthesis. Anchored Oligo $(dT)_{12}$ primers ($T_{12}MN$, $M \neq T$, N = A/C/G/T, DingGuo Bio.) were used to initiate the first strand cDNA synthesis, using 2 U M-MLV reverse transcripase (Bebco) in 1 × RT buffer containing 10 μmol/L DTT, 10 μmol each dNTP, 2 U RNase inhibitor, and 1 μmol/L $T_{12}MN$ for 1 h at 37 ℃ in a total 20 μl reactional system. The reaction was stopped by incubation at 95 ℃ for 5 min.

For PCR amplification, 16 arbitrary primers (AP) (DingGuo Bio.), coded 1 through 16, the sequences of which are given in Table 1, combining the above four $T_{12}MN$ primers were used to amplify target sequences of the generated cDNAs. PCR was performed in 0.5 ml-microcentrifuge reaction tubes in a final volume of 20 μl containing 1 × PCR buffer, 10 μmol each dNTP, 4 U *Taq* polymerase, 1 μmol/L $T_{12}MN$, 1 μmol/L AP and 1 μl of the RT reaction products. PCR amplification was performed with a LITTLE GENIUS temperature cycler (Ferrotec, Japan) set for 2-min at 94 ℃ and for 40 cycles of 30 s at 94℃, 2 min at 40 ℃ and 30 s at 72℃ and one final 5-min cycle at 72 ℃.

Seven-μl aliquots of PCR products were separated by 6% polyacrylamide (Arc: Bis=29.2:0.8), 20 cm-length, 0.75 mm-thick gels in a vertical electrophoresis cell (DingGuo Bio.). Molecular markers were included in each run. Gels were silver-stained according to the following protocol: the gels were fixed in 10% ethanol and 0.5% acetic acid for two 6-min times, and stained with 0.2% silver nitrate for 10 min. The gel then was washed completely in distilled water and color development was processed with 1.5% sodium hydroxide and 0.4% formaldehyde (cold). The reaction was stopped with 0.75% sodium carbonate.

Take photos and analyze the results with Image Master VDS (Pharmacia Biotech).

Table 1 The sequences of anchored and arbitrary primers used in this study

Name of Primers	Sequences of Primers
Anchored primer A: T12MA	5′ACGACTCATAGGGCTTTTTTTTTTTT (A/C/G)A 3′
Anchored primer C: T12MC	5′ACGACTCATAGGGCTTTTTTTTTTTT (A/C/G)C 3′
Anchored primer G: T12MG	5′ACGACTCATAGGGCTTTTTTTTTTTT (A/C/G)G 3′
Anchored primer T: T12MT	5′ACGACTCATAGGGCTTTTTTTTTTTT (A/C/G)T 3′
Arbitrary primer 1	5′ACAATTTCACACAGGACGACTCCAAG 3′
Arbitrary primer 2	5′ACAATTTCACACAGGAGCTAGCATGG 3′
Arbitrary primer 3	5′ACAATTTCACACAGGAGCTAGCATGG 3′
Arbitrary primer 4	5′ACAATTTCACACAGGAGCTAGCAGAC 3′
Arbitrary primer 5	5′ACAATTTCACACAGGAATGGTAGTCT 3′
Arbitrary primer 6	5′ACAATTTCACACAGGATACAACGAGG 3′
Arbitrary primer 7	5′ACAATTTCACACAGGATGGATTGGTC 3′
Arbitrary primer 8	5′ACAATTTCACACAGGATGGTAAAGGG 3′
Arbitrary primer 9	5′ACAATTTCACACAGGATAAGACTAGC 3′
Arbitrary primer 10	5′ACAATTTCACACAGGAGATCTCAGAC 3′
Arbitrary primer 11	5′ACAATTTCACACAGGAACGCTAGTGT 3′
Arbitrary primer 12	5′ACAATTTCACACAGGAAGGTACTAAGG 3′
Arbitrary primer 13	5′ACAATTTCACACAGGAGTTGCACCAT 3′
Arbitrary primer 14	5′ACAATTTCACACAGGATCCATGACTC 3′
Arbitrary primer 15	5′ACAATTTCACACAGGACTTTCTACCC 3′
Arbitrary primer 16	5′ACAATTTCACACAGGATCGGTCATAG 3′

1.4 The isolation and re-amplification of differentially expressed cDNA bands

The differentially expressed cDNA bands of interest were isolated from polyacrylamide gel to precipitate DNA with alcohol and sodium acetate according to Sambrook et al. (1999) and re-amplified. The second step PCR amplification was performed with the appropriate pairs of primers under the same conditions used for first-step cDNA amplifications, but the dNTPs doubled. After re-amplification, the size of PCR products were estimated by agarose gel electrophoresis with EB stained and sequenced by Shanghai Sangon Biological Engineering Technology and Service Co. The data of sequence were compared with the databases of NCBI in GenBank to determine the gene segments, which were important for the development of sexual dimorphism of RA.

2 Results

2.1 The characteristic of RNA sample

The ratio of $O.D_{260}/O.D_{280}$ of the RNA sample isolated in this experiment was about 2.0 and the bands of 18S and 28S rRNAs were clear and not smeared. The concentration of the total RNA sample was $1-1.5$ μg/μl based on the fact that $O.D_{260}=1$ is equal to RNA of 40 μg/ml.

2.2 mRNA differential display

Total RNA, extracted from RA and treated with DNase, was used as a substrate for T_{12}MN-primed, RT-dependent cDNA synthesis. The generated RT products were then PCR-amplified employing the 16 arbitrary primers in all the 64 pair combinations and the reaction products were separated by polyacrylamide gel electrophoresis, silver-stained and then photographed (two representative examples of this analysis are given in Fig.1). The first PCR amplification analysis yielded 1 158 bands in total, 18 bands for each primer pair (Table 2), of which 273 bands were differentially generated. There were 161 differential bands between 100 bp and 500 bp, and in which 61 bands were co-expressed in females at P35 and in males only at P45; 45 differential bands were detected only in females at P35; 14 bands that appeared to be expressed in females and males at P45; 13 bands were only expressed in males at P45; 9 bands were co-expressed in females at P35 and P45; 19 bands were only expressed in females at P45 (Table

Table 2 Number of cDNA amplified

Primers	Number of cDNAs from 16 APs	Percentage of cDNAs per AP (%)	Number of differential bands in all	Percentage of differential bands per AP (%)
T_{12}MA	368	23.00	60	3.75
T_{12}MT	327	20.43	99	6.19
T_{12}MC	287	17.94	68	4.25
T_{12}MG	176	11.00	46	2.88

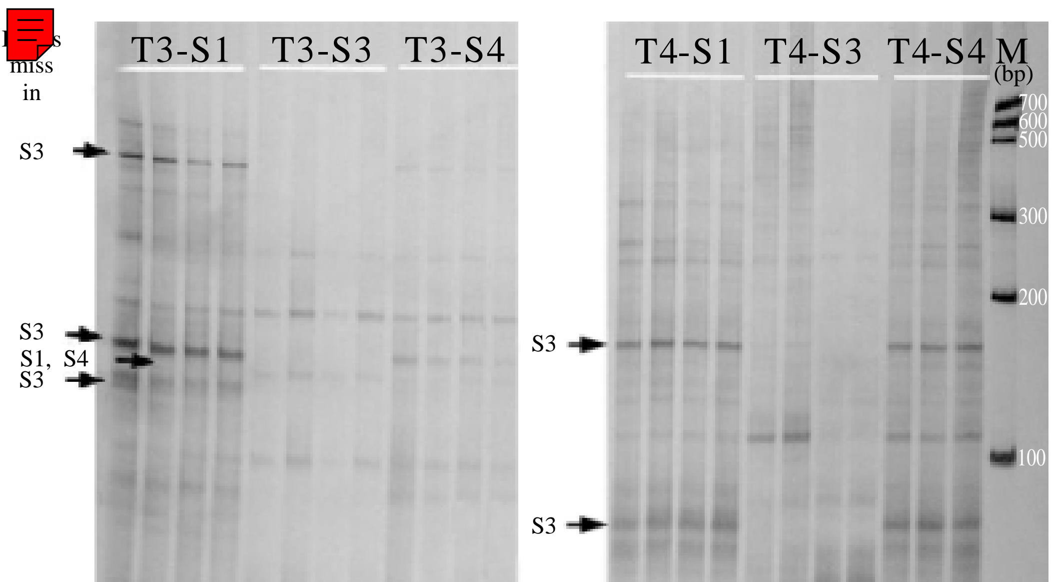

Fig.1 Two representative differential display of total RNA from three samples using multiple primer sets

Total RNAs from three samples (S1, S3 and S4) were transcribed by T_{12}MN-primed RT and the generated cDNAs were PCR-amplified by arbitrary primers; the reaction products were then separated by polyacrylamide gel electrophoresis and silver-stained. A portion of a 6% polyacrylamide gel, obtained with arbitrary primers T/3 (left panel) and T/4 (right panel), is shown. Arrowheads indicate the missing differentially amplified products. Molecular markers (in bp) are shown on the right panel (M). S1, S3 and S4 stand for female RA at P35, female and male RA at P45, respectively.

Table 3 Differentially generated bands in the three examples

Primer set	Number of bands			Number and size (bp) of differentially generated bands[a]		
	P35F	P45F	P45M	S1	S3	S4
A7	36	40	48	6(>600[S4], 100[S4])	2(>600, >600)	2(>600[S1], 100[S1])
A9	20	20	20	1(>200[S4])	1(200)	1(>200[S1])
A10	25	20	24	6(>600[S4], 180[S4], 250[S4], 300[S4])	1(700)	5(>600[S1], 180[S1], 250[S1], 300[S1])
A11	8	5	8	3(500[S4], >300[S4], >300[S4])		3(500[S1], >300[S1], >300[S1])
A17	9	9	8	1	1(>600)	
A20	10	9	10	1(490[S4])		1(490[S1])
A22	24	23	25	4(200[S4], 350[S4], 600[S4])	3(200)	4(200[S1], 350[S1], 600[S1])
T8	15	17	17	2(>500[S4])	1	1(>500[S1])
T9	14	10	12	4(>300[S4], >100[S4])		3(>300[S1], >100[S1])
T10	11	24	29	3(180[S4], 250[S4], 250[S4])		3(180[S1], 250[S1], 250[S1])
T11	16	15	16	1(>300[S4])		1(>300[S1])
T15	12	12	12	1(400[S4])	1(700)	1(400[S1])
T16	29	23	29	7(150[S4], >400[S4], >400[S4], >700[S4], >700[S4], >700[S4], >700[S4])	1(300)	7(150[S1], >400[S1], >400[S1], >700[S1], >700[S1], >700[S1], >700[S1])
T18	11	11	12	2(300[S4], <300[S4])	1	3(300[S1], <300[S1])
T21	29	29	29		1(3 000)	1(600[S1])
T22	47	46	47	1(600[S4], 350[S4])		1(350[S1])
C11	22	19	21	3(400[S4], >600[S4])		3(400[S1], >600[S1])
C12	22	21	22	1(>200[S4])		1(>200[S1])
C14	10	9	10	1(250[S4])		1(250[S1])
C16	28	31	28		3(160, >200, 500)	
C18	13	10	11	3(>300[S4])		1(>300[S1])
C21	35	37	35	1(550[S4])	3	1(550[S1])

The table summarized two types of bands which we were interested. One was expressed both in S1 and S4, and the other was expressed only in S3. The band sizes only from 100 bp to 700 bp were showed. S1, S3 and S4 represent female RA at P35, female and male RA at P45, respectively. The symbols ">" or "<" indicated the size of the bands that is closed to the value behind. M: Male. F: Female.

a: Size of bands which only expressed in S3 or both expressed in S1 and S4, expressed in bases (bp), is given in parentheses.

3). Most of the differential cDNAs exhibited one single band after the second amplification and agarose gel electrophoresis and few showed some weak bands besides the major band. After the second PCR amplification, sixteen differential bands were screened out, of which eleven were co-expressed in female P35 and male P45, five were only expressed in female at P45.

2.3 The sequence of the differentially expressed band, T3

With mRNA DD, using primer combination T_{12} MT and AP3, we detected a 220 bp cDNA, which was named T3 and co-expressed in female at P35 and male at P45, not expressed in female at P45. Its full sequence was the following: AACCCTTACA ACTGCATATC TTCCGGAGCA CAGAGGCAGT GACGGGCTGC CAGGAAGCCG TTTCGTTCAT GCTCAGTAGG AAGGATGGAC TGCAGGAAGC AGAACTGTGA CCCAAGAACC GCTGGCACTG ACTGTGGAAG CAGATGGACA ACCATGCCCC CTTCACTTCA GCTTACTGAA AAGCTTGTGA GCAACGCAAT GGTCTCCTGT GTGAAATTGT. A search for homologous nucleic acid or protein sequences was performed in the databases of NCBI (http://www.ncbi.nlm.nih.gov) showed no nucleic acid sequence homology, but low amino acid sequence homology with Bcl complex in chicken *Gallus gallu s* (Sequence No. 1048228857-015527-19305). 13 of the 16 amino acids recognized were homologous.

3 Discussion

3.1 The reliability of our methods

The mRNA DD, a potentially powerful methodology originally developed in eukaryotic systems by Liang and Pardee (1992), makes it possible to identify and isolate genes that are over- or under-expressed in one cell (tissue) type relative to others. Recently, a sexual dimorphic gene in the developing zebra finch telencephalon, for the first time, was identified for the first time by using ddRT-PCR method (Veney, et al., 2003). In the present study, we initiated the first strand cDNA synthesis for two times and repeated every DD reaction for two times in order to confirm the differentially expressed segments.

The 161 differential cDNA segments distributing 100 – 500 bp were re-amplified and most of them exhibited one single band after the agarose gel electrophoresis and few showed some weak bands besides the major band. We guessed that there were binding sites of arbitrary primers within the cDNA segments.

3.2 The specific genes controlling the appearance of sexual dimorphism in RA

Considering that the significant amplification bands mostly distributed in the range between 100 – 500 bp, we focused our attention particularly onto these size bands. The 161 differential bands were detected in this range.

Of the 161 differential bands, 61 differential segments were co-expressed in females at P35 and in males at P45. Our previous study identified that the size of RA volumes of male and female munia were the same at P35, while their sexual dimorphism in RA volume appeared at P45(Zeng et al., 2001; Zuo et al., 2002). Considering the characteristic of the sexual dimorphism of RA between P35 and P45, we suggest that these gene segments might contribute to the maintenance of the RA volume.

There were 45 segments detected in female only at P35. It was inferred that these cDNAs might be related to a specific age, P35, because these genes were expressed neither in the female RA nor in the male RA at P45.

14 segments were detected in both sexes only at P45. It was thought that these genes were related to a specific age, P45, because sex differences in RA volume became very markedly at P45. The only common ground of these two samples was that their ages were the same.

The gene segments that were only expressed in male at P45 (13 bands) or were co-expressed in female at P35 and P45 (9 bands) might be contributive to the gender, male or female. These, only expressed in males, might be related to the development of the male brain region. Similarly, these only expressed in females might be also involved in the development of the female brain.

There were 19 cDNA segments, which were only expressed in the females at P45. If some of these 19 segments could be translated into functional protein or where they were specifically expressed, we should consider the possibility that these cDNAs were related to shrinkage of the RA volume in females.

The most significant finding in the present study was the cDNAs co-expression in females at P35 and males at P45 as well as the expression in females only at P45, which might be critical for regulating changes of cell morphology, cell survival, or even apoptosis and anti-apoptosis of RA cells. After the second PCR amplification, sixteen differential bands were screened out, of which eleven were co-expressed in female at P35 and male at P45, five were only expressed in female at P45, which underlay the genetic mechanism of the appearance of the RA sexual dimorphism in white-rumped munia.

3.3 The possible role of T3: anti-apoptosis

The differential gene segment, T3, was co-expressed in the RAs of P35 female and P45 male, but not in the female at P45. The sizes of RA volume were similar in the female and male birds at P35; but sexual dimorphism in RA volume became very markedly at P45. Taken together, this fact could lead us to propose that the T3 segment might be related to the retaining of RA volume. Moreover, our previous study (Zeng et al., 2001) revealed that the cell apoptosis ratio within female RA was about five times higher than that within male RA in white-rumped munia at P45. Similarly, previous reports indicated that the sex difference of RA volume in zebra finch *Poephila guttatta* could be a result of sex differences in apoptosis when females lose more neurons than males at juveniles (Nordeen and Nordeen, 1988; Kirn and DeVoogd, 1989). Cell apoptosis could be one important factor that might give rise to RA sexual dimorphism. Therefore, the genes related to apoptosis might play a role in this process. T3 segment was differentially expressed just at this period and might be involved in apoptosis.

The result from the comparison of the T3 sequence with the data from gene bank testified our guess, too. The theoretic amino acid sequence of T3 segments had 81% homology (13 of the 16 amino acids recognized were homologous) with Bcl complex in chicken. It is known that the role of Bcl-2 protein family was inducing or inhibiting apoptosis (Adams and Suzanne, 1998). So, it was inferred that the T3 was possible to function as anti-apoptosis in the development of RA and the members of Bcl-2 protein family were likely to control the development of RA at some pivotal stages. Furthermore, the T3 segment

might be a new gene segment because no homologous gene sequence was found.

In the present study, our results confirmed that more than 100 differential display bands were detected in both sexes at the same age of white-rumped munia during their development. At present, we do not know how many genes were involved in the differential modulation of RA volume and neuron number during a relative narrow developmental window, but the timing and pattern of its expression, taken together in context of the differentiation of the song control nuclei, is worth elaborating. This is the first time by using mRNA DD method to reveal the sexual dimorphism of vocal-control nuclei in songbirds. We can use these cDNA segments as probes to screen the full-length cDNA from a cDNA library or design the specific primers based on these cDNA segments to obtain the full-length cDNA with 5'RACE (rapid amplification of cDNA end), it will now be possible to study the function of these genes.

References

Adams JM, Suzanne C, 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281 (28): 1 322 - 1 326.

Agate RJ, Grisham W, Wade J, Mann S, Wingfield J, Schanen C, Palotie A, Arnold AP, 2003. Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch. Proc. Natl. Acad. Sci. USA 100 (8): 4 873 - 4 878.

Arnold A, 1975. The effects of castration and androgen replacement on song, courtship, and aggression in zebra finch *Poephila guttata*. J. Exp. Zool. 191: 309 - 326.

Bottjer SW, 1997. Developmental plasticity in neural circuits for a learned behavior. Annu. Rev. Neurosci. 20: 459 - 481.

Bottjer SW, Halsema KA, Brown SA, Meisner EA, 1989. Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. J. Comp. Neurol. 279: 312 - 326.

Gahr M, Metzdorf R. 1999. The sexually dimorphic expression of androgen receptors in the song nucleus hyperstriatalis ventrale pars caudale of the zebra finch develops independently of gonadal steroids. J. Neurosci. 19 (7): 2 628 - 2 636.

Gurney ME, 1982. Behavioral correlates of sexual differentiation in the zebra finch song system. Brain Res. 231: 153 - 172.

Jiang JC, Xu ML, Chen H, Zhang H, 1992. A study on the acoustic behavior in budgerigar *Melopsittacus undelafu*. Acta Zool. Sin. 38 (3): 286 - 297 (In Chinese).

Johnson F, Bottjer SW, 1992. Growth and regression of thalamic efferents in the song-control system of male zebra finches. J. Comp. Neural. 326: 442 - 450.

Kirn JR, DeVoogd TJ, 1989. Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. J. Neurosci. 9 (9): 3 176 - 3 187.

Li DF, Zuo MX, Lan SC, Hu J, 1992. Sex differences of vocal control nuclei in the forebrain of songbirds. Acta Zool. Sin. 38 (3): 298 - 301 (In Chinese).

Liang P, Pardee AB, 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967 - 971.

Nixdorf-Bergweiler BE, Wallhausser-Franke E, DeVooge TJ, 1995. Regressive development in neuronal structure during song learning in birds. J. Neurobiol. 27 (2): 204 - 215.

Nordeen EJ, Nordeen KW, 1990. Hormones and structuring of neural pathways controlling avian song. Comp. Physiol. 8: 92 - 103.

Nordeen KW, Nordeen EJ, 1988. Projection neurons within a vocal motor pathway are born during song learning in zebra finches. Nature 334: 149 - 151.

Nottebohm F, Arnold AP, 1976. Sexual dimorphism in vocal control areas of the songbird brain. Science 194: 211 - 213.

Nottebohm F, Nottebohm ME, Crane LA, Wingfield JC, 1987. Seasonal changes in gonadal hormone levels of adult male canaries and their relation to song. Behav. Neural. Biol. 47: 197 - 211.

Reisert I, Pilgrim C, 1991. Sexual differentiation of monoaminergic neurons—genetic or epigenetic? Trends Neurosci. 14 (10): 468 - 473.

Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, Medina L, Paxinos G, Shimizu T, Striedter G, Wild M, Ball GF, Durand S, Gütürkün O, Lee DW, Mello CV, Powers A, White SA, Hough G, Kubikova L, Smulders TV, Wada K, Dugas-Ford J, Husband S, Yamamoto K, Yu J, Siang C, Jarvis ED, 2004. Revised nomenclature for avian telencephalon and some related brainstem nuclei. J. Comp. Neurol. 473 (3): 377 - 414.

Rindi L, Lari N, Garzelli C, 1999. Search for genes potentially involved in mycobacterium tuberculosis virulence by mRNA differential display. Biochem. Biophys. Res. Commun. 258: 94 - 101.

Sambrook J, Fritsch EF, Maniatis T, 1999. Molecular cloning: A Laboratory Manual. 2nd edn. New York, USA: Cold Spring Harbor Laboratory Press.

Smith GT, Brenowitz EA, Beecher MD, Wingfield JC, 1997. Seasonal changes in testosterone, neural attributes of song control nuclei, and song structure in wild songbirds. J. Neurosci. 17 (15): 6 001 - 6 010.

Stein DG, 2001. Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? Trends Neurosci. 24 (7): 386 - 391.

Veney SL, Peabody C, Smith GW, Wade J, 2003. Sexually dimorphic neurocalcin expression in the developing zebra finch telencephalon. J. Neurobilo. 56: 372 - 386.

Zeng SJ, Zhang XW, Zuo MX, 2001. Sexual dimorphism of song control nucleus RA in the forebrain of songbird *Lonchura striata*. Acta Zool. Sin. 47 (5): 535 - 541 (In Chinese).

Zeng SJ, Zhang XW, Peng WM, Zuo MX, 2004. Immunohistochemistry and neural connectiveity of the Ov shell in the songbird and their evolutionary implications. J. Comp. Neurol. 470: 192 - 209.

Zuo MX, Zeng SJ, Peng WM, Zhang XW, 2002. Neural development of vocal behavior in striated manikin *Lonchura striata swinhoei*. Acta Zool. Sin. 48 (1): 50 - 57 (In Chinese).

Zuo MX, Zeng SJ, Shi Y, Huang ZY, 1998. The sexual dimorphism of vocal control nuclei in the telecenphalon of songbird *Lonchura striata swinhoei*. Acta Zool. Sin. 44 (3): 302 - 208 (In Chinese).

蒋锦昌，徐慕玲，陈　浩，张　弘，1992. 虎皮鹦鹉声行为的研究. 动物学报 38 (3): 286 - 297.

李东风，左明雪，蓝书成，胡　建，1992. 鸣禽前脑发声控制核团的雌雄差别. 动物学报 38 (3): 298 - 301.

曾少举，张信文，左明雪，2001. 鸣禽白腰纹鸟前脑古纹状体粗核性双态发育的神经机制. 动物学报 47 (5): 535 - 541.

左明雪，曾少举，彭卫民，张信文，2002. 白腰纹鸟发声行为的神经发育. 动物学报 48 (1): 50 - 57.

左明雪，曾少举，时　宇，黄之瑜，1998. 鸣禽端脑发声控制核团的雌雄差别. 动物学报 44 (3): 302 - 308.