COMPARATIVE GENE ANALYSIS OF *CRABP-II* GENE UPREGULATED BY ZIC1 USING AFFYMETRIX GENECHIP MICROARRAY

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Zic1 is a highly conserved zinc finger transcription factor that plays a very early developmental role in establishing different regions of the future nervous system. Studies from several model organisms suggest important roles for *zic1* gene in patterning the neural plate, in formation of the neural crest, and in cerebellar development (Kuo *et al*., 1998; Li *et al.,* 2006).

Retinoids are natural or synthetic derivatives of vitamin A (retinol). There is increasing evidence that retinoids regulate cell differentiation and pattern formation during vertebrate embryogenesis and the neoplastic cells development (Kochhar, 1967). Vitamin A (retinol) is essential for vision, reproduction, normal embryonic development, and the regulation of the growth and differentiation of diverse cell types in adult tissues. In addition, there is a strong evidence for beneficial retinoid responses in preventing or treating clinical tumors based on preclinical, epidemiological, and clinical findings (Nason-Burchenal and Dmitrovsky, 1999).

The best known biologically active retinoid, alltrans retinoic acid (RA), has long been known to be teratogenic in humans (Kochhar, 1967) and reported to cause severe birth defects when administered to pregnant women (Lammer *et al.,* 1985). The spectrum of birth defects caused by RA includes cleft palate, specific central nervous system (CNS) defects and congenital heart and limb defects, and is conserved in rodents, chickens and frogs suggesting the existence of a well-defined class of RA-sensitive events during vertebrate embryogenesis (Sive *et al.,* 1990).

CRABPs (cellular retinoic acid binding proteins) belong to members of a superfamily of lipid-binding proteins that are thought to act by maintaining tolerable concentrations of intracellular RA, as modulators of RA catabolism and as intracellular transporters for RA from the cytoplasm to the nuclear receptor (Mansfield *et al.,* 1998). Based on their significance roles in binding lipids, *CRABPs* are thought to be candidate genes for meat

quality-related traits in domestic animals. The mapping of the porcine *CRABPI* and *CRABP-II* genes is one step towards further investigation on their possible roles in meat quality traits (Lee *et al.,* 2006).

Interestingly, the gene for *CRABP-II* is located on human chromosome 1q21-23, which is a region that has been linked with disorders such as familial combined hyperlipidemia (FCHL), type 2 diabetes mellitus, and partial lipodystrophy, all of which are characterized by dyslipidemia (Salazar *et al.,* 2007).

CRABP-II has been proved to be upregulated by RA during neural differentiation of *Xenopus laevis* (Ho *et al.,* 1994). It was also proved to be a direct target of human embryonal carcinoma through the use of cDNA-based microarray (Freemantle *et al.,* 2002). There is growing evidence suggesting that *CRABP-II* may be regulated by factors other than RA. *CRABP-II* is directly regulated by estrogen in the rat uterus through an estrogen receptor (ER) binding site in the promoter region of *CRABP-II* (Li *et al.,* 2003). *CRABP-II* is overexpressed in a wide variety of cancers. In breast cancer cell lines, estrogen induces *CRABP-II* expression, possibly through retinoic acid receptor In addition, overexpression of *CRABP-II* has been observed in several breast cancer cell lines and primary tumors (Lu *et al.,* 2005). From analysis of the published microarray data, *CRABP-II* was found to be expressed in these cell lines, irrespective of the estrogen status. A recent study identified both *MycN* and *CRABP-II* overexpression in Wilms tumors (Li *et al.,* 2005), and expression of *MycN* correlates with CRABP-II levels in these tumors. *CRABP-II* expression is upregulated in parathyroid adenomas in correlation with tumor suppressor gene *HRPT2* downregulation (Zebracka *et al.,* 2007).

To understand the molecular mechanisms through which Zic1 acts, it is necessary to identify genes that are directly regulated by Zic1 and to determine their individual roles. *CRABP-II* proved to be upregulated by Zic1 from our previous study (Cornish *et al.,* 2009, under review). *CRABP-II* seemed to have a great importance since it was found to be over expressed during several cancer diseases. Considerable conservation on the levels of gene structure and amino acid sequence was observed between amphibian and mammalian *CRABP-II* family genes (Ho *et al.,* 1994). Therefore, the objective of this study is to determine the degree of functional relatedness present among CRABP-II proteins of phylogenetically distant organisms. In addition, we describe the expression patterns using real-time PCR and *in situ* hybridization in addition to the results of comparative gene analysis for *CRABP-II* gene versions isolated from different organisms.

MATERIALS AND METHODS

In vitro transcription

RNA was synthesized from *zic*1GR, and dn*zic*1 constructs in pCS2 plasmid as previously described (Kuo *et*

al., 1998; Merzdorf and Sive, 2006). *Zic1GR* is a fusion construct of the zic1 coding region with the human glucocorticoid receptor binding domain; the resulting fusion protein only enters the nucleus in the presence of dexamethasone (Kuo *et al.,* 1998). The dominant interfering *zic*1 construct (dn*zic1*) comprises the zinc finger domain and the C-terminus of *zic1* (Merzdorf and Sive, 2006).

Microinjection and RNA isolation

Xenopus laevis embryos were injected at the 2-cell stage into both blastomeres with a total of 200 pg *zic1*GR and 1 pg noggin to neuralize the animal caps (ectodermal explants) or with 200 pg β-globin and 1 pg noggin for controls (Li *et al.,* 2006). Animal caps were dissected at stage 9 and cultured. When the caps reached the equivalent of stage 12, they were treated with 10 μg/ml cycloheximide (C-7698 Sigma, St Louis, MO) to prevent further protein synthesis and 30 min later with 10 μM dexamethasone (D-4902 Sigma St Louis, MO) to activate preformed *zic*1GR. After a further 3 h in culture, the animal caps were harvested and total RNA was isolated (Li *et al.,* 2006). Small samples of each batch of RNA were tested by RT-PCR with muscle actin primers to ensure that mesoderm was not present and minus RT (–RT) samples were used to confirm that genomic DNA had been removed.

Microarray experiment

Total RNA was converted to biotin-labeled cRNA using the GeneChip Eukaryotic Expression 3' Amplification One-Cycle Target Labeling and Control Reagents kit (#900493) and hybridized to Affymetrix GeneChip *Xenopus laevis* Genome Arrays (#900491, Affymetrix, Santa Clara, CA) as described in the user's manual (Affymetrix GeneChip Expression Analysis Technical Manual, November 2004). Briefly, total RNAs (approximately 2 μg per sample) were reverse transcribed to cDNA using a T7-oligo(dT) primer. Following second-strand cDNA synthesis, the double-stranded cDNA was purified as a template for the subsequent *in vitro* transcription reaction. Biotin-labeled complementary RNA (cRNA) was synthesized in the presence of a biotinylated nucleotide analog/ribonucleic acid mix. The labeled cRNA (single stranded) was purified, fragmented, and hybridized to the arrays at 45°C for 16 h with constant rotational mixing at 60 rpm. Independent triplicates of experimental and control samples were hybridized to separate arrays. Washing and staining of the arrays were performed using the Affymetrix GeneChip Fluidics Station 450. Arrays were scanned using an Affymetrix GeneChip Scanner 7G and GCOS software version 1.4.

Microarray data were analyzed using GeneSpring software version 7.3 (Agilent Technologies, Palo Alto, CA). The Affymetrix CEL files were imported and normalized using Robust Multichip Averaging with median polishing (Bolstad *et al.,* 2003) and genes were filtered for threshold signal intensities of at least 50 in one array. Flag data (present, marginal or

absent calls) was imported from array CHP files and was used as an additional filter (present or marginal call in at least two arrays). The gene list was further trimmed to identify genes with fold-change differences of at least 1.5 fold changes. Statistically significant differentially expressed genes were identified through ANOVA using a Student's t-test (p-value $\leq$ 0.05) and Benjamini and Hochberg multiple test correction. Gene expression data were deposited with Gene Expression Omnibus at the National Center for Biotechnology Information, and can be accessed through accession number GSE12128.

Quantitative RT-PCR

To confirm the results obtained in microarray studies for *CRABP-II* gene, independent sets of animal cap samples were generated as described above for the microarray. Gene-specific primer set for *CRABP-II* gene was used for qRT-PCR. A SYBR Green mastermix (Eurogentec) was used in combination with a Corbett Rotor Gene 3000 (no. 204243). PCR products were further examined by melt curve analysis and gel electrophoresis. Differences in transcription between *zic1* injected and control samples of RNA were analyzed by the ΔΔCT method (Livak and Schmittgen, 2001).

The following primer pairs were used: muscle actin (Stutz and Spohr, 1986); EF1-α (Gammill and Sive, 1997); *CRABP-II*-forward: 5'-TGGAAAAGAGCATGGGAAAG-3' and *CRABP-II*-reverse: 5'-TCTGCCTAGTAAGGGGTTTGTATG-3'.

Whole mount in situ hybridization

Expression of *CRABP-II* was determined in albino embryos by *in situ* hybridization (Harland, 1991). *CRABP-II* expression was studied in uninjected embryos and in embryos injected in one cell at the 2-cell stage with 100 pg full-length *zic1* (Kuo *et al.,* 1998) or dn*zic1* RNA (Merzdorf and Sive, 2006) together with 25 pg lacZ RNA as tracer. β-galactosidase staining (Kolm and Sive, 1995) preceded *in situ* hybridization using a digoxygenin-labeled *CRABP-II*, or *zic1* antisense RNA probe, an antidigoxigen-AP antibody (#1093274, Roche Diagnostics, Mannheim, Germany) and the alkaline phosphatase substrate NBT/BCIP (B1911 Sigma, St. Louis, MO) for color detection. *CRABP-II* antisense RNA probe was synthesized from an XhoI digestion of the pExpress-1 plasmid containing *CRABP-II* (RZPD Deutsches Ressourcenzentrum fuer Genomforschung GmbH), transcribed with SP6 polymerase. *Zic1* probe (Kuo *et al.,* 1998).

AlignX and ClastalW

Clustal W (Higgins and Sharp 1988; Larkin *et al.,* 2007) is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be

seen via viewing Cladograms or Phylograms.

AlignX® Module: Rapid Multiple Sequence Alignment With Minimal Preparation AlignX® uses a modified Clustal W algorithm to generate multiple sequence alignments of either protein or nucleic acid sequences for similarity comparisons and for annotation. The power of AlignX® is that it maintains annotated features within the alignment for easy visualization and localization of regions of interest.

Determination of phylogenetic relationships

The DNA and protein sequences for *CRABP-II* gene from different organisms were aligned using AlignX module of the Vector NTI software package, which uses the multisequence alignment CLUSTAL program (Higgins and Sharp 1988). The percentages of similarity were determined using the AlignX module of the Vector NTI software package (Invitrogen).

Nucleotide sequence accession numbers

The GenBank accession numbers for *CRABP-II* nucleotide sequences data reported in this article are as follows: BC142037 for *Bos Taurus*, NM_001008670 for *Bos Taurus,* XM_001500415 for *Equus caballus,* XM_001925466 for *Sus scrofa*, BC164791 for *Danio rerio,* NM_182859 for *Danio rerio*, NM_001006862 for *Xenopus (Silurana) tropicalis*, NM_007759 for *Mus musculus,* NM_017244 for *Rattus norvegicus*, XM_001099615 for *Macaca mulatta*, XM_001116699 for *Macaca mulatta*, CR450357 for *Homo sapiens*, NM_001878 for *Homo sapiens*, XM_525223 for *Pan troglodytes*, XM_849822 for *Canis familiaris*, XM_001367482 for *Monodelphis domestica*, NM_001140880 for *Salmo solar* and NM_001085780 for *Xenopus laevis.*

RESULTS AND DISCUSSION

CRABP-II is a direct target of Zic1

In order to identify downstream target genes of Zic1, we conducted an Affymetrix GeneChip microarray screen. Zic1-induced and uninduced *Xenopus* ectodermal explants (animal caps) served as experimental and reference samples. To limit the screen to the identification of direct transcriptional targets of Zic1, a hormone-inducible version of Zic1 (Zic1GR; Kuo *et al.,* 1998) was employed and the animal caps were treated with the translational inhibitor cycloheximide. A direct target of activin signaling was discovered by a very similar approach (Meek *et al.,* 2004) and animal caps in combination with cycloheximide were used to identify direct BMP targets on *Xenopus* microarrays (Peiffer *et al.,* 2005). Then, differentially expressed genes identified in the screen included a *CRABP-II* gene with UniGene cluster Xl.12210 (3.68-fold change). *CRABP-II* encodes a protein of 138 amino acids with one lipocalin domain. Lipocalins are transporters for small hydrophobic molecules, such as

lipids, steroid hormones, bilins, and retinoids (Marchler-Bauer *et al.*, 2007).

Animal caps were isolated from injected embryos at late blastula stage 9 and were treated with CHX and dex as described above (all control animal caps were also treated with CHX and dex). Animal caps were harvested after an additional 3 h of culture and each sample was subjected to quantitative real-time RT-PCR analysis.

First, we confirmed that *CRABP-II* expression is upregulated by Zic1GR in the presence of CHX. The averages of eleven independent experiments demonstrate that animal caps taken from control embryos injected with β-globin plus noggin RNAs show basal levels of *CRABP-II* expression, while caps taken from *zic*1GR plus *noggin*-injected embryos showed a significant increase in *CRABP-II* expression. The approximately 4.23±1.32 fold induction of *CRABP-II* expression by Zic1GR in the presence of CHX suggests that *CRABP-II* is a direct target of the transcription factor Zic1. In comparison, direct targets of other *Xenopus* transcription factors are induced 2- to 36-fold.

Spatial expression of CRABP-II

The expression patterns of *CRABP-II* were determined by whole-mount *in situ* hybridization using stage 17 (mid neurula stage). *CRABP-II* is expressed in dorsal-lateral view. Staining was found in the presumptive hindbrain and tail regions. There are also small lateral and anterior patches of staining, which possibly correspond with the future nasal pit and branchial arch regions (Fig. 1A). Expression patterns of *CRABP-II* were previously found to begin at the onset of gastrulation, *CRABP-II* mRNA is localised at the dorsal side of the embryo, in the ectoderm and in invaginating mesoderm. xCRABP expression then rapidly resolves into two domains; a neural domain, which becomes localised in the anterior hindbrain, and a posterior domain in neuroectoderm and mesoderm. These two domains were already evident by the mid-gastrula stage (Ho *et al.*, 1994).

Zic1 is expressed at the lateral edges of the neural plate and in the dorsal neural tube. During early neurula stages, zic1 is expressed in midbrain and hindbrain regions and later extends along the dorsal spinal cord (Kuo *et al.*, 1998). *Zic1* is expressed in a larger domain that overlaps with *CRABP-II* expression domain. The overlap between the expression domains of these two genes is consistent with our finding that *CRABP-II* expression is activated by Zic1.

Zic1 regulates the expression of CRABP-II in whole embryos

In order to investigate whether Zic1 is able to induce ectopic expression of *CRABP-II* in whole embryos, RNA for full-length *zic1*GR was injected into one cell of two cell albino *Xenopus* embryos. lacZ RNA was co-injected as tracer. The embryos injected with *zic1*GR RNA were treated with dexamethasone (dex) at mid-gastrula stage 11. Embryos were harvested

at neurula stages and the expression of *CRABP-II* was assayed by whole mount *in situ* hybridization. Zic1GR induced significant ectopic *CRABP-II* expression (Fig. 1B). These data demonstrate that *CRABP-II* is upregulated by Zic1 in whole embryos.

Assessment of phylogenetic relationships

Eighteen different *CRABP-II* gene sequences were found in 14 different organisms. Sequences for each gene were downloaded from the national center for biotechnology information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Only coding sequences were used to build the multi sequence alignment (MSA). While protein sequences were obtained by translating the coding sequence (CDS) within each NCBI record.

DNA sequence similarity matrix results

The lowest calculated similarity (67%) was found between *Mus musculus* (House mouse) and *Danio rerio* (Zebra fish) with accession numbers NM_007759 and BC164791, respectively. While the highest similarity (~100%) was obtained from the two *Danio rerio* (Zebra fish) sequences with accession numbers BC164791 and NM_182859 with only two nucleotide differences at positions 428 and 429. In addition, two sequences belonging to *Homo sapiens* (Human) and one belonging to *Pan troglodytes* (Chimpanzee) with accession numbers CR450357, NM_001878 and XM_525223, respectively, showed almost 100% similarity with only one nucleotide difference between the human sequences and chimpanzee at position 111 and another difference between the three sequences at position 144. A phylogenetic tree is created using a pairwise distance matrix and nearest-neighbor algorithm. The constructed dendrogram showing these relationships is presented in Fig. (2).

Protein sequence similarity matrix results

The similarity matrix for all proteins in this study showed almost the same trend as DNA sequence similarity matrix, however, in all cases the protein sequence comparisons showed same or higher similarity than the DNA sequence comparisons. According to these results, the lowest similarity was 68% between *Salmo solar* (Atlantic Salmon) accession number NM_001140880 and *Macaca mulatta* (Rhesus monkeys). While the highest similarity was 100% calculated from the two *Danio rerio* (Zebra fish) sequences accession number BC164791 and NM_182859 with no amino acid deference. Also two *Homo sapiens* (Human) and one *Pan troglodytes* (Chimpanzee) accession numbers CR450357, NM_001878 and XM_525223, respectively, showed 100% similarity. Also, similarity between *Equus caballus* (wild horse) accession number XM001500415 and *Bos Taurus* (caw) accession number NM001008670 was 100%. This group also includes *Sus scarofa* (pig) accession number XM001925466 and another version of the cow CRAPB2 accession number BC142037 with only one amino

acid difference than *Equus caballus* (wild horse) accession number XM001500415 and *Bos taurus* (caw) accession number NM001008670. The constructed dendrogram demonstrates that data is shown in Fig. (3).

Alignment of the complete open reading frames (ORFs) of *CRABP-II* genes through different vertebrate organisms showed significant sequence identity (67-100%) at the nucleic acid level and (68-100%) at the amino acid level, indicating an absolute degree of evolutionary conservation within this gene. The high degree of structural relatedness between the lower vertebrate *Xenopus CRABP-II* gene and their mammalian counterparts correlated well with the abilities of this *Xenopus CRABP-II* to function as cancer-related gene in a mammalian cell transformation system.

SUMMARY

The retinoids are natural and synthetic derivatives of vitamin A (retinol). Retinol is essential for vision, reproduction, normal embryonic development, and the regulation of the growth and differentiation of diverse cell types in adult tissues. A strong rationale exists for the use of retinoids in cancer therapy and chemoprevention based on preclinical, epidemiological, and clinical findings. In the cell, retinoic acid (RA) can be mediated by two cellular retinoic acid binding proteins (CRABP-I and CRABP-II). CRABP-II proved to be expressed in several cancers and upregulated directly by RA in addition to many other transcription factors including Zic1 transcription factor in early neural development using Affymetrix GeneChip Microarray, real-time PCR and *in situ* hybridization. Comparisons of *CRABP-II* genes through different vertebrate organisms showed significant sequence identity (67%-100%) at the nucleic acid level and (68%-100%) at the amino acid level, indicating considerable degree of evolutionary conservation within this gene. The high degree of structural relatedness between the lower vertebrates and mammalian *CRABP-II* gene versions, correlated well with the ability of *Xenopus CRABP-II* to function as cancer-related gene in a mammalian cell transformation system to gain more understanding about cancer cell biology.

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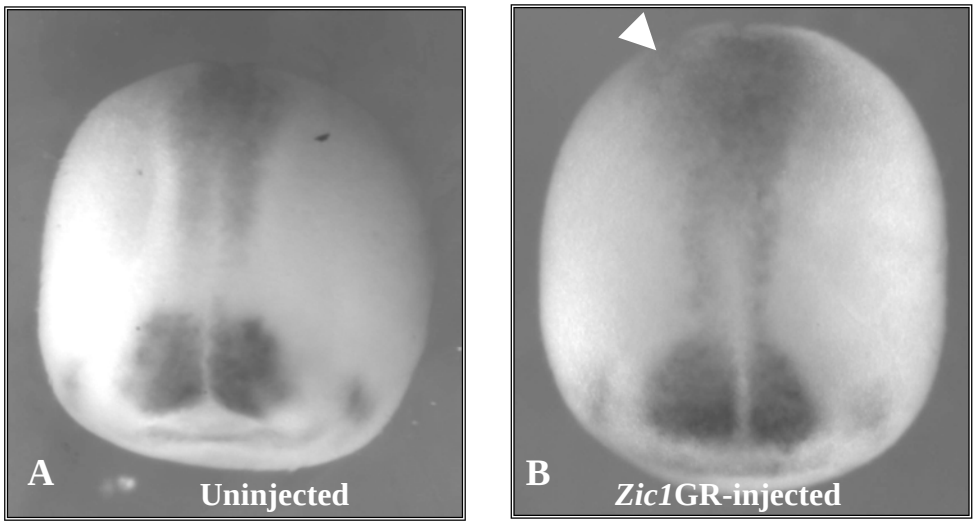

Fig. (1): *CRABP-II in situ* hybridization at stage 17 (mid neurula stage). (A): Dorsal view for uninjected embryo, staining was found in the presumptive hindbrain and tail regions. There are also small lateral and anterior patches of staining, which possibly correspond with the future nasal pit and branchial arch regions, (B): Dorsal view for *zic1*GR-injected embryo, Zic1 induces *CRAPII* expression in the injected side (white arrow head).

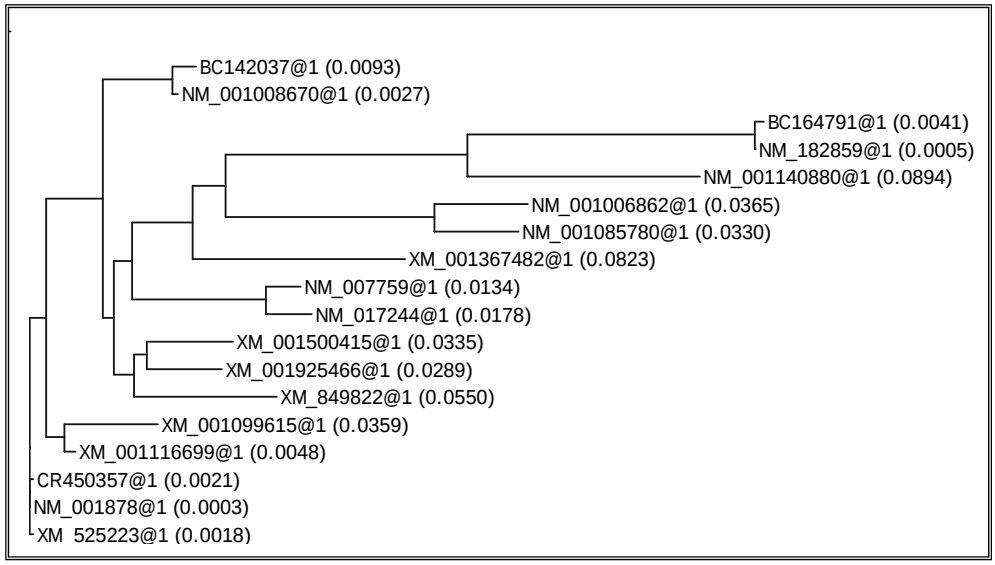

Fig. (2): Dendrogram shows the relation between eighteen deferent DNA sequences for CRABP-II genes represent fourteen different organisms.

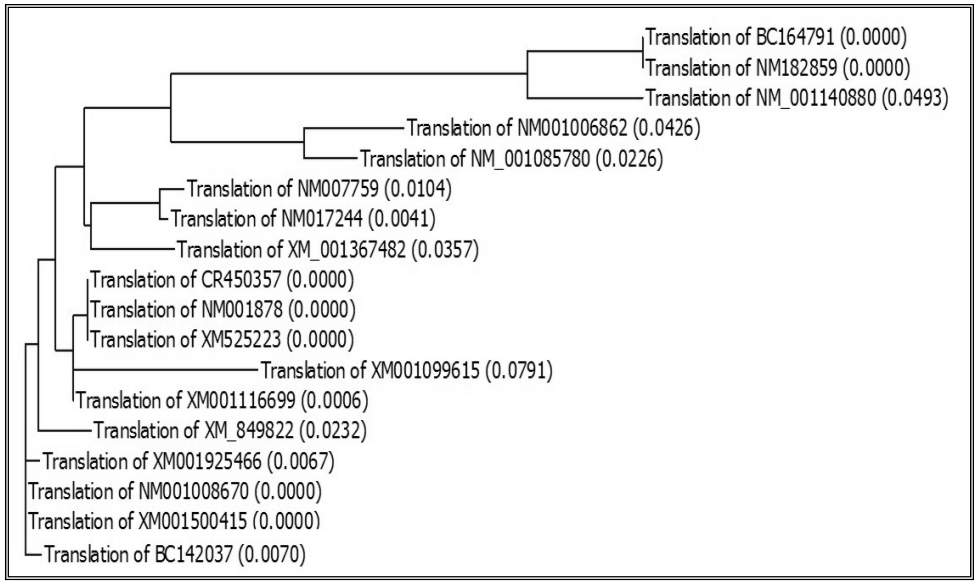

Fig. (3): Dendrogram shows the relation between eighteen deferent protein sequences for CRABP-II genes represent fourteen different organisms.