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Phylogeny of Vertebrate Nuclear Receptors – Analysis of Variance Components in Protein Sequences

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A B S T R A C T

Nuclear receptors (NR) constitute a large family of proteins and play a crucial role in regulating mineral metabolism and physiological homeostasis of various organ systems. The aim of this study was to elucidate whether the variance among NRs of estrogen, androgen and vitamin-D in various vertebrate species including humans is attributed to differences between the taxonomic groups within a specific receptor (i.e. between orthologous) or between the different proteins within the taxon (i.e. between paralogous genes). Published data on 57 protein sequences of the above NRs were used for phylogenetic analysis. The results showed that in DNA- and ligand-binding regions, 94% and 70% of variance is due to differences between the three proteins. However, in non-binding regions, 47% of the variance results from differences between the three paralogous proteins. Human sequences consistently clustered with their mammal orthologous within the three groups of NR sequences, clearly indicating that evolution of human sequences is not distinct from mammal sequence evolution.

Key words: *nuclear receptors, protein sequences, phylogeny, vertebrate, analysis of variance*

Introduction

Nuclear receptors are profoundly important for the regulation of growth and differentiation of many tissues in metazoic animals. Among their numerous functions, nuclear receptor proteins play a crucial role in regulating mineral metabolism, bone metabolism, homeostasis and other biological processes[1–4]. These functions are carried out by transcription control, mediated by heterodimerization and homodimerization of the receptors[5]. Nuclear receptors are characterized by a li-

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gand-binding region; a DNA-binding region and several hinge regions. The protein sequences of the ligand- and DNA-binding regions are well conserved in evolutionary terms[6].

The nuclear receptors super family includes five major subfamilies[7]: 1) thyroid hormone receptors, 2) steroid receptors, 3) retinoic acid receptors, 4) peroxisome proliferator-activated receptors and 5) vitamin D receptor group. However, many nuclear receptors are žorphan', having no known ligand. Nuclear receptors are found in metazoans, as simple as cnidaria (jellyfish)[5,8]. Fragments of steroid receptor sequences have been detected in lampreys[8]. One may hence suppose that with the emergence of the first vertebrates about 830 million years ago[9], all members of the steroid receptor family already existed. It is assumed that nuclear receptors arose from a single ancestor that has evolved into an entire family via gene duplications and exon shuffling[5].

The present paper concerns steroid receptors and the vitamin D receptor. These receptors are unique to vertebrates[5] and are crucial in bone turnover, and in particular in calcium metabolism regulation. This circumstance led to »an explosion« of the genetic research of the corresponding receptor hormone genes in humans. Vitamin D receptor (VDR) estrogen receptor (ER) and androgen receptor (AR) genes belong to the most prominent candidate genes that are assumed to influence bone mass and mineral density[10–13]. The main reason for this interest is a growing problem of osteoporosis, affecting the elderly, both sexes and all racial groups. Genetic risk factors are the major contributors to the development of osteoporosis, due to their involvement in bone mass loss. The above receptor genes have been implicated among the most plausible candidate genes in the regulation of bone mass. It is therefore of great interest to reveal the major pattern in the evolution of human nuclear receptors since their first appearance in primitive vertebrates. It is of special interest to determine whether and to what extent the dramatic changes in ecology and skeletal anatomy of the vertebrates associated with a transition from the water to the land, to air breathing and to a tetrapod movement, etc., affected the structure of the steroid receptors and vitamin D receptor.

Molecular data suggest that steroid receptor genes arose from a single ancestor by way of 3 duplications, two of which occurred before the divergence of jawed vertebrates from the agnatha and another duplication before the divergence of teleost fish from cartilagenous fish[8,14]. Estrogen receptor is likely to have appeared first, then diverging into subtypes alpha and beta[15]. Progesterone receptor, androgen receptor, and mineralocorticoid and glucocorticoid receptors diverged afterwards.

ER binds 17β estradiol (E2) and AR binds testosterone. Both proteins belong to the steroid receptor group[14]. Estradiol and testosterone are powerful steroids and have many target organs, including the male and female reproductive tracts, mammary glands, and skeletal and cardiovascular systems. The combined action of estradiol and testosterone is responsible for the onset of puberty, gonad maturation and maintaining bone density and vascular elasticity in both males and females[16]. Estradiol especially strongly affects bone density[17]. VDR binds vitamin D, which is converted to calcitriol. Calcitriol induces the synthesis of osteocalcin, the most abundant noncollagenous protein in bone. Vitamin D deficiency in humans and other mammals causes severe bone metabolism disorders. Divergence of vertebrate species gave rise to numerous orthologues of steroid receptors and vitamin D receptors. Estradiol and testosterone have similar structure and differ in position C3, where estradiol

has an –OH group while testosterone has a ketone (Figures 1a and 1b). Vitamin D (calciferol, 1,25-dihydroxy-vitamin D3) is also an active steroid. As seen in Figure 1(c), vitamin D possesses several similarities to steroids but contains a different group in position C17 and contains three rings rather than four.

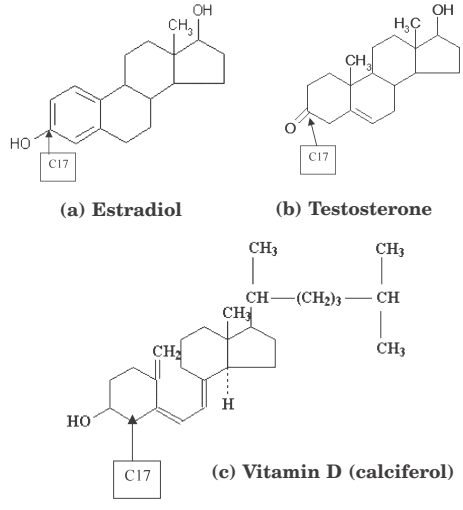

Fig. 1. Molecular structure of estradiol (a), testosterone (b), and vitamin D (calciferol) (c).

The environmental requirements of mineral metabolism in various taxonomic groups and species – fish, homeothermic and isothermic tetrapods and avians – are vastly different. Gene duplications and long divergence time provide proteins ample opportunities for accumulating dissimilarities. Therefore, we may assume that proteins that operate under different requirements will be dissimilar. On the other hand, however, these proteins maintained similar functional roles throughout vertebrate evolution. A remarkable similarity is maintained among nuclear receptor sequences in general and steroid receptors in particular, despite the long divergence time[7]. What, then, are the forces that influence the amino-acid sequences of nuclear receptors, specifically nuclear receptors of *Homo sapiens*? Can we expect greater similarity between nuclear receptors of the same ligand in various taxonomic groups, or do the different requirements of receptors in various taxonomic groups effect sequences in a certain taxonomic groups in a specific way?

The major aim of this study was to elucidate how much of the total variation among nuclear receptors is attributed to differences between the taxonomic groups for the specific receptor (i.e. orthologous) and how much to difference between the different proteins within the taxon. To achieve the goal of the present study, analysis of molecular variance was performed on two steroid receptors, AR and ER, and an intracellular receptor VDR. By determining whether the variance is greater between orthologous or between different proteins, we will be able to shed some light on the evolutionary processes that created current similarities and dissimilarities between proteins.

Materials and Methods

Sequences, alignment and pyhlogenetic analysis

Publicly available protein sequences of nuclear receptors were obtained for all major vertebrate groups: mammals, reptiles/avians, amphibia and teleost fish. All sequences were extracted from Entrez database (www.ncbi.nlm.nih.gov/entrez/). The sequences included are listed in Table 1. In total, information of 57 protein sequences was available. The sequences were aligned by ClustalX[18] using default parameters and phylogenetic trees were viewed by TreeView program. (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

TABLE 1
NUCLEAR RECEPTOR SEQUENCES INCLUDED IN THIS STUDY

Estrogen receptor		Androgen receptor		Vitamin D receptor	
Mammals:		Mammals:		Mammals:	
Human	P03372	Human	P10275	Human	NM_000376
Mouse	NM_010157	Mouse	P19091	Mouse	NM_009504
Rat	NM_012754	Rat	P15207	Rat	S24174
Macaque	AF119229	Macaque	O97952	Tamarin	AAK48863
Cow	Y18017	Chimpanzee	O97775		
Horse	AF124093	Baboon	O97960		
Sheep	AY0333393	Lemur	O97776		
Pig	AF164957	Pig	BAB33376		
Hamster	AF181077	Dog	Q9TT90		
		Rabbit	P49699		
Birds:		Birds:		Birds:	
Quail	AF434713	Canary	L25901	Quail	I50451
Chicken	X03805				
Zebra finch	L79911				
Reptiles:		Reptiles:		Reptiles:	
Lizard	AB055221	N/A		Snake	AJ286886
Crocodile	AB055220			Turtle	AJ286870
Iguana	AF095911			Crocodile	AJ011391
Amphibia:		Amphibia:		Amphibia:	
Frog	P81559	Frog		Frog	U91846
Fish:		Fish:		Fish:	
Goldfish	AY055725	Eel	JG0194	Halibut 1	BAA95016
Zebrafish	AF349414	Trout	BAA32784	Halibut 2	BAA95015
Salmon	AY049952	Haliochoeres	AAG48340	Zebrafish	AF164512
Trout	AJ289883	Red seabream	BAA33451		
Catfish	AF253506	Nile Tilapia	AB045212		
Croaker	AF298183	Haplochromis	AAD25074		
Gilthead seabream	AJ006039				
Red seabream	AB007453				
Medaka	P50241				
Nile tilapia	Q9YH33				
Blue tilapia	P50240				

Reconstruction of ancestral sequences

Since we aimed to compare taxonomic groups rather than individual sequences, and since for each given protein, data on different species for each taxonomic group were available, we chose to reconstruct the common ancestor of each taxonomic unit (mammals, reptiles, birds, amphibians and fish). The ancestral sequences of the respective taxonomic groups were reconstructed by PAML software[19]. PAML uses a maximum-likelihood matrix of the substitution rate of the 20 amino acids and a phylogenetic tree of the sequences which ancestors we aim to reconstruct, to determine the most likely amino acid in each node of the tree, for all the positions in the protein. The result of this process is one reconstructed ancestral sequence for each taxonomic group – mammalian AR, mammalian ER, and so forth. These

sequences were aligned by ClustalX[18], a distance matrix was created for each alignment, and a Multi Dimensional Scaling (MDS) plot[20] and an Analysis of Molecular Variance (AMOVA) analysis[21] were performed. A more detailed account of AMOVA is provided below.

Multidimensional Scaling (MDS) is used for plotting distance matrices, creating a graphical representation of similarities and dissimilarities between sequences. Two 2-dimensional MDS plots have been created for the reconstructed ancestral sequences by SPSS software (release 10.0.5, standard version). Analysis of molecular variance was performed by AMOVA software[21], in order to determine the variance components in protein sequences attributable to two main factors: 1) taxonomic group and 2) type of nuclear receptor. AMOVA is based on the classic Analysis of Variance (ANOVA). In one -way ANOVA, variance *among* groups is compared to variance *within* groups. The null hypothesis in ANOVA is that the variance among groups is equal to the variance within groups. AMOVA is similar to ANOVA, with the same null hypothesis, but the variance used for computations is molecular distance rather than simple arithmetic means and standard deviations and testing is done by permutation. The molecular distance was computed by a software for analysis of phylogenetic data, ClustalX[18], which aligns the sequences while grading the distance between each pair of sequences according to the number of gaps, mismatches in the alignment, and provides a pairwise distance matrix for the 14 consensus sequences. In relation to their functional role, the available protein sequences, for each AR, ER and VDR can be divided into three groups of sequences (see below). The null hypothesis was, as previously stated, that the variance among the three types of protein sequences within each taxonomic group (orthologous proteins) is not larger than the variance between the different proteins within the group (paralogous proteins). We intended to check whether the null hypothesis is valid, or whether the variance among groups is indeed larger than the variance within groups. To test the above null hypothesis, four AMOVA analyses were performed on sequences, namely,

a) full-length sequence;

b) DNA-binding region;

c) ligand-binding region;

d) non-binding region.

Results

Phylogeny of ancestral sequences

The phylogenetic tree of the entire set of 57 vertebrate ER, AR and VDR sequences is shown in Figure 2. Three clusters can be clearly distinguished, corresponding to the three proteins and showing that sequences of each specific protein are located on a different branch. Within each protein-specific cluster, fish and terrestrial vertebrates are located on different branches. Other sub-clusters such as reptiles and mammals can also be distinguished on each branch. *Homo sapiens* proteins consistently cluster with mammal proteins, tending to neighbor other primates. Interestingly, human and rat VDR sequences are virtually identical.

Figure 3 shows the phylogenetic tree of the reconstructed ancestral sequences of the different taxonomic groups. Basically this figure is clearly reminiscent of Figure 2 and AR, ER and VDR sequences are seemingly monophyletic. For all three proteins, orthologous proteins are clustered together, i.e. sequences cluster by protein rather than by taxonomic group. Again, human protein sequences when examined as a separate taxon were consistently attached to the corresponding mammalian node (not shown).

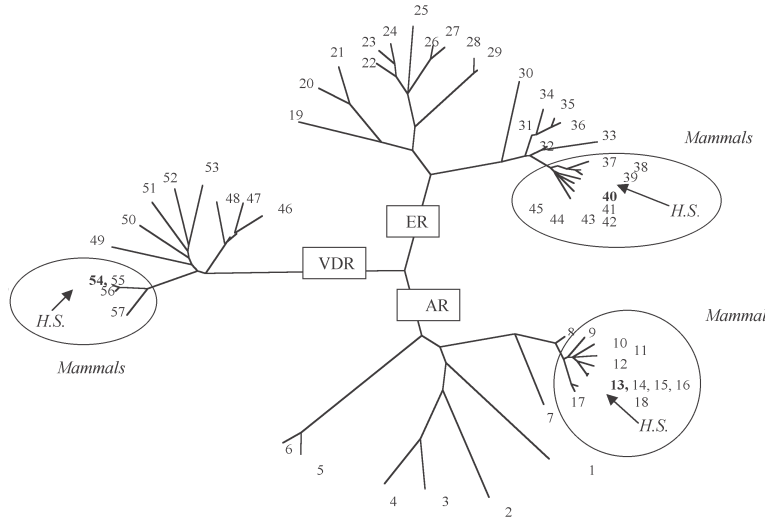

Fig. 2. Phylogenetic tree of AR, ER and VDR sequences. AR = androgen receptor, ER = estrogen receptor, VDR = vitamin D receptor H.S. = Homo sapiens

AR

Fish
1 AR Eel
2 AR Trout
3 AR Halichoeres
4 AR Red seabream
5 AR Nile tilapia
6 AR Haplochromis

Amphibia
7 AR Frog

Birds
8 AR Canary

Mammals
9 AR Rabbit
10 AR Pig
11 AR Dog
12 AR Lemur
13 AR Human
14 AR Baboon
15 AR Chimpanzee
16 AR Macaque
17 AR Rat
18 AR Mouse

ER

Fish
19 ER Catfish
20 ER Zebrafish
21 ER Goldfish
22 ER Croaker
23 ER Gilthead seabream
24 ER Red seabream
25 ER Medaka fish
26 ER Nile tilapia
27 ER Blue tilapia
28 ER Trout
29 ER Salmon

Amphibia
30 ER Frog

Reptiles
31 ER Crocodile
32 ER Lizard
33 ER Iguana

Birds
34 ER Quail
35 ER Chicken
36 ER Zebra finch

Mammals
37 ER Hamster
38 ER Mouse
39 ER Rat
40 ER Human
41 ER Macacque
42 ER Pig
43 ER Sheep
44 ER Horse
45 ER Cow

VDR

Fish
46 VDR Halibut1
47 VDR Halibut2
48 VDR Zebrafish

Amphibia
49 VDR Frog

Reptiles
50 VDR Crocodile
51 VDR Snake
52 VDR Turtle

Birds
53 VDR Quail

Mammals
54 VDR Human
55 VDR Rat
56 VDR Tamarin
57 VDR Mouse

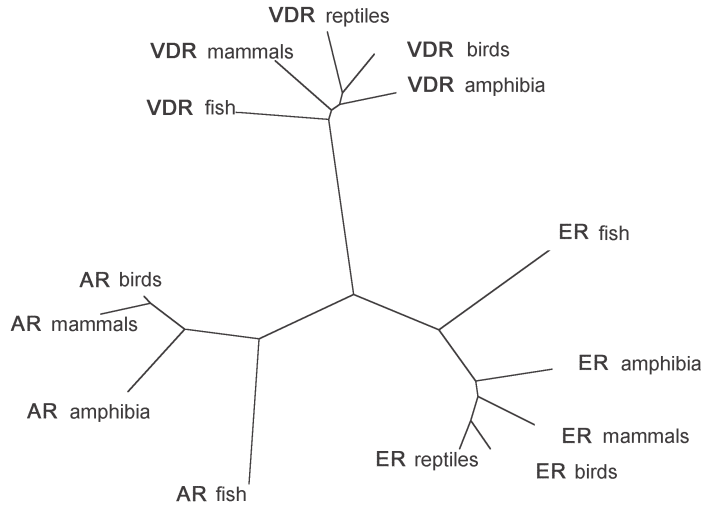

Fig. 3. Phylogenetic tree of the ancestral sequences of AR, ER and VDR.

TABLE 2
GROUPING BY PROTEINS – AR, ER, VDR

	Sequence	Variance components	
		Among paralogous proteins	Among orthologous proteins
a	Full-length	62.47%	37.53%
b	DNA-binding region	93.78%	6.22%
c	ligand-binding	70.21%	29.79%
d	Regions that do not bind DNA or ligand	47.14%	52.86%

Analysis of molecular variance (AMOVA)

To quantify the effect of taxonomic group and type of protein, we performed AMOVA on complete protein sequences and on binding and non-binding regions (see Materials and Methods). The results shown in Table 2 indicate that when complete protein sequences are compared, nearly 63% of the variance is attributable to a variance between the proteins and about 37% is accounted for a variance among taxons within proteins. When only the DNA- and ligand-binding regions are examined, 94% and 70% of the variance, respectively, were attributable to a variance among proteins and only 6% and 30% result from the variance within the proteins. That is to say, that the majority of the protein sequences variation was attributable to paralogous differences and much less to orthologous differences. In the non-binding regions of the studied sequences, the situation tends to be altered: 47% of the variance was explained by paralogous differences among proteins and 53% – by taxonomic differences within each protein in average. The human sequences in all instances, when examined separately, followed the general pattern, always clustering by a protein and with mammals.

MDS plot

To illustrate these findings, we provide Figures 4a and 4b that show the MDS plots for the DNA-binding region and non-binding regions, respectively. In the first case (Figure 4a) it is clearly seen that each group of proteins is distinct from the others. ER protein sequences are clustered in the upper right quadrant, AR proteins are clustered in the lower right quadrant while VDR proteins are to the left and spread along the 0 score of the dimension 2. As seen, there is no overlapping between the three groups of proteins. On the contrary, within each protein specific group, there is a remarkable overlap between molecules belonging to different taxonomic groups.

The picture is very different when distances between non-binding regions are

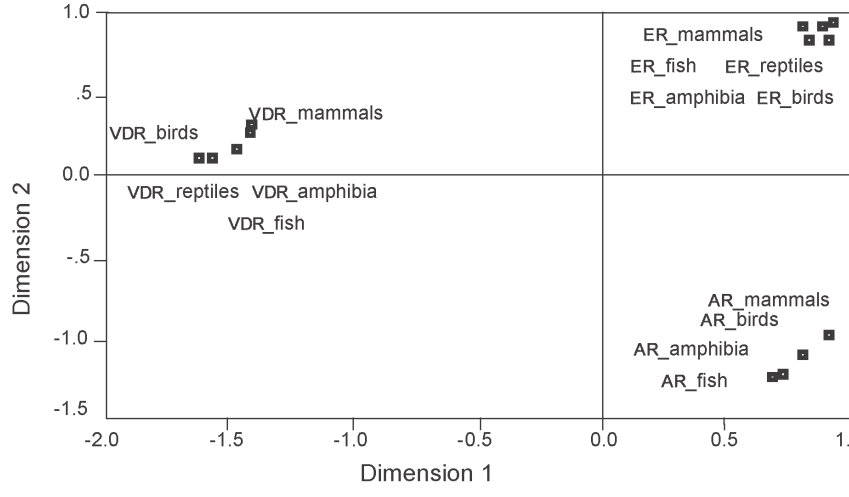

Fig. 4a. 2-dimensional MDS plot of nuclear receptor anceptor proteins – DNA binding region.

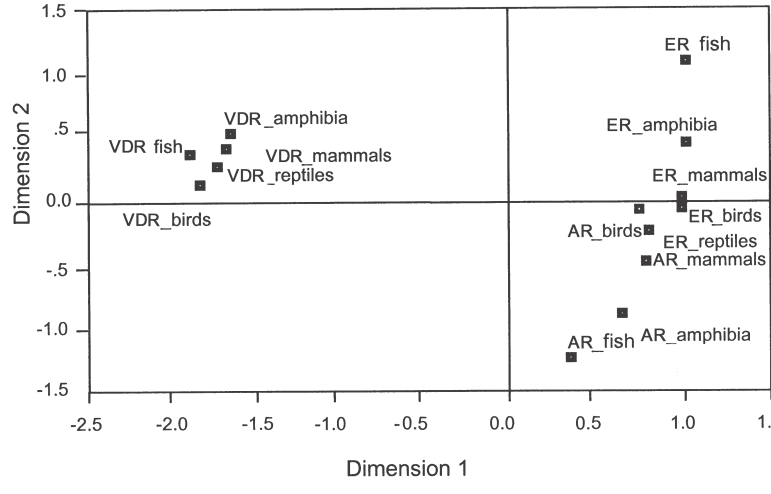

Fig. 4b. 2-dimensional MDS plot of nuclear receptor anceptor proteins – non-binding region.

plotted (Figure 4b). ER and AR protein sequences are located in the right upper and lower quadrants. However, the distances between the sequences within each protein are much larger than in Figure 4a and the sequences are not clustered into two distinct groups but are spread in one large cluster. VDR sequences, however, located separately in the upper left quadrant, although again the distances between the taxonomic groups are larger than those seen in Figure 4a.

Discussion

As mentioned in the Introduction, there are six known steroid receptors: esterogen receptors (ER) α and β, progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)[14]. These receptors apparently emerged from a single ancestral receptor by a series of gene duplications[5,8,14]. Each receptor has a unique role in bone metabolism, in regulating reproductive functions, or both. Baker[22] describes the most recent advances in the evolution of these proteins.

In the present paper we examined the major evolutionary patterns of AR, ER and VDR in 57 representatives of the main taxonomic groups of vertebrates. Data on protein sequences included teleost fish, amphibians, reptiles, birds and mammals. The phylogenetic trees of the protein sequences and their reconstructed ancestral sequences, as well as the MDS plots, showed three distinct clusters of sequences, each cluster containing only AR, ER or VDR sequences. Analysis of molecular variance in nuclear receptors showed that the similarity between vertebrate orthologs of each receptor is much greater than the similarity between different receptors in the same taxonomic class.

The phylogenetic tree of 57 nuclear receptors shown in Figure 2 is in agreement with previous molecular studies[5,6,8,23] and with the fossil record[24]. The sequences are clustered into three groups in accordance to the type of nuclear receptor. In each cluster, the topology of the branches of taxonomic groups conforms to the known vertebrate phylogeny. In all three clusters, human sequences unexceptionally cluster with other mammal sequences, within the branches of their respective nuclear receptors. Hence, human sequences behave according to the same constraints that affected the evolution of sequences in other species.

Since the tree constructed of the entire set of vertebrate sequences complies with known vertebrate evolution, and since each taxonomic group according to a given protein contained different species, we reconstructed 'ancestor' sequences representing the corresponding taxonomic groups for each type of nuclear receptor. The phylogenetic tree of the 'ancestor' sequences, shown in Figure 3, also conforms with currently acceptable data. This tree also demonstrates three distinct clusters of sequences and a typical topology of the taxonomic groups within each cluster.

As demonstrated in the MDS plot (Figure 4a), DNA binding regions of VDR sequences are much less »clustered« than sex steroid receptor sequences, i.e., within each group of ortholog receptors, similarity within AR and ER receptors is stronger than within VDR receptors. This can be explained by the fact that sex (and also adrenal) steroid receptors form dimers and complexes with other proteins, namely heat shock protein 90[5, 25]. Hence, we may assume that the molecular evolution of these sequences is influenced by protein-protein interactions in addition to other evolutionary constraints. These interactions are likely to pose a strong selective pressure on sex steroids that does not apply for VDR. Figure 4b illustrates the distances between non-binding regions of AR, ER and VDR. Here distances

within groups are larger than the distances in Figure 4a. Also, AR and ER sequences do not form two distinct clusters but merge as the distances within the groups become larger and the distances between the groups are smaller. This may suggest that selective pressure on non -binding regions of nuclear receptors is weaker than on DNA- and ligand-binding regions, more amino-acid replacements are tolerated in these regions, hence the larger distances between sequences.

The results of AMOVA varied substantially when the analysis was performed on different domains of nuclear receptors. In DNA- and ligand-binding domains, the similarity between orthologs is very high, 93% and 70% respectively (Table 2). In non-binding domains, only about 50% of the variance can be attributed to differences among paralogous proteins. The AMOVA results can be explained by the fact that the DNA- and ligand binding regions are much more conserved than non -binding regions. The different orthologs of each receptor essentially bind the same ligand and recognize a conserved DNA sequence. Hence, similarities between nuclear receptor proteins can be attributed to:

a) Stabilizing selective pressure due to the same ligand (which can explain similarities among all ortholog receptors of a specific molecule) or similar ligands (which can explain similarities between receptors of ligands with similar structure, such as steroid receptors).
b) Slow rate of evolution of these specific genes as a result of slow accumulation of neutral replacements[8].
c) Convergent evolution due to environmental constraints.

The latter option seems not very feasible, since the environmental requirements are overwhelmingly different for fish homeothermic and isothermic tetrapods and avians. The choice between the two other possibilities is difficult. Thornton[8] suggested that ER was the first steroid receptor. A series of genome-wide duplications created several copies (paralogs) of this sequence, of which one conserved its function as estrogen receptor and the others evolved into androgen, progesterone, glucocorticoid and mineralocorticoid receptors. The rate of evolution of the conserved estrogen receptor was lower than the rate of evolution of the other descendents. According to Baker[26], nuclear receptor evolution has slowed considerably in land vertebrates, and in addition to the relatively short divergence time of mammals, not many changes have accumulated in the human protein in relation to other mammal proteins.

However, the comparison of variation in binding (especially DNA – binding) and non-binding regions showed much lower variation in the former, regardless of the type of the protein and its evolutionary age. One can, therefore, assume that because the evolutionary age of the whole molecule is the same, then if the basic mutation rate is independent of the corresponding DNA sequence (e.g. the same in average for the whole sequence of the receptor protein), then binding and non-binding regions are under the different selective pressure. In other words, the binding region of each of the 3 tested proteins was and still probably is exposed to a much stronger stabilizing selection pressure. Non-binding regions showed less clear pattern in discrimination between the proteins as well as between the taxonomic groups. This situation may be indicative of their relatively random changes, likely due to an accumulation of mutations and random genetic drift.

In conclusion, in the nuclear receptor evolution, most of the variance between protein sequences can be attributable to differences between the DNA- and ligand

-binding regions of different receptors and is not affected by affiliation of a sequence to a certain taxonomic group. In addition, the evolution of human nuclear receptors takes the same course as the evolution of other mammal nuclear receptor sequences.

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FILOGENIJA NUKLEARNIH RECEPTORA KRALJEŽNJAKA – ANALIZA KOMPONENTI VARIJANCE U SEKVENCAMA PROTEINA

SAŽETAK

Nuklearni receptori (NR) čine veliku obitelj proteina i igraju ključnu ulogu u regulaciji metabolizma minerala i fiziološkoj homeostazi različitih organskih sustava. Cilj ove studije bio je razlučiti može li se varijanca između nuklearnih receptora estrogena, androgena i vitamina D u različitih vrsta kralježnjaka uključujući ljude, pripisati

razlikama između taksonomskih skupina unutar specifičnog receptora (odnosno, između ortologa) ili između različitih proteina unutar istog taksona (tj. između paralognih gena). Publicirani podaci o sekvencama 57 proteina ispitivanih nuklearnih receptora korišteno je za filogenetske analize. Rezultati su pokazali da u DNK i »ligand-binding« regijama, 94% i 70% varijance se može pripisati razlikama između tri paralogna proteina. Sekvence čovjeka, zajedno s ortolozima sisavaca, konzistentno se klasteriraju unutar tri skupine sekvenci nuklearnih receptora, što jasno pokazuje da evolucija sekvenci čovjeka se ne razlikuje od evolucije sekvenci drugih sisavaca.