FULL PAPER *Wildlife Science*

Dioxin Sensitivity-Related Two Critical Amino Acids of Arylhydrocarbon Receptor May Not Correlate with the Taxonomy or Phylogeny in Avian Species

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(Received 7 April 2013/Accepted 20 July 2013/Published online in J-STAGE 2 August 2013)

ABSTRACT. There are two arylhydrocarbon receptor (AhR) isoforms in birds, AhR1 and AhR2. The varying sensitivity of AhR is reported to be related to two critical amino acids at positions 325 and 381 in the AhR1 ligand-binding domain. In this study, seven avian species whose *in vivo* dioxin sensitivity was known, and 13 species with no data regarding their *in vivo* dioxin sensitivity were examined. The two critical amino acids in the ligand-binding domain were investigated in avian species, and the results were compared with the taxonomy or phylogenetic trees for the bird AhR proteins. We found that the two critical amino acids did not correlate with the taxonomy or phylogeny of these proteins, suggesting that dioxin sensitivity was independent of taxonomy.
KEY WORDS: aryl hydrocarbon receptor, avian species, dioxin, ligand-binding domain.

doi: 10.1292/jvms.13-0179; *J. Vet. Med. Sci.* 75(12): 1577–1583, 2013

Environmental pollutants, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs), can induce serious toxicity in avian species. The types of toxicity induced include teratogenic, immunotoxic and reproductive toxicity [1, 2, 19, 23, 31]. The dioxin concentration required to induce these types of toxicity varies significantly between birds [3]. The large difference in dioxin sensitivity among avian species is reported to be dependent on the arylhydrocarbon receptor (AhR) protein [11, 17], which has a role in the induction of toxicity [10, 21, 27].

AhR is a basic-helix-loop-helix/*Per Arnt Sim* (PAS) family protein and a transcription factor activated by ligand binding [6]. When not bound to a ligand, AhR remains in the cytosol, forming a complex with heat shock protein 90 (HSP90), AhR-associated protein (XAP2 or ARA9) and p23 [7, 24]. Once bound to a ligand, AhR is translocated to the nucleus [32] where it forms a heterodimer with an AhR nuclear translocator (Arnt), which then binds to the xenobiotic responsive element (XRE) [20, 28]. After binding to XRE, transcription of the CYP1A1, CYP1A2 and AhR repressor (AhRR) genes is activated [13].

Avian species have two AhR isoforms, AhR1 and AhR2 [33, 34], whereas most mammals possess only one. The dominant isoform of AhR differs among bird species [18], and there are large differences in function, even within the same AhR isoform. For example, although avian AhR1s are highly conserved (>90%) among species, there are large interspecies differences in their sensitivity to dioxins, which can be explained by differences in their ligand-binding affinities and transactivation abilities.

It is reported that AhR sensitivity can be predicted from the two amino acids at positions 325 and 381 of AhR1 [17]. Chicken is well known to be the only avian species which has a sensitive type of AhR, however, the sensitivity of AhRs in broad avian species is still unclear. In this study, several kinds of avian species, which were chosen from phylogenetic tree of bird, were investigated to determine their dioxin sensitivity. The amino acid sequences of AhR1 and AhR2 were determined for each species, and compared to their taxonomic and phylogenetic classifications.

MATERIALS AND METHODS

Animals: Bird species analyzed in this study were selected considering clade of phylogenetic tree based on DNA sequences [12]. A one-year-old female blue-eared pheasant (*Crossoptilon auritum*), a male ruddy shelduck (*Tadorna ferruginea*), a one-year-old male mallard (*Anas platyrhynchos*), two male great horned owls (*Bubo virginianus*), two male and one female bar-headed goose (*Anser indicus*), one male Indian peafowl (*Pavo cristatus*), one 12-year-old male goose (*Anser anser*), one 19-year-old female black-headed ibis (*Threskiornis melanocephalus*), one female swan goose (*Anser cygnoides*), two male snowy owls (*Bubo scandiacus*), one female Chilean flamingo (*Phoenicopterus chilensis*), one eight-year-old male Humboldt penguin (*Spheniscus humboldti*), one female cape barren goose (*Cereopsis novaehollandiae*) and one gender-undetermined black-crowned night

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Table 1. Primers for avian AhR

	Forward 5'-sequence-3'	Reverse 5'-sequence-3'
Avian AHR Full	CAGGATGAACCCCAATGTCAC	GTCACATAAATCCACTAGATGCCAAA
Avian AHR1-1	GGATGAACCCCAATGTCACCTA	ATCGTCCTTGAAAATTCATA
Avian AHR1-2	TCATCTGCAGGTTACGATGCCT	ACACAGACTCATCTTGCCTTA
Avian AHR1-3	TGCCCTTCATGTTTGCCACTGGTGA	TCCAATTTGTGAACATCCCAT
Avian AHR1-4	CAGCTCTGTCAAAAGATGAAA	TTACATAAATCCACTAGA

heron (*Nycticorax nycticorax*), were provided by Maruyama Zoo (Sapporo, Japan). These animals died due to accidental injury or disease, such as enteritis. Their livers were immediately frozen in liquid nitrogen and stored at −80°C until use. All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

cDNA cloning and sequencing of AhR: Hepatic total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.) and reverse-transcribed to cDNA using Oligo(dT). Partial AhR1 and AhR2 DNA sequences were amplified by PCR using the primers listed in Table 1. The PCR parameters for the amplification of AhR1 were as follows: 94°C for 2 min, then 94°C for 30 sec, 66°C for 45 sec and 72°C for 3 min for 35 cycles, followed by 72°C for 5 min. PCR products were subject to direct-sequencing using primers Avian AhR1-1 to 4 and an annealing temperature of 50°C; the forward and reverse AhR2 primers were used in pairs with an annealing temperature of 63°C. For obtaining DNA sequence information for the ligand-binding domain, the primers: AhR2-LBD-F 5'-TCTCCAGACAAAGCACAAGCTGGAC-3' and AhR2-LBD-R 5'-GTACAGGACTGCTTCCCCCGTG-3' were used. Reproducibility of sequence was confirmed at least three times.

Phylogenetic tree and amino acid sequence alignment of AhR1 and AhR2: DNA sequences of avian AhR1 were aligned by CLUSTAL W using Molecular Evolutionary Genetics Analysis (MEGA) 5 [30]. The accession numbers for the sequences included in this analysis were: ostrich AhR1 (AB820092), blue eared-pheasant AhR1 (AB820094, AB820095), Indian peafowl AhR1 (AB820097), swan goose AhR1 (AB820099), bar-headed goose AhR1 (AB820101), goose AhR1 (AB820103), mallard AhR1 (AB820105, AB820106), ruddy shelduck AhR1 (AB820108), cape barren goose AhR1 (AB820110), black-headed ibis AhR1 (AB820112), Humboldt penguin AhR1 (AB820113), Chilean flamingo AhR1 (AB820115, AB820116), black-crowned night heron AhR1 (AB820118, AB820119), snowy owl AhR1 (AB820121), great horned owl AhR1 (AB820122, AB820123), peregrine falcon AhR1 (AB560859), common cormorant AhR1 (AB109545), black-footed albatross AhR1 (AB106109), common tern AhR1 (AF192503), chicken AhR (AF192502), ostrich AhR2 (AB920093), blue eared-pheasant AhR2 (AB820096), Indian peafowl AhR2 (AB820098), swan goose AhR2 (AB820100), bar-headed goose AhR2 (AB820102), goose AhR2 (AB820104), mallard AhR2 (AB820107), ruddy shelduck AhR2 (AB820109), cape barren goose AhR2 (AB820111), Humboldt penguin AhR2 (AB820114), Chilean flamingo AhR2 (AB820117), black-crowned night heron AhR2 (AB820120), great horned owl AhR2 (AB820124), peregrine falcon AhR2 (AB560860), black-footed albatross AhR2 (AB106110), common cormorant AhR2 (AB287294) and chicken AhR2 (XM421887). Human AhR (L19872) was added as an outgroup. For AhR2, an alignment was performed using ~250 bases of DNA of the ligand-binding domain and excluded areas containing gaps. A phylogenetic tree was constructed by the Maximum likelihood method based on MEGA5 program. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed [9]. Tamura-Nei model [29] was applied into the nucleotide sequences.

RESULTS

Two critical amino acids in the AhR1 and AhR2 proteins: Based on the two critical amino acids in the ligand-binding domain of AhR1 [17], the avian species we examined could be divided into three groups. The first group, with amino acids 325-Ile and 381-Ser, consisted of the ostrich and chicken. The chicken is reported to be a highly sensitive species to TCDD [14, 17, 18, 34]. The blue-eared pheasant, Indian peafowl, black-footed albatross and swan goose composed the second group, possessing amino acids 325-Ile and 381-Ala. Other avian species, including the bar-headed goose, goose, mallard, ruddy shelduck, cape barren goose, snowy owl, great horned owl, peregrine falcon, black-headed ibis, Humboldt penguin, Chilean flamingo, common cormorant and the black-crowned night heron, belonged to the last group, harboring amino acids 325-Val and 381-Ala (Figs. 1 and 2; Table 2).

The species could also be divided into three groups according to the amino acids in the AhR2 ligand-binding domain. The group possessing the amino acids 325-Leu and 381-Ala comprised the ostrich, blue-eared pheasant and Indian peafowl. The chicken is the only one species to constitute the group of species to possess 325-Val and 381-Ser AhR2. Other avian species belonged to the group harboring the amino acids 325-Val and 381-Ala (Figs. 1 and 2; Table 2).

AhR1 and AhR2 phylogenetic analyses: The phylogenetic tree constructed from the avian AhR1 sequences indicated that the ostrich was distinct from the other avian species. Also, *Galliformes*, including chicken, pheasant and peafowl, *Ciconiiformes*, including albatross, tern, cormorant, penguin, flamingo, falcon and heron and *Strigiformes,* including the snowy owl and great horned owl, grouped close together

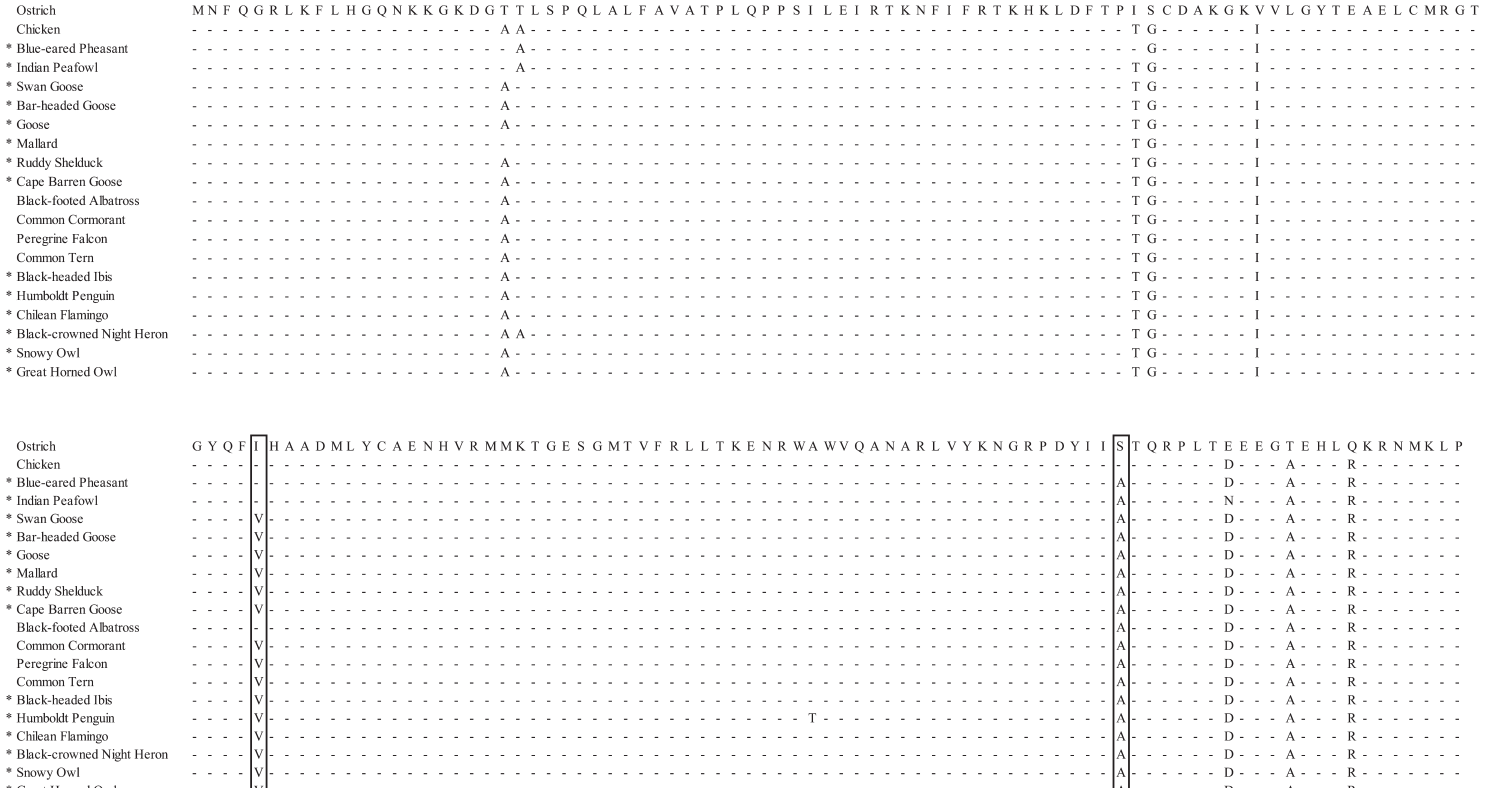

Fig. 1. Amino acid sequence alignment of AhR1. The amino acid sequences of the ligand-binding domain from AhR1s were aligned using the ClustalX2 software, and the accession numbers are listed in the Materials and Methods. Boxes indicate the two critical amino acids at positions 325 and 381. * indicates the species whose AhRs are cloned and sequenced in this study.

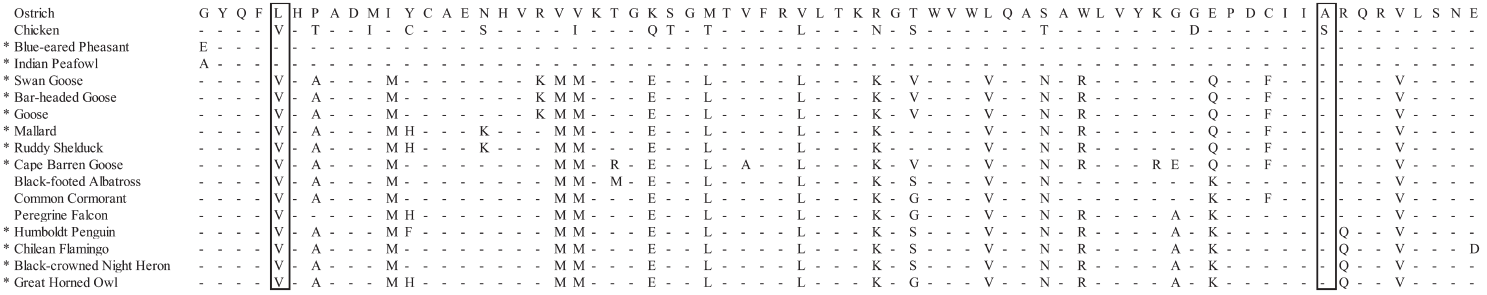

Fig. 2. Amino acid sequence alignment of AhR2. The amino acid sequences of part of the ligand-binding domain from AhR2s were aligned using the ClustalX2 software, and the accession numbers are listed in the Materials and Methods. Boxes indicate the two critical amino acids at positions 325 and 381. * indicates the species whose AhRs are cloned and sequenced in this study. Snowy owl, common tern and black-headed ibis are not shown in this figure.

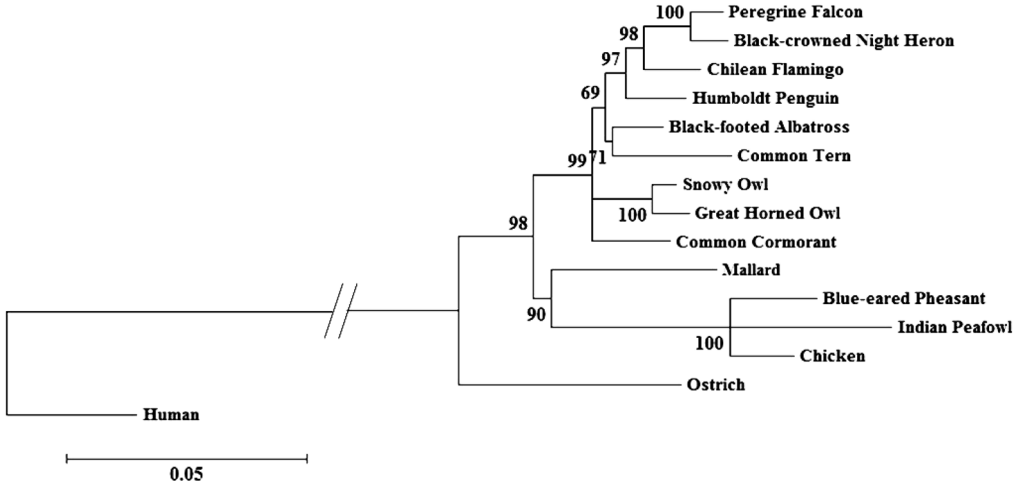

Fig. 3. Phylogenetic analysis of avian AhR1. DNA sequences of AhR1s were aligned by CLUSTAL W using the MEGA5 program. Human AhR (L19872) was added as an outgroup. Alignment was performed with a length of about 2,000 bases, including the functional domains, PAS-A, PAS-B and the Q-rich domains and excluded the regions containing gaps. The phylogenetic tree was constructed by ML method using MEGA5. The number of bootstrap replications was set to 500. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Tamura-Nei model was applied in nucleotide sequences. Swan goose, bar-headed goose, goose, ruddy shelduck, cape barren goose and black-headed ibis are not shown in this figure.

Table 2. The critical amino acids in ligand-binding domains of AhR1 and AhR2 with *in vivo* sensitivity

	AhR1		AhR2		Order	*In vivo* Sensitivity
	325	381	325	381		
Ostrich	I	S	L	A	St	
Chicken	I	S	V	S	G	High [16]
*Blue-eared Pheasant	I	A	L	A	G	Middle [3, 22][a)]
*Indian Peafowl	I	A	L	A	G	
*Swan Goose	I	A	V	A	A	
*Bar-headed Goose	V	A	V	A	A	
*Goose	V	A	V	A	A	low [5]
*Mallard	V	A	V	A	A	low [3, 5, 16]
*Ruddy Shelduck	V	A	V	A	A	
*Cape Barren Goose	V	A	V	A	A	
Black-footed Albatross	I	A	V	A	C	
Common Cormorant	V	A	V	A	C	low [26][b)]
Peregrine Falcon	V	A	V	A	C	low [15][c)]
Common Tern	V	A	–	–	C	low [4, 15]
*Black-headed Ibis	V	A	V	A	C	
*Humboldt Penguin	V	A	V	A	C	
*Chilean Flamingo	V	A	V	A	C	
*Black-crowned Night Heron	V	A	V	A	C	
*Snowy Owl	V	A	V	A	Sg	
*Great Horned Owl	V	A	V	A	Sg	

The two amino acids in AhR1 and AhR2 at positions 325 and 381 of each avian species were indicated. I: isoleucine, S: serine, A: alanine, V: valine, L: leucine. * indicates the species whose AhRs are cloned and sequenced in this study. Abbreviations for each order are; St: *Struthioniformes*, G: *Galliformes*, A: *Anseriformes*, C: *Ciconiiformes* and Sg: *Strigiformes*. a) *In vivo* sensitivity of common pheasant or ring-necked pheasant (*Phasianus colchicus*). b) *In vivo* sensitivity of double-crested cormorant (*Phalacrocorax auritus*). c) *In vivo* sensitivity of American kestrel (*Falco sparverius*).

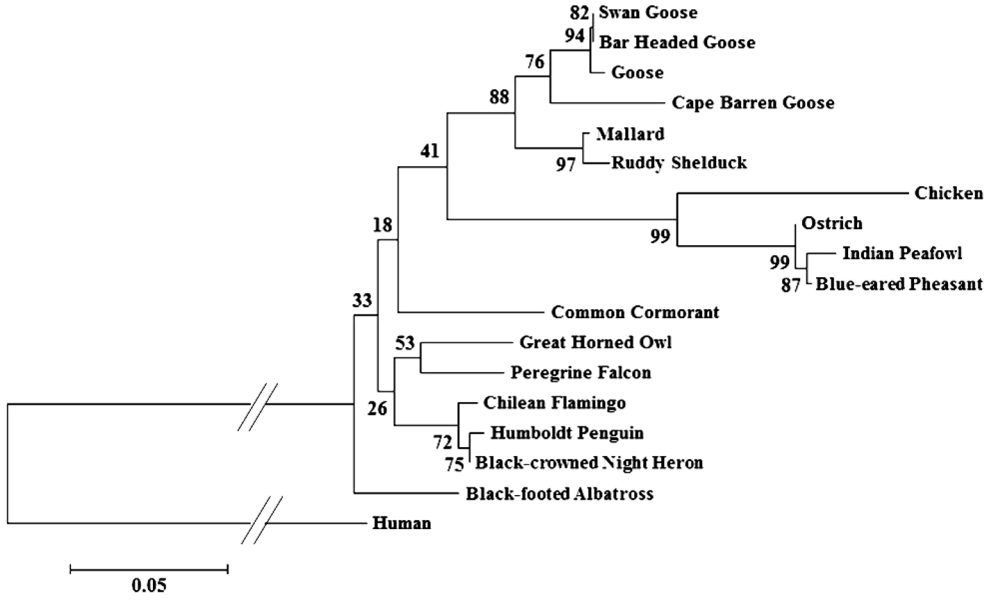

Fig. 4. Phylogenetic analysis of avian AhR2. DNA sequences of AhR2s were aligned using the by CLUSTAL W using MEGA5 program. Human AhR (L19872) was added as an outgroup. Alignment was performed at a length of about 200 bases, a region of the ligand-binding domain, and also excluded regions containing gaps. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The phylogenetic tree was constructed by ML method using MEGA5. Tamura-Nei model was applied in nucleotide sequences. Snowy owl and black-headed ibis are not shown in this figure.

on the tree. These groupings were consistent with the taxonomic groupings of the avian species (Fig. 3).

In the case of AhR2, the phylogenetic tree was also linked to the taxonomy of the species with the exception of the ostrich and great horned owl. The orders *Galliformes, Ciconiiformes* and *Anseriformes* in phylogenetic tree were all assembled the same as that of AhR1 (Fig. 4).

DISCUSSION

In mammals, several factors have been reported to determine the ligand-binding affinity or dioxin sensitivity to AhR-induced toxicity. In C57BL/6 and DBA/2 mice, large differences in AhR ligand-binding affinity result from AhR point mutations at codon 375 in the ligand-binding domain [8]. Similarly, Han/Wistar rats, which are insensitive to dioxin, harbor a point mutation at position 497 in AhR [25].

Avian species are distinct in that they possess two AhR isoforms, AhR1 and AhR2, and the type of AhR dominantly expressed varies among avian species [18]. Most species dominantly express AhR1, and AhR1 ligand-binding affinity is reported to directly correlate with CYP1A transactivation ability [18]. Critical mutations which decide dioxin sensitivity have been found in avian species at positions 325 and 381 of AhR1 [17]. Head *et al.* [14] showed these two amino acids are effective for predicting avian dioxin sensitivity. In the study, avian species are divided into three groups according to the key amino acids in AhR1. The most sensitive group is with AhR1 of 325-Ile and 381-Ser, middle for 325-Ile and 381-Ala, and the least sensitive for 325-Val and 381-Ala. In this study, the blue-eared pheasant, goose and mallard are newly classified according to the two amino acids, and we found that the grouping corresponds with *in vivo* sensitivity also in these cases.

In the current study, we investigated whether similar avian species would have similar levels of dioxin sensitivity. Indeed, the phylogenetic trees constructed from the amino acids sequences of AhRs gave results in-keeping with the evolutionary history of these proteins [12]. Unexpectedly, even though the amino acids sequences of AhRs highly reflected the taxonomy, the identity of the two critical amino acids, at positions 325 and 381, did not correspond to the phylogenetic trees of AhR or taxonomy. In fact, these two amino acids were not conserved in the orders, *Galliformes* and *Ciconiiformes* [14, 34]. Therefore, our new findings suggested that these key amino acids at positions 325 and 381 are independent from the other amino acids sequences of AhRs, so that they cannot be predicted from the phylogenetic tree or from taxonomy. That is, the ligand-binding affinity or the dioxin sensitivity of each avian AhR protein cannot be determined from the taxonomy.

The amino acid sequence of the AhR1 ligand-binding domain was highly conserved among different species. In the ostrich, multiple amino acid changes were found throughout the ligand-binding domain, compared with other avian species. This ostrich AhR1 was reported to possess high trans-

activation ability in our previous study, similar to chicken AhR1 [11].

Regarding the two critical amino acids, avian species harboring isoleucine at position 325 in AhR1 were chicken, peafowl, pheasant, albatross, swan goose and ostrich. All species in the order *Galliformes* examined in this study, including chicken, peafowl and pheasant, possessed isoleucine at position 325. However, albatross and swan goose were the only species to possess this amino acid in their respective orders, *Ciconiiformes* and *Anseriformes*. Only two of the avian species, chicken and ostrich from the orders *Galliformes* and *Struthioniformes*, respectively, possessed a serine at position 381. Species within the order *Galliformes*, such as peafowl and pheasant, did not harbor this amino acid. Taken together, these findings indicate that the two critical amino acids are independent of taxonomy or even phylogeny of the full-length amino acid sequence of AhR1. Therefore, we conclude that it is difficult to predict the dioxin sensitivity of avian species from taxonomy or evolutionary history.

In the case of avian AhR2, the amino acid sequence of the ligand-binding domain was not as highly conserved as that of AhR1. In terms of the two critical amino acids in the ligand-binding domain, the only species to possess 381-Ser in AhR2 was the chicken. This amino acid was not conserved in the AhR1 and AhR2 of ostrich. In addition, we identified the amino acid leucine at position 325 in pheasant, peafowl and ostrich. This amino acid has not previously been reported at this position, and its corresponding AhR function is therefore unknown. It will be of interest to investigate the function or ligand-binding affinity of this type of AhR protein. Future researches are also required to fully investigate the avian AhR2 protein and its role in avian dioxin sensitivity.

In conclusion, the two critical amino acids at positions 325 and 381 in the ligand-binding domain of AhR were investigated in several bird species, and the results were compared with the taxonomy or phylogenetic trees for the AhR proteins. The two critical amino acids did not correlate with the taxonomy or phylogeny of these proteins, and dioxin sensitivity was independent of taxonomy.

ACKNOWLEDGMENTS. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, which was awarded to M. Ishizuka (No. 24248056). We sincerely thank Mr. Noboru Takaesu in Sapporo Maruyama Zoo for the collection of animal samples.

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