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# Red squirrel (*Sciurus vulgaris*) and Alpine accentor (*Prunella collaris*) ALAD gene molecular genetic characteristics

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## Introduction

In recent years, in the alpine and sub alpine area of the High Tatra Mts., threshold concentration of heavy metal - lead has often been detected. Recent research studies contain data of alpine vegetation affected by the airborne lead and other metals containing pollutants (Janiga 2002). It is well known, that especially in the Tatra Mountains there are significant correlations between the orography and rainfall composition (Gazda and Hanzel 1978, Konček *et al.* 1973).

Long-term exposure to the limit values for lead in the environment has adverse impact on the organisms of vertebrates. In birds, there are changes in the kidneys, especially mitochondrial alteration and loss of enzyme activity involved in the synthesis of heme - ALAD (δ-aminolevulinic acid dehydratase) (Hutton 1980). Studies focused on bioaccumulation of heavy metals in birds show the same enzymatic ALAD activity inhibition and increasing metal accumulation in the tissues (Vanparys *et al.* 2008). The reduction of ALAD activity correlates with the accumulation of lead, which was recorded in waterfowl, bird raptors or passerine species (Dieter *et al.* 1979, Beyer *et al.* 1988, Hoffman *et al.* 1981, Henny *et al.* 1994).

In mammals, including humans, chronic exposure to lead causes the changes in haematopoiesis, gastrointestinal tract, liver and kidneys and accumulation in bone tissues is also detected. The most significant lead intoxication effect is decrease of heme synthesis. The synthesis process is affected by high erythrocyte membrane permeability for lead, which is ten times higher than for calcium. Final effect of lead accumulation in erythrocytes is interference with heme synthesis degrees. Inhibition starts at blood lead concentration 15μg/100 ml. The ferochelatase, coproporphyrinogen oxidase and ALA – dehydrogenase are inhibited. The most sensitive indicator of lead intoxication is the heme biosynthesis enzyme – ALAD (Ava and Luz 2000).

*ALAD polymorphism and lead susceptibility*

It has been recognized that ALAD, the second enzyme in the heme biosynthetic pathway, plays a role in the pathogenesis of lead poisoning (Bergdahl *et al.* 1997). The inhibition of erythrocyte ALAD activity is a sensitive indicator of exposure to lead and has been used as a diagnostic tool (Sakai and Morita 1996, Secchi *et al.* 1974).

Animal models of variants in the ALAD gene may help in defining the role of the enzyme in lead toxicity. It has been discovered that two common laboratory strains of mice differ in their expression of the ALAD gene (Ava *et al.* 2000).

Structure of murine ALAD gene was analyzed by Bishop *et al.* (1996). Comparison of murine and human ALAD gene showed 70% similarity in nucleotide sequence within 250 bp long fragments. This comparison confirms the presence of conservative and functionally important areas such as ALAD gene promoters in the area (Bishop *et al.* 1996). Human ALAD gene is located on 9q34 chromosome and characterized by the presence of two co dominant forms - ALAD1 and ALAD2 (Hsieh *et al.* 2000, Petrucci *et al.* 1982, Onaja and Claudio 2000, Süzen *et al.* 2004).

The essence of these two ALAD gene forms is transversion G/C on position 177 in ALAD2 gene sequence (Wetmur *et al.* 1991a). The presence of this nucleotide substitution offer Msp-1 restriction site, which is used to distinguish the forms of this gene. Similarly, works conducted in human population have identified substitutions on position 168 (Rsa-1 restriction site), 397 (G/A transition, Cfr-10 restriction site). There are several restriction sites for Msp-1 in ALAD gene, especially in intron sequence around exon 4. For lead sensitivity change is exon 4 transversion crucial (Wetmur *et al.* 1991b). The size of the human ALAD gene is about 16 kilobytes (Wetmur 1994). Phylogenetic studies indicate a high conservatism of ALAD protein (Shaik *et al.* 2009).

*Chromosome mapping and gene localisation in Sciuridae and Passeriformes*

It is important to point out that triggering of this mechanism allowed a catastrophic increase in the rate of chromosome evolution in rodents. This event obviously took place after Sciuridae (tree squirrels, chipmunks, marmots, and ground squirrels) split from the main lineage of Rodentia. Detailed localization of human chromosome probes on chromosomes of many squirrels and reciprocal painting showed that the squirrel genomes are highly conserved, are similar to the human and ancestral genomes, and have several signatures suggesting a common origin for Rodents and Lagomorphs (Graphodatsky 2007, Li *et al.* 2004, 2006, Richard *et al.* 2003, Stanyon *et al.* 2003).

Recently the use of human whole-chromosome painting probes to detect regions of homology with

squirrels (Richard *et al.* 2003a, Stanyon *et al.* 2003, Li *et al.* 2004, 2006) has demonstrated that non-murid rodents display more conserved genomes, confirming earlier conclusions based on comparative banding analysis (Petit *et al.* 1984, Viegas-Pequignot *et al.* 1986). These findings suggest that the use of non-murids for higher-order systematic and comparative genomic studies will be fruitful, but at this point the extent of the retention of a conserved genome in other rodent lineages is unknown. In an attempt at redress it were performed cross species painting experiments using human chromosome painting probes on representatives of three main non-murid lineages represented by Pedetidae, Castoridae, and Dipodidae. This allows a direct comparison with previously published data on Sciuridae and expands the taxonomic coverage to include representatives of four of the five major evolutionary lineages recognized in Rodentia. These experiments had two main aims: first to clarify phylogenetic relationships among Sciuridae, Pedetidae, Castoridae and Dipodidae (i.e., the Rodentia suborders) and, secondly, to provide insights into the putative ancestral karyotype of Rodentia, thus facilitating comparative genomic studies among placentals (Graphodatsky *et al.* 2008).

Whole genome is known in the Sciuridae family member Ground squirrel (*Spermophilus tridecemlineatus*), or North American Kangaroo rat (*Dipodomys ordine*) of the suborder Sciurognathi.

ALAD gene of Ground squirrel contains 13 exons, the length is 1059 bp and translation product comprises 352 amino acids. Kangaroo rat gene contains also 13 exons, 1,074 bp long transcript which is subsequently translated into 357 amino acids long product. Rodents ALAD gene localisation has been demonstrated on chromosome 4 in *Mus musculus* and chromosome 5 in *Rattus norvegicus*.

Localisation of ALAD gene is known, for example in the field of Passeriformes representative – Zebra finch (*Taeniopygia guttata*). ALAD gene in this species is localized on chromosome 17 in area 1,275,364-1,278,801 bp from start of the gene. Gene contains 8 exons, is 720 bp long and length of translation product is 240 amino acids.

*Comparative genomics of ALAD gene structure and genome conservation level of Squirrel and Prunella*

PCR and subsequent sequencing of ALAD gene selected areas of the animals is possible only in case we have specific primers for this gene available. For primer design is necessary to know the sequence before and behind area of our interest. In cases of the red squirrel and alpine accentor there are two Sciuridea or Passeriformes related organisms with known sequences available.

But it still remains an open question to what extent the zebra finch genome coincides with the alpine accentor and ground squirrel structure of the genome with red squirrel genome.

Indirect answer on this question can give a exon segments sequence comparison of ground squirrel and kangaroo rat, which shows a high degree of similarity. In this case it should be noted that Kangaroo rat is rated within the suborder Sciurognathi, which includes a wide range of rodents. The suborder Sciurognathi includes Sciuridae. Based on the findings above, high coincidence in exon areas of Ground squirrel and Red squirrel with unknown genome sequence should be considered. Similarly, in the alpine accentor case genome of as phylogenetically related animal as possible is essential.

*ALAD gene investigation approaches*

Sequenation of an unknown area, which contains gene of our interest, includes several options. If we know amino acids sequence in protein that is coded by investigated gene, it may be examinated by reverse process when DNA sequence is established according to nucleotide sequence of mRNA. Another option is isolation of mRNA from the tissues and using RT-PCR approaches to obtain cDNA. These approaches are closely connected with complicated biological material collection from living animals. Long-term collection and subsequent genomic DNA isolation from samples offers classical way to find nucleotides sequence via desired DNA segment amplification and sequenation. Here we get again to the question which primers we should select for this purpose. In case we investigate next unknown sequence of ALAD gene, a „walking" primer can be used. To obtain specific products by this technique it's necessary to run more parallel tests, incorporating the amount of random primers in combination with a specific primer (Čikoš *et al.* 2001).

For the purpose of establishing the ALAD gene sequences of alpine accentor and red squirrel I have therefore proposed a procedure based on finding the gene sequences of the most phylogenetically related species in the databases. In cDNA of these related species there are designed primers and obtained products sequenced. In this phase we have exon fragments with their specific sequence. Further primer design is already based on known sequences with gradually bridging individual exons. Obtained nucleotide sequences of introns in the fields allow complete mosaic of ALAD gene (Fig. 1).

Red squirrel is rather common animal and its living environment spans from the plains to the sub alpine level. She expands from the West Europe to East Asia. Rather easy capture with the possibility of isolation of DNA, together with a wide distribution gives a precondition of effective monitoring of the burden of heavy metals in many locations.

Alpine accentor can be found in the mountains of southern Europe to Asia at an altitude of about 2,000m.a.s.l. Similarly, a wide distribution of the animal within the alpine zone makes a prerequisite for monitoring of the accumulation of lead contamination caused by aerosols transferred to the mountains.

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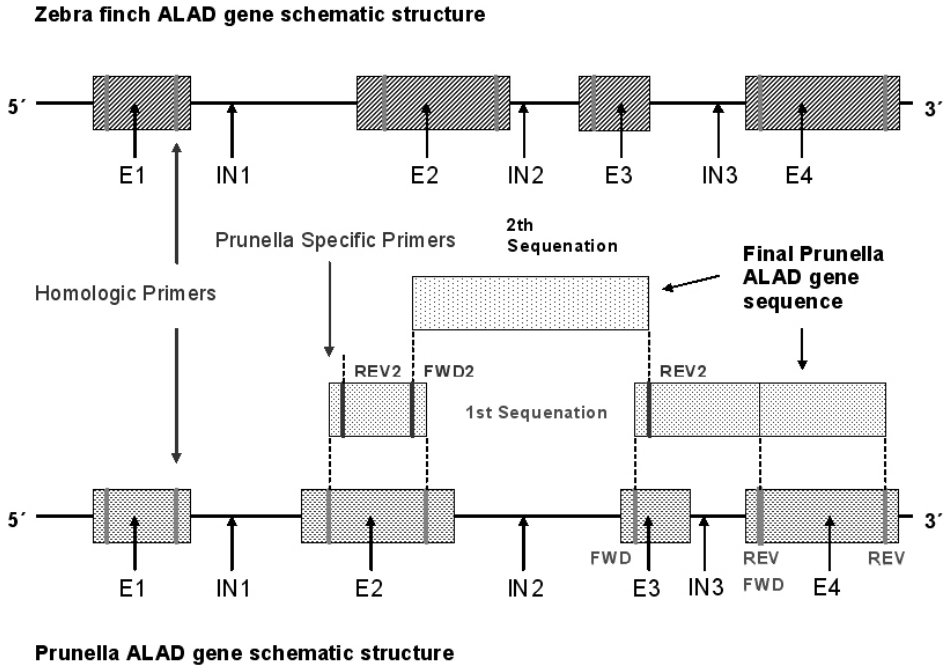


**Fig. 1.** Scheme of a possible sequenation procedure of an unknown ALAD gene sequence of *Prunella collaris* or *P. modularis*. The first step is homologous primer design to the known ALAD gene sequence of zebra finch. In the case of successful amplification of selected sections of the exon sequence are then designed primers in already known gene sequence of *Prunella* (first sequencing). Segments amplificated this way are also sequenced (second sequencing). Overlapping section of thus obtained segments will enable to obtain the ALAD gene sequence throughout its length.

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