Collie eye anomaly (CEA) is a hereditary ocular disorder which is characterized by regional hypo-
plasia of choroids, the highly vascularized layer of the eye that supplies blood and nutrients to the reti-
a, and resulting in an opthalmoscopically detectable window defect in the ocular fundus located tempo-
ally to the optic nerve. Occasionally, tortuous retinal vessels and multiple retinal folds are also observed (Roberts, 1960, 1966; Lowe et al., 2003). The clinical symptoms can vary greatly among af-
fected dogs within one breed, between parent and offspring and within a litter. No medical treatment of the disease is available. The genetic cause of this variability is unknown. Yakely (1972) reported CEA in 70–97% Rough and Smooth Collies in the USA, likewise Bedford (1982a) in Great Britain.

Wallin-Hakanson et al. (2000) observed CEA in approximately 68% of Rough Collies in Sweden. The frequency of CEA in Border Collie breed is lower. Bedford (1982b) reported an approximate 2–3% incidence in Great Britain. However, CEA also affects some other breeds, such as Australian Shepherds (Curtis et al., 1991), Lancashire Heelers (Bedford, 1998) and Shetland Sheepdogs (Barnett and Stades, 1979).

CEA inheritance is autosomal recessive with variable expression and pleomorphism (wide range in the clinical expression of the defect), the genetic ba-

Co: CEA; polymorphism; indel; Piko™ Thermal Cycler; allele frequencies

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colobomas, can develop retinal detachments leading to blindness. In these cases, subretinal and preretinal neovascularization and intraocular haemorrhage can occur (Lowe et al., 2003). In some affected pups older than 7 weeks of age, a previously ophthalmoscopically detectable choroidal hypoplastic defect becomes masked by ingrowths of the tapetal layer or by pigmentation of the retinal pigment epithelium in the nontapetal region. This apparent reversion of the hypoplastic lesion was recognized and termed the “go normal” phenotype (Bjerkas, 1991). Because variation of the expression of CEA disease is recognized and was observed, individuals with any ophthalmoscopic evidence of choroidal hypoplastic lesion are genetically classified as affected, regardless of whether they subsequently appeared unaffected or if they exhibited secondary manifestation of the disease, like coloboma (Yakely et al., 1968; Lowe et al., 2003). Segregation studies of Lowe et al. (2003) indicated that penetrance of the affected phenotype in dogs homozygous for choroidal hypoplasia, the primary CEA phenotype, was less than 100%. Their data also suggested that there was both incomplete penetrance of the CEA phenotype in the homozygous affected dogs and partial penetrance in some heterozygous dogs.

Sequence analysis revealed that all affected dogs shared a homozygous deletion of 7 799 bp in the \textit{NHEJ1} gene (Parker et al., 2007). The entire 7 799-bp deletion comprises nucleotides 28,697,542 to 28,705,340 on chromosome 37 based on CanFam2 assembly (http://genome.ucsc.edu/). It is located within the 67-kb intron 4 of the gene, ~ 460 bp from exon 5. The authors reported that this intronic deletion spans a highly conserved binding domain to which several developmentally important proteins bind. Their work established that the primary CEA mutation arose as a single-disease allele in a common ancestor of herding breeds and highlighted the value of comparative population analysis for refining regions of linkage.

The principal goal of our study was to analyze populations of six purebred dog breeds in the Czech Republic and to introduce an easier, faster and suitable method for routine testing of the \textit{NHEJ1} genotypes for practical purposes of dog breeders.

**MATERIAL AND METHODS**

In total, 379 samples of unclotted blood (approx. 0.5 ml) from purebred dog breeds (Australian Shepherd Dog, Border Collie, Rough Collie, Smooth Collie, Shetland Sheep Dog, and Nova Scotia Duck Tolling Retriever) in the Czech Republic were collected in K3EDTA test tubes.

For the amplification of the \textit{NHEJ1} gene fragment harbouring the indel, PCR analysis was carried out using two sets of primers as described by Parker et al. (2007). The first set of primers: \textit{NHEJ1-F17}, 5’-TCTCACAGGCAGAAAGCTCA-3’, and \textit{NHEJ1-R17}, 5’-CCATTTCATTCTTTGCCAGT-3’, amplified within the insertion, and the second set of primers, \textit{NHEJ1-F20}, 5’-TGGGCTGTT-GAACATTGTGTA-3’, and \textit{NHEJ1-R23}, 5’-CCTTTTTGTTTGCCCTCAGA-3’, amplified across the deletion.

A direct PCR amplification using the whole unclotted blood samples was performed using a Piko\textsuperscript{TM} Thermal Cycler (Finnzymes Instruments Oy, Espoo, Finland). The reaction set-up recommended by the producer was modified in that 8 μl of 20-times diluted blood, 10 μl Phusion\textsuperscript{TM} Flash High Fidelity PCR Master Mix, 1 μl Primer F and 1 μl Primer R (10 pmol/μl) were used. After initial denaturation at 98°C for 5 min, the samples were amplified for 30 cycles at 98°C for 10 s, 60°C for 5 s and 72°C for 15 s; final extension was at 72°C for 1 min. The amplicons were analysed by electrophoresis on 1.5% agarose gels.

**RESULTS AND DISCUSSION**

Amplicons obtained with the two sets of PCR primers were analyzed by agarose gel electrophoresis and genotypes of \textit{NHEJ1} in the individual dogs were identified. Using the first set of primers (F17 and R17), PCR fragments were amplified within the insertion in healthy homozygotes and in carriers. No PCR fragment was amplified in affected homozygotes. When the second set of primers (F20 and F23; amplification across the deletion) was used, no PCR fragment was obtained in healthy homozygotes. PCR fragments were observed in carriers and in affected homozygotes only. Results of PCR analysis and identification of genotypes are presented in Figure 1.

Genotype and allele frequencies in the six purebred dog breeds are presented in Table 1.

High frequencies of the mutant allele were found in Collie breeds. In the Rough Collie we did not identify any healthy individual among 37 analyzed dogs. On the contrary, Australian Shepherd had a
low frequency of the disease allele. In the population of 33 individuals only 3 were carriers and no affected individual was observed.

With this information about the genetic state breeders of Rough Collies in the Czech Republic can improve genetic health as regards the CEA disease, even if it can be very difficult to find a single genetically healthy homozygote partner for breeding at present. In the first instance, it might be useful to mate a carrier to a carrier and produce on average 25% healthy, 50% carriers and 25% affected puppies. When genetic testing and breeding of the genetically healthy homozygotes with the carriers or the affected partners are progressing,

Table 1. Genotype and allele frequencies of NHEJ1 in analyzed dog breeds; “healthy” allele is designated CEA, mutant allele responsible for the disease is designated cea

<table>
<thead>
<tr>
<th>Breed</th>
<th>n</th>
<th>Genotype frequencies</th>
<th>Allele frequencies</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>healthy CEA/CEA</td>
<td>carrier CEA/cea</td>
</tr>
<tr>
<td>Australian Shepherd</td>
<td>33</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>Border Collie</td>
<td>80</td>
<td>51</td>
<td>27</td>
</tr>
<tr>
<td>Rough Collie</td>
<td>37</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Smooth Collie</td>
<td>45</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Shetland Sheep Dog</td>
<td>141</td>
<td>43</td>
<td>75</td>
</tr>
<tr>
<td>Nova Scotia Duck Tolling Retriever</td>
<td>43</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>379</td>
<td>166</td>
<td>158</td>
</tr>
</tbody>
</table>
the frequency of the disease in the population will decrease and the number of genetically healthy animals will increase without loss of some other valuable traits in Collies. Yakely (1972) reported decreased occurrence of affected and carrier individuals through similar selective breeding in Collie breeds in the USA.

Selective breeding in Smooth Collie and Shetland Sheep Dog populations can be used more easily. It is possible to mate one genetically healthy partner with the carrier or affected one and continually test the progeny. This will certainly increase the frequency of healthy individuals and decrease the frequency of carriers and affected individuals in the population.

The low frequencies of the disease allele in Australian Shepherd and Border Collie breeds indicate that affected individuals should be excluded from breeding completely. Bedford et al. (1982b) estimated the low frequency of the mutant allele (cea) at approximately 2–3% in Great Britain, and this is in agreement with our findings.

Selection against CEA disorder in Nova Scotia Duck Tolling Retrievers (frequency of the mutant allele is 0.244) is complicated by the occurrence of another inherited eye disease (progressive rod-cone degeneration) at a higher frequency (unpublished results). The selection against both inherited diseases requires a thoroughly defined breeding policy and preference of the population needs, and not the pursuit of breeders’ individual interests.

The results obtained by the described method were confirmed by the analysis of a randomly selected set of 30 blood samples from clear (genetically healthy), carrier (heterozygotes) and affected (homozygotes with the disease) dogs genotyped in OPTIGEN laboratory (New York, Ithaca, USA) before they were analyzed in our laboratory, with excellent agreements.

CONCLUSIONS

The direct PCR method using a Piko™ Thermal Cycler was applied for the analysis of the NHEJ1 gene, which is responsible for Collie Eye Anomaly (CEA). The method is simple and a very low amount of whole blood is used in PCR analysis (0.5 μl for PCR reaction). The method is very convenient, particularly for the analyses of small and toy breeds of dogs such as Toy Poodle or Yorkshire Terrier puppies. In total, 379 individuals of six dog breeds were genotyped and frequencies of genotypes and alleles were estimated. Suggestions to control the breeding process aimed at the elimination of the disease allele have been proposed.

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