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THESIS

GENOTYPIC AND PHENOTYPIC INVESTIGATION OF PROGRESSIVE RETINAL ATROPHY IN DOGS

METITA SUSSADEE

A Thesis Submitted in Partial Fulfillment of
The Requirements for the Degree of
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Progressive retinal atrophy (PRA) is a group of inherited retinal disorders in domestic dogs. To evaluate outer retinal function, normal parameters of electroretinogram (ERG) was established from 56 normal eyes of four dog breeds, Poodle, Labrador Retriever, Thai Ridgeback and Thai Bangkaew. Phenotypic characterizations and genetic study of PRA in dogs were described. One form of PRA, progressive rod-cone degeneration (PRCD) was chosen for genetic testing in Poodles using the PCR-RFLP technique. In addition, the PRA suspected Thai Bangkaew dogs were screened with 4 candidate gene mutation; *PRCD*, *RPGR, RHO* and *CCDC66* genes. The normal ERG parameters some significant differences among dog breeds were reported. Genetic testing confirmed the *PRCD* gene mutation in suspected Poodles in Thailand. A prevalence of carriers in 50 Poodles and 58 Labrador Retrievers were 12% and 3.45% respectively while the allele frequency of mutant allele were 0.1 and 0.02 respectively. In suspected Thai Bangkaew dogs, the ophthalmoscopic and ERG alterations were compatible with retinal degeneration. The 4 candidate gene mutations were excluded as causes of this disease in this breed. The normal ERG parameters would be useful for evaluation of retinal diseases in dogs. As well as, the genetic testing to identify disease status before breeding should assist to avoid the transmission of affected allele to offspring.

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Student's signature Thesis Advisor's signature

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Metita Sussadee
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LIST OF ABBREVIATIONS

α	=	α subunits of cGMP phosphediesterase
ADPRA	=	autosomal dominant progressive retinal atrophy
β	=	β subunits of cGMP phosphediesterase
bp	=	base pair
^{o}C	=	Degree Celsius
CBC	=	complete blood cell count
cd.s/m^{2}	=	candela per square metre
CCDC66	=	coiled-coil domain containing 66
CD	=	cone degeneration
CFA	=	*Canis familiaris*
CMR	=	canine multifocal retinopathy
CORD1	=	cone-rod degeneration 1
CRD	=	con-rod dystrophies
CRD3	=	cone-rod dystrophy 3
CRD2	=	cone-rod dystrophy 2
CSNB	=	congenital stationary night blindness
cGMP	=	cyclic guanosine monophosphate
CRD	=	Cone-rod dystrophies
DNA	=	deoxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
ERD	=	early retinal degeneration
ERG	=	electroretinography
EtBr	=	ethidium bromide
γ	=	γ subunits of cGMP phosphediesterase
H&E	=	hematoxylin and eosin
Hi-int R&C	=	scotopic higher intensity responses
HMsERG	=	Handheld Multi-species ElectroRetinoGraph
Kb	=	Kilobase
$MgCl_2$	=	magnesium chloride

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LIST OF ABBREVIATIONS (Continued)

ml	=	millitre
mRNA	=	messenger RNA
ms	=	milliseconds
ORF	=	open reading frame
PCR	=	polymerase chain reaction
PD	=	photoreceptor dysplasia
PDC	=	Phosducin
PDE6β	=	phosphodiesterase β-subunit gene
PLR	=	pupillary light reflex
PRA	=	progressive retinal atrophy
PRCD	=	Progressive rod-cone degeneration
R	=	rhodopsin
RCD	=	rod-cone dysplasia
RFLP	=	restriction fragment length polymorphism
RP	=	retinitis pigmentosa
RPE	=	retinal pigment epithelium
RPE65	=	retinal pigment epithelium-specific protein 65kDa
RPGR	=	retinitis pigmentosa GTPase regular gene
SD	=	standard error
sec	=	second
SINE	=	short interspersed nuclear element
SSCP	=	single-strand conformation polymorphism
Std R&C	=	scotopic standard intensity responses
TB	=	Thai Bangkaew
TE	=	Tris-EDTA
Tα	=	α subunit of transducing
Tβ	=	β subunit of transducing
Tγ	=	γ subunit of transducing
µm	=	micrometer

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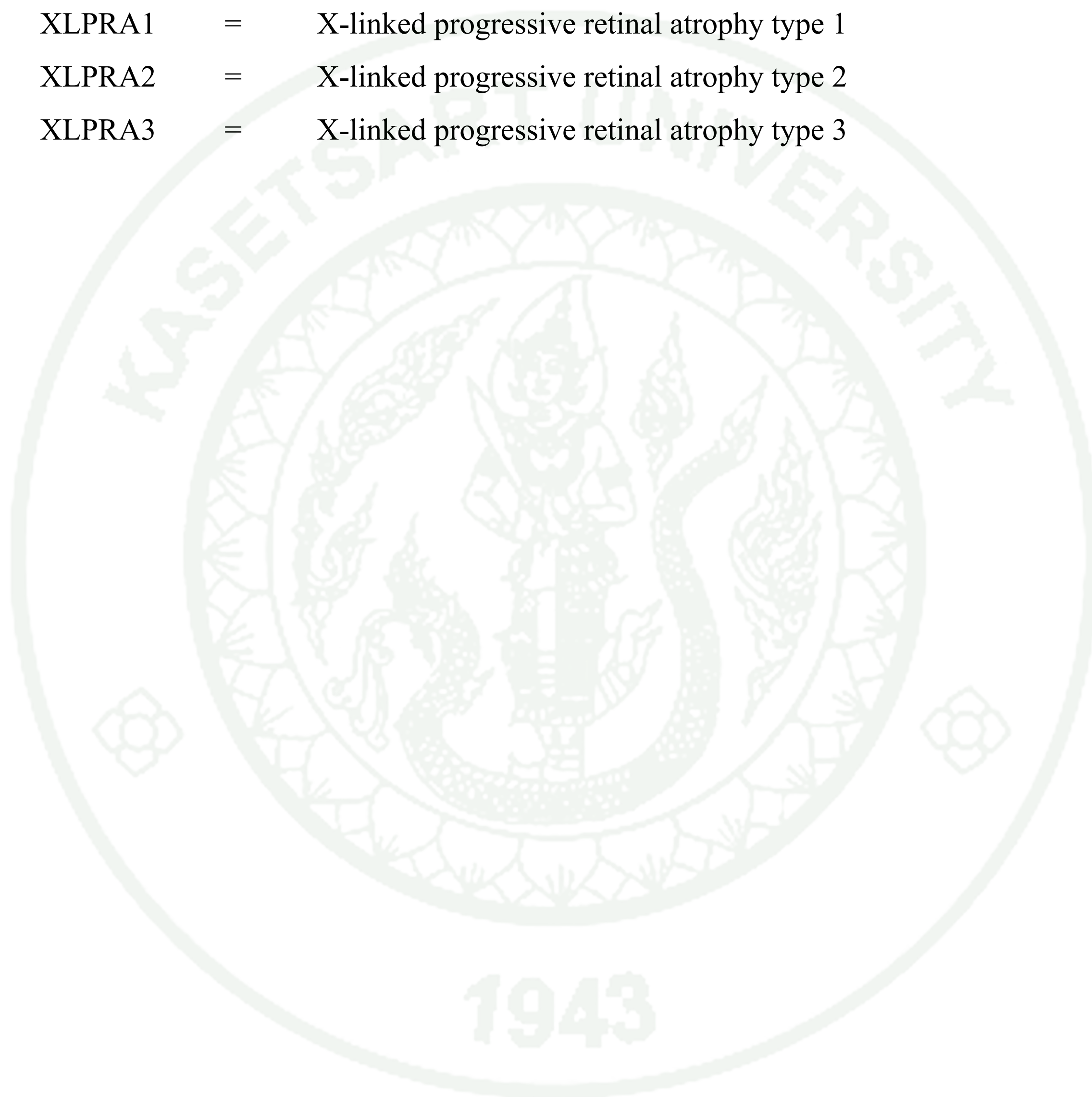

μV	=	microvolts
UV	=	ultraviolet
XLPRA1	=	X-linked progressive retinal atrophy type 1
XLPRA2	=	X-linked progressive retinal atrophy type 2
XLPRA3	=	X-linked progressive retinal atrophy type 3

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GENOTYPIC AND PHENOTYPIC INVESTIGATION OF PROGRESSIVE RETINAL ATROPHY IN DOGS

INTRODUCTION

Canine progressive retinal atrophy (PRA) or degeneration is a group of inherited retinal disorders in domestic dogs (*Canis familiaris*) which involved with several modes of transmission and a large number of genes and mutations (Zangerl *et al.*, 2006; Andre *et al.*, 2008). It has been described in more than 100 dog breeds worldwide. PRA eventually leads to night blindness and followed by permanent blindness in both eyes of affected dogs. The affected dogs resulting in blindness are the causing stress for their owners. The disease has been broadly divided into two main categories; the degenerative conditions or late-onset form which the retina develops normally and then degenerates during the dog's lifetime, and the dysplastic or early-onset form in which the retina develops abnormally. (Narfström and Petersen-Jones, 2007). Most forms of PRA that have been investigated are autosomal recessive. However, there are X- linked and dominantly inherited forms which have been described relatively (Petersen-Jones, 2005). PRA shares many phenotypic and clinical features with human retinitis pigmentosa, in which patients typically lose night vision in young adulthood and progressively lose of rod and cone photoreceptor cells in their later lives (Hartong *et al.*, 2006).

Diagnosis of PRA is based on history taking, clinical signs, ophthalmic examination, electroretinography (ERG), histological finding, and molecular genetic testing (Ofri, 2008). There are some potential difficulties in making an early diagnosis of PRA because of their enormous variation in ophthalmoscopic appearance and the progression of vision loss varies between types of PRA (Narfström and Petersen-Jones, 2007). ERG is an important tool for diagnosis of retinal function in living animals. It usually provides much earlier diagnosis than does an ophthalmoscopic or behavioral examination. However, the problems of variation in each laboratory, individual breed, machine and age affect the ERG parameters. Therefore normal ERG

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in each breed especially certain dog breeds in Thailand which have never reported before, are needed to be established. For molecular genetic testing, identification of mutations responsible for different forms of PRA allows carrier and pre-degenerate animals to be detected. The confirmation and identification of gene mutation including the analysis of gene frequency for the database of the country are substantially beneficial for the success to reduce numbers of PRA affected dogs.

As there are many phenotypic variations of clinical diagnosis and there has never been an in depth study and survey on PRA of dog breeds in Thailand. This research aimed to identify normal ERG parameters in certain dog breeds, Poodle, Labrador Retriever, Thai Ridgeback and Thai Bangkaew and describe the significant differences in ERG parameters of each breed. The characterization, identification of responsible gene mutations in Poodle as well as survey of allele frequency of the disease in Poodle and Labrador Retriever were performed. Moreover, identification of phenotypic characterization and the responsible gene mutations of retinal degeneration in Thai Bangkaew were attempted. These databases of disease diagnosis and genetic frequency will be beneficial for veterinarians and breeders for future diagnosis and proper awareness with this disease.

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OBJECTIVES

1. To establish normal ERG parameters in Poodle, Labrador retriever, Thai Bangkaew and Thai Ridgeback at the age of 2-8 years old.

2. To confirm phenotypic and genotypic of responsible gene mutation of PRA in Poodle in Thailand

3. To analyze the allele frequencies of PRA in Poodle and Labrador Retriever in Thailand

4. To identify phenotypic characterization and the responsible gene mutations of retinal degeneration in Thai Bangkaew.

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LITERATURE REVIEW

1. The dog retina and biochemical pathway

The dog retina is a part of the eye that lies against the back. It is a complex structure and its formation and continued function are controlled by a large number of genes. Potentially mutations in any of the genes that govern retinal structure and function could cause a disease such as progressive retinal atrophy (PRA) (table 1). The retinal functions are collection the light and then transfer to the brain where it is interpreted as vision. There are 10 layers of retina, containing specialized cells called photoreceptors and nerve cells. The retinal photoreceptors are composed of rods, for vision in dim light (night vision), and cones for vision in bright light (day and color vision) (Ofri, 2008). The most common disease of inherited retinopathies is progressive retinal degeneration which usually affects the rods initially, and then cones in later stages of the disease (Andre *et al*., 2008). This disease is related with the mutations in genes coding for proteins in visual pathways. In 2005, Petersen-Jones described the two biochemical pathways which are important for vision. The first pathway is the phototransduction cascade (Figure 1), which converts photons of light that reaches the photoreceptors into an electrical signal for transmission to the inner retina. The second pathway is the visual cycle (Figure 2), which is responsible for regeneration of a vitamin A derivative within the retinal pigment epithelial layer (RPE) prior to transport to the photoreceptor (Petersen-Jones, 2005). The visual transduction process is central to photoreceptor function, and investigation of the genes involved in photoreceptor structure and phototransduction have helped to increase the understanding of retinal degeneration in dogs.

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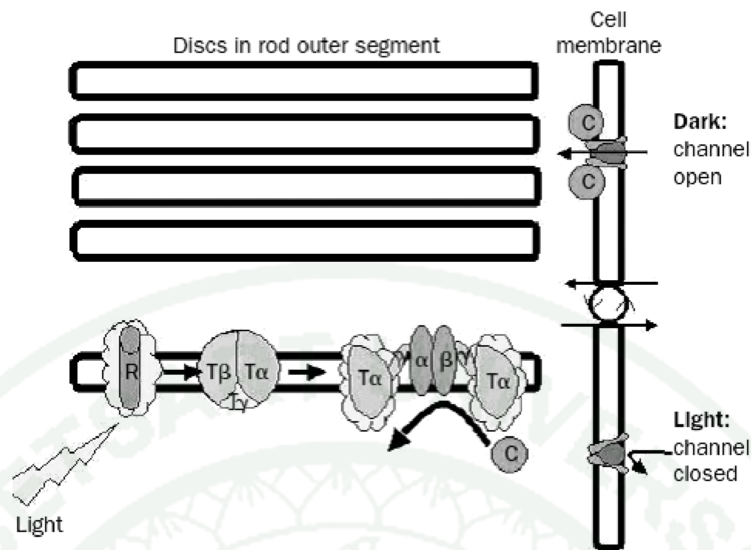

Figure 1 The phototransduction cascade occurs in rod cells. In dim light, more of the cyclic guanosine monophosphate (cGMP) gated channels in the outer segment cell membrane are open, resulting in an influx of cations causing depolarization of the cell. After light initiates the visual transduction cascade, the cGMP is hydrolysed and its concentration decrease. The cGMP channels close and the cell membrane becomes hyperpolarized; R: Rhodopsin, Tα Tβ and T γ: subunit of transducing, α β and γ: subunits of cGMP phosphediesterase, C: cyclic GMP (Petersen-Jones, 2005).

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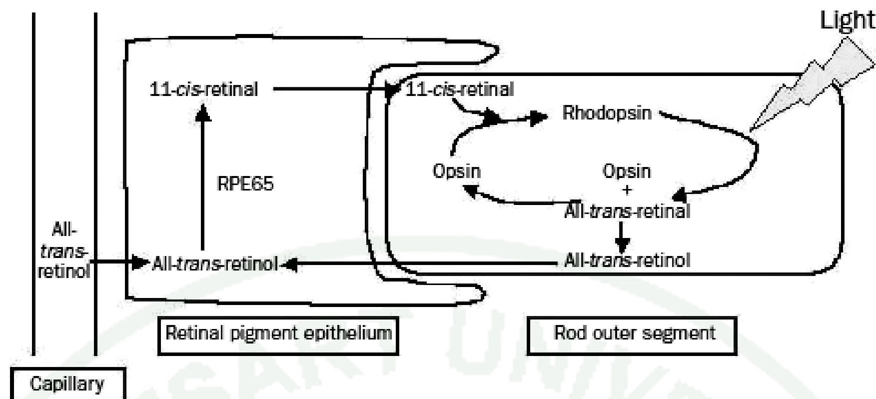

Figure 2 The visual cycle occurs following phototransduction in the rod outer segment. The rhodopsin is converted to opsin and all-trans-retinal and returned to the retinal pigment epithelium for conversion back to 11-cis-retinal. Additional all-trans-retinol is supplied from the capillaries of the choriocapillaris. Note the RPE65 protein plays a role in the conversion in the retinal pigment epithelium (Petersen-Jones, 2005).

2. Classification of progressive retinal degenerations

Retinal disorders can be categorized in various forms, which are summarized in Figure 3 (Ostrander and Ruvinsky, 2012). The degenerative retinal disorders are divided into the stationary and progressive retinal disorders. The stationary retinal conditions are characterized by a pathology which does not deteriorate throughout life. These conditions include the congenital stationary night blindness (CSNB) in Briard, the cone degeneration (CD) in Alaskan Malamute and German Shorthaired Pointer and the canine multifocal retinopathy (CMR) in Pyrenean Mountain Dog.

The other conditions, progressive retinal disorders, are characterized by increasing severity and decreasing visual function over time. Progressive retinal changes during the dog's lifetime invariably lead to complete blindness. These forms are also divided into the progressive retinal atrophy (PRA) and the con-rod dystrophies (CRD). The CRD are disorders predominantly of cones, with rods becoming affected later. These progressive retinal forms include the cone-rod

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degeneration 1 (CORD1) in Miniature Long-haired Dachshund, Cone-rod dystrophy in Standard Wire-haired Dachshund, the cone-rod dystrophy 3 (CRD3) in Glen of Imaal Terrier and the cone-rod dystrophy 2 (CRD2) in Pit Bull Terrier.

For PRAs, they are characterized by initial loss of rod photoreceptor function, followed by that of the cones, and for this reason night blindness is the first significant clinical sign for most affected dogs. The classification of PRA is an issue of different opinions and discussion. Narfström and Petersen-Jones have been subdivided PRA mainly into retinal dysplasia and degeneration. Retinal dysplasia manifests with abnormalities in the photoreceptor that develops prior to maturation of retina, approximately 8 weeks of age in the dog (Gum *et al*, 1984), and the rate of progression along with loss of photoreceptors in the disease process are most often rapid. Whereas, the degenerative diseases also manifest with outer segment degeneration but detected after the retina is fully mature. The affected dog usually diagnosed when older than 8 weeks (Narfström and Petersen-Jones, 2007).

PRA have been broadly divided in early and late-onset. In the form of early-onset photoreceptor degenerations have been described in dogs including Rod-cone dysplasia (RCD) in Irish Setter, Collie and Cardigan Welsh Corgi, Early retinal degeneration (ERD) in Norwegian Elkhound, Photoreceptor dysplasia (PD) in Miniature Schnauzer and X-linked progressive retinal atrophy type 2 (XLPRA2) in Mixed-breed dogs. Whereas, the forms of late-onset includes Progressive rod-cone degeneration (PRCD) in multiple breeds, Autosomal dominant progressive retinal atrophy (ADPRA) in English Mastiff, X-linked progressive retinal atrophy type 1 (XLPRA1) in Siberian Husky and Samoyed, X-linked progressive retinal atrophy type 3 (XLPRA3) in Border Collie and unclassified PRA form of coiled-coil domain containing 66 (CCDC66) in Schapendoes dogs. The age onset and the responsible genes mutation of PRAs are shown in Table1.

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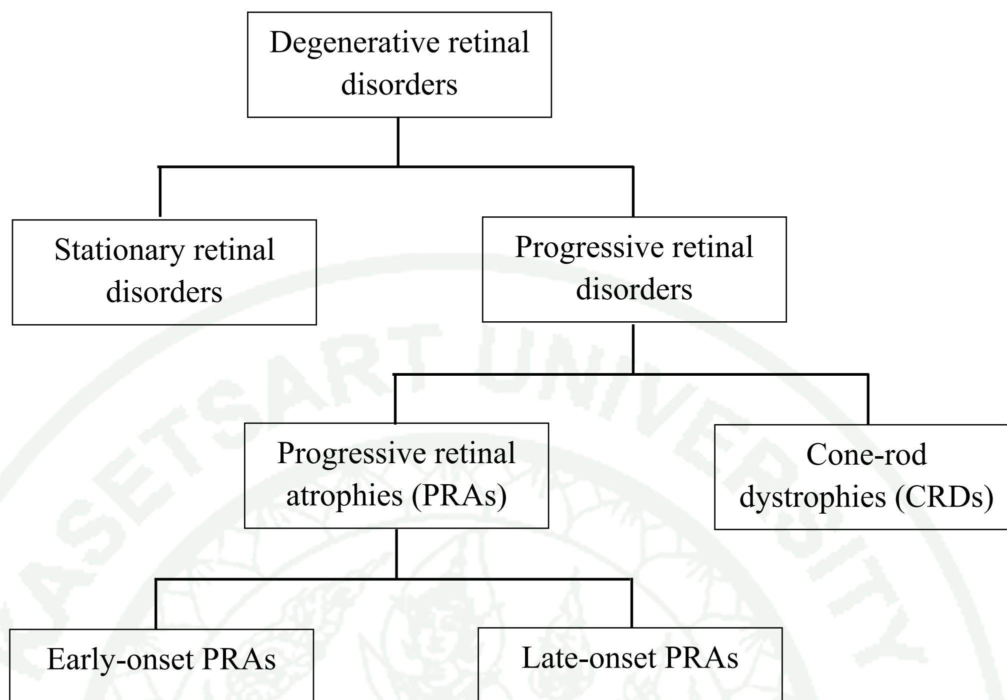

Figure 3 Categorization of canine retinal disorder. (Modified from Ostrander and Ruvinsky, 2012).

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Table 1 Genes associated with progressive retinal atrophy and the age onset of affected dogs.

PRA forms	Locus/ Chromosome	Gene	Breed	ERG abnormalities	Behavioral signs	Ophthalmo-scopic signs
Early onset PRA						
Rod-cone dysplasia	RCD1/CFA3	*PDE6β*	Irish Setter Sloughi	3-6 wk	6-8 wk	12-16 wk
	RCD2/CFA7	*RD3*	Collie	6 wk	6 wk	3.5-4 m
	RCD3/CFA4	*PDE6α*	Cardigan Welsh Corgi	NR	NR	6-16 wk
Early retinal degeneration	ERD/CFA27	*STK38L*	Norwegian Elkhound	5-6 wk	6 wk	6-12 m
Photoreceptor dysplasia	PD/CFA7	*PDC*	Miniature Schnauzer	6-8 wk	6-12 m	1-2 y
X-linked PRA	XLPRA2/ CFA X	*RPGR*	Mixed-breed dogs (Mongrel dogs)	5-6 wk	NR	4 m
Late onset PRA						
Progressive rod-cone degeneration	PRCD/CFA9	*PRCD*	Multiple breeds*	6 m-3 y**	3-5 y**	3-8 y**
Autosomal dominant PRA	ADPRA/ CFA20	*RHO*	English Mastiff Bullmastiff	13 m	NR	NR

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Table 1 (Continued)

PRA forms	Locus/ Chromosome	Gene	Breed	ERG abnormalities	Behavioral signs	Ophthalmo-scopic signs
X-linked PRA	XLPRA1/ CFA X	*RPGR*	Siberian Husky Samoyed	1 y	2-4 y	1.5-2 y
X-linked PRA	XLPRA3/ CFA X	unknown	Border Collie	2 y	3 y	3 y
Unclassified	CCDC66/ CFA20	*CCDC66*	Schapendoes	2-5 y	NR	NR

*American Cocker Spaniel, American Eskimo Dog, Australian Cattle Dog, Australian Shepherd, Australian Stumpy Tail Cattle Dog, Chesapeake Bay Retriever, Chinese Crested Dog, Cockapoo, English Cocker Spaniel, Entelbucher Mountain Dog, Finnish Lapphund, Golden Retriever, Kuvasz, Labradoodle, Labrador Retriever, Lapponian Herder, Miniature and Toy Poodle, Nova Scotia Duck Tolling Retriever, Portuguese Water Dog, Spanish Water Dog, Swedish Lapphund. ** Difference in each breed, NR not record.

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3. PRA Diagnosis

3.1 History and ophthalmoscopic examination

The onset and progression of vision loss varies between the types of PRA. Although, some forms result in behavioral changes of night-blindness in puppies followed by total blindness in the first few years of life, the late-onset forms have the clinical signes in middle-age and result in blindness several years later. Moreover, secondary cataract is common with the later-onset forms of PRA (Clements *et al*., 1996). Thus owners sometimes wrongly assume that the loss of vision is due to the formation of cataracts and take the patients to the veterinarian when they are in the advance stage. The history of disease onset is important to diagnosis and classification the form of PRA. For example, progressive rod-cone degeneration in poodle may be detected by ophthalmoscopic and electroretinographic abnormalities at 3-5 years and 6-9 months respectively. Whereas for rod cone dysplasia in Irish setter is detected at 12-16 weeks and 3-6 weeks of age respectively (Ofri, 2008).

The Ophthalmic examination consists of behavioral testing by photopic and scotopic obstacle tests and pupillary light reflex (PLR) examination, to assess functional retina and postretinal transmission of signals. The menace response (the reaction to a sudden, threatening object coming into the near of view) is used to assess vision. The other tests, such as small cotton balls test, visual placing reflex and dazzle reflex might be included (Narfström and Petersen, 2007). When the retina becomes thinned due to PRA, it allows even more reflection of light back from the tapetum starting at the periphery of the retina. The next changes are a thinning of the retinal blood vessels and the optic nerve head atrophy as the disease progresses (Petersen-Jones, 1998). Therefore, clinical signs of PRA are often remarkable similar including visual impairment, especially in dim light, and ophthalmoscopic signs of retinal degeneration such as bilateral tapetal hyperreflection, attenuation of retinal blood vessels, pale of optic disc, and uneven distribution of the pigment in the nontapetal fundus.

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3.2 Electroretinography

3.2.1 Electrodiagnostic and protocol

Electroretinography (ERG) is a technique to measure electric potentials occurring in the retina in response to light stimulation. It is a reliable diagnostic tool for assessing the function of the retina and commonly used to diagnose retinal disorders before cataract extraction or in cases of unexplained visual loss without ophthalmoscopic abnormalities (Slatter, 2008). The electrochemical changes result in membrane hyperpolarization, reduction of inhibitory neurotransmitter release and initiation of the visual signal to the brain. Recently, an important indication for ERG recording in the dog is the early diagnosis of PRA. In most of the 20 canine breeds in which the disease has been studied electrophysiologically, changes in the ERG appear long before the appearance of clinical signs (Ofri, 2002).

The rod and cone function should be precisely evaluated using separate testing procedures by the use of full-field conditions, such as the Genzfeld stimulator, in order to obtain a uniform distribution of light across the retina. Rod function can be tested through studying the process of dark adaptation (20 min), by stimulating the dark-adapted retina with low levels of light intensity (0.02-0.03 cd/m^2/sec). Whereas, cone function is tested by light adapting , 10 min using white background light with an intensity of 30-40 cd/m^2, in order to desensitize the rod system and then stimulating the cone with high intensity light stimuli using standard flash (2-3 cd/m^2/sec). Cone function is also studied using multiple flashes (flicker) of bright light at a minimum frequency of 30 Hz (Narfström *et al*., 2002, Marmor *et al*., 2009).

The electroretinogram, during retinal stimulation by difference those light intensity, registers the difference of potential captured by electrodes arranged over the cornea (active electrode), next to the lateral canthus (reference electrode) and on the occipital crest (ground electrode). The signals that are captured by electrodes must go through pre-amplifiers and differential amplifiers before they are recorded

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and show different parts of the ERG wave corresponding to different structures within the retina (Figure 4). The main waves are the a-wave for the rods and cones, the b-wave for the bipolar and Muller's cells, and the c-wave for the pigment epithelium (Ekesten, 2007; Ofri, 2008). Typical ERG wave form and ERG parameter measuring are showed in Figure 5.

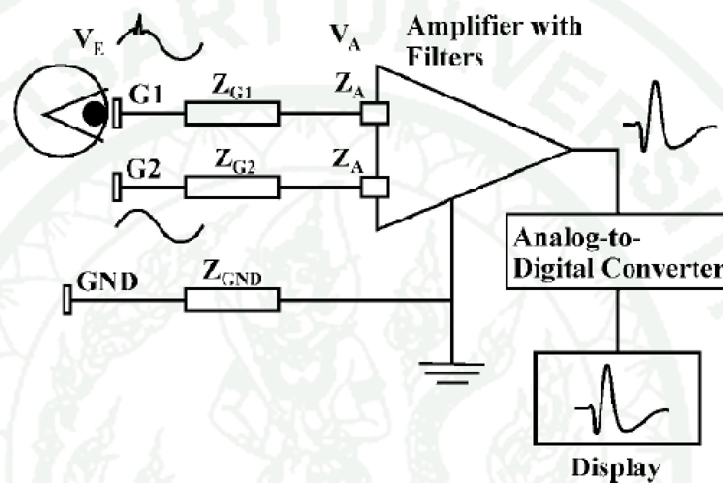

Figure 4 ERG recording set-up. Three electrodes are used to record electrical signal from stimulated retina: an active (G1); a reference (G2); and a ground electrode (GND). The active electrode is placed closer to the eye, whereas the reference electrode is placed a little bit farther away. Signals of the retina pass through the active electrode (V_E). A background sine wave noise appears at both active and reference inputs. The difference between the active and reference input, is amplified by the ERG amplifier. The ground electrode serves as zero reference. The signal is amplified and filtered before it is digitized by an analog-to-digital converter and displayed. Z_{G1}, Z_{G2}, and Z_{GND} are the electrode impedances. Z_A is the amplifier input impedance and V_A the voltage of the signal at the amplifier input (Modified from Komaromy *et al.*, 2002).

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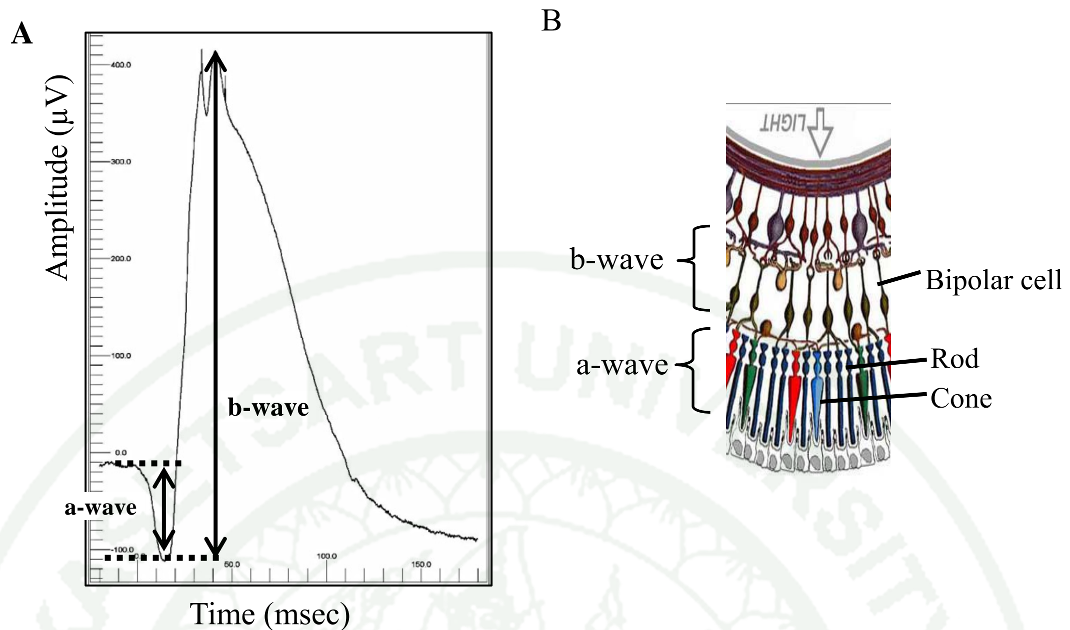

Figure 5 (A) Typical ERG wave form and ERG parameter measuring, a-wave amplitude is measured from the baseline to the wave trough, b-wave amplitude is measured from the a-wave trough to the b-wave peak, a- and b-wave implicit times are measured from the stimulus to the a-wave trough and b-wave peak, respectively; (B) The approximate sites of generation of ERG components, the a-wave mainly from rods and cones and the b-wave from bipolar cells.

3.2.2 ERG parameters

In order to interpret the ERG, three parameters must be considered: latency, implicit time and amplitude. The reports of ERG results include the normal values for the specific breed and age-group, preferably indicating the median values and the limits of normality using the 5th and 95th percentiles (Narfström *et al*., 2002). There are numerous factors which can distort the accuracy and reliability of an ERG recording. Many researchers have focused on these factors including the stage of retinal adaptation (Tuntivanich *et al*., 2005; Yu *et al*., 2007), the electrode type and position (Mentzer *et al*., 2005; Steiss *et al*., 1992), the anesthetic technique (Jeong *et al*., 2009; Lin *et al*., 2009; Norman *et al*., 2008), the age, species and breeds of the

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animal (Itoh *et al*., 2013), the environment and body temperature (Mizota and Adachi-Usami, 2002), the pupil diameter (Marmor *et al*., 2009), eye movement (Nair *et al*., 2011), intraocular pressure (Grozdanic *et al*., 2010), diabetic condition (Safatle *et al*., 2010), the condition of the anterior segment; cataract stage and lens-induced uveitis (Maehara *et al*., 2007) , and the effects of hypercapnia (Varela *et al*., 2010). Moreover, environmental illumination, excessive ambient electrical noise, the intensity of the flash, retinal conditions, the quality of recording equipments and the quality of the solution used between the contact lens electrode and the cornea are all important factors for ERG parameters (EKesten, 2007; Hansho *et al*., 2004; Komaromy *et al*., 2002; Marmor *et al*., 2009). Thus, to use ERG results diagnostically, each laboratory or clinic should establish technical procedures that permit to obtain reproducible ERG result under specific conditions.

The ERG parameters of a few dog breeds have been previously reported. Normal ERG parameters of 15 healthy Beagle dogs were determined using an ERG contact lens electrode with built-in diode light sources (LED-electrode) (Meahara *et al*., 2005). In addition, reference ERG parameters in Shih-Tzu were established using an ERG Jet contact lens electrode and portable mini-Ganzfeld ERG unit (HMsERG) (Lee *et al*., 2009). Itoh and colleges found that the ERG amplitudes in healthy Shi-Tzu were lower than in Beagle dogs after stimulation with the same light intensities (Itoh *et al*., 2010; Itoh *et al*., 2013). Therefore, it is important to determine normal ERG values for each dog breed when using ERG diagnosis.

3.2.3 The study of ERG on PRA affected dogs

In several breeds affected with inherited PRA, such early diagnosis is made possible by ERG. Herbert B. Parry (1953) reported the development of the ERG in affected Irish setter puppies. This report demonstrate a diminished b-wave in dogs 22 days old and correlate the reduction in amplitude with rod degeneration (Parry *et al*., 1995). Sandberg and others reported full-field electroretinograms in Miniature Poodles with progressive Rod-cone degeneration in 1986. ERG was

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recorded from 8 Miniature French Poodles (4-34 months old) with inherited PRCD and from 11 normal Miniature Poodles. These affected dogs lost 7.2% and 2% of remaining rod and cone amplitude per month respectively. These declined waves separate affected dogs into 3 stages. First, Rod and cone b-wave amplitudes were both normal. Second, rod b-wave amplitudes were reduced below normal but cone b-wave amplitudes were still normal. And finally, rod amplitudes were extremely declined or nondetectable while cone amplitudes were subnormal. Interestingly, affected Poodles had normal rod and cone b-wave implicit times over all three stages (Sandberg *et al.*, 1986). The study in the Poodle reported ophthalmoscopic and behavioral visual deficits associated with PRA typically appear at 3-5 years of age (Ofri, 2002). However, impaired rod function which expressed as increased scotopic b-wave implicit times and abnormal dark-adaptation curves can be documented electroretinographically at the age of 6-9 months. This early diagnosis is a vital tool in efforts to eradicate the disease through preventive breeding (Ofri, 2002). However as more genetic tests become available, ERG can confirm the diagnosis and evaluate function of the retina.

3.3 Histological examination

Diagnosis of PRA can be confirmed by histological examination to indicate the progressive loss of retina; however this technique is only possible after eye removal. Histological finding usually concur with the ERG abnormalities and vary in difference forms of PRA. For example, in Rod-cone dysplasia type1, the rod development becomes arrested and photoreceptor morphology appears abnormal on 16 days of age. The rod inner segments remain diminutive, with short, disorientated and disorganized outer segment discs (Aguirre *et al*., 1982). For Progressive rod-cone degeneration in Poodle, the photoreceptors develop normally. The first identifiable change is the appearance of vascular profiles in the outer portion of the photoreceptor and rod outer segments show lamellar disorientation and disorganization. As the disease progress, the inner segment and cell body degeneration occurs. Cone degeneration also occurs but at a slower rate than rods. The rate of photoreceptor

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degeneration varies in a characteristic way between different areas of the retina (Aguirre *et al*., 1982).

4. The genes associated with different forms of PRA

The majority of described canine disease display a recessive mode of inheritance (70%) and all of the canine progressive retinal degenerations characterized are recessive with the exception of three X-linked forms (Zhang *et al*., 2002; Zangerl *et al*., 2006; Karlsson *et al*., 2007 and Vilboux *et al*., 2008). PRA are also highly heterogeneous genetically, with several modes of transmission and large number of genes and mutation involved. Some forms of PRA are common to multiple dog breeds, while others are specific in just a single breed. Several forms of PRA are similar to their equivalent retinitis pigmentosa (RP) in humans, a group of the most common human inherited retinopathies (Petersen-Jones, 2005). To date, mutation in 11 loci have been described that account for this disease in dogs. Based on disease onset, these mutation genes can divide into early and late onset PRA.

4.1 Early-onset PRA

4.1.1 Rod-cone dysplasia 1 (RCD1) of *PDE6β* gene

RCD1 which affects Irish Setters is an autosomal recessive resulting from an abnormality in retinal cGMP metabolism (Aguirre *et al*., 1999). This biochemical abnormality results in arrested postnatal development and subsequent degeneration of rod photoreceptor displaying markedly elevated levels of retinal cGMP. In 1993, Suber identified the *PDE6β* (phosphodiesterase β-subunit gene) mRNA defect early in the disease process and indicated that affected dogs carry a nonsense mutation at codon 807 with G to A transition at nucleotide 2420 of *PDE6β* gene (Suber *et al*., 1993). Dekomien and colleagues identified an 8-bp insertion after codon 816 of PDE6B in Sloughi dog breed at the same locus. In addition, they were excluded as *PDE6β*, a candidate gene for PRA in other breeds such as Labrador Retriever, Tibetan Mastiff, Dachshund, Tibetan Terrier, Miniature Poodle, Australian

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cattle dog, Cocker Spaniel, Collie, Saarloos Wolfhounds, Chesapeake Bay Retriever and Yorkshire Terrier (Dekomien *et al*., 2000).

4.1.2 Rod-cone dysplasia 2 (RCD2) of *RD3* gene

RCD2 is an autosomal recessive disorder in Rough and Smooth Collie dogs. Kukekova (2009) identified the mutation of *RD3* gene involves in retinal function and development, although the function of this gene is not well understood. The candidate region, C1ORF 36 of CFA7 showed a different pattern of splicing. The insertion of this region was demonstrated to cosegregate with RCD2, and predicted to alter the last 61 codons of the normal open reading frame and further extend the ORF (Kukekova *et al*., 2009).

4.1.3 Rod-cone dysplasia 3 (RCD3) of PDE6α gene

RCD3 of Cardigan Welsh corgi, first discovered in Australia is an autosomal recessive gene leading to blindness in young adult dogs as in RCD1 (Keep, 1972). Petersen-Jones and colleagues successfully in identified this causal gene mutation by single-strand conformation polymorphism (SSCP) analysis. This was a 1 bp adenine deletion at position 1939-1940 (GenBank Accession number Z68340) (codon 616) of *PDE6α* gene encoding the alpha subunit of cyclic GMP phosphodiesterase. This mutation results in a frame shift and premature stop codon (Petersen-Jones *et al*., 1999). Three years later, they reported an improved DNA-base test for detection of the codon 616 mutation that was used earlier for diagnosis (Petersen-Jones and Entz, 2002).

4.1.4 Early retinal degeneration (ERD) of *STK38L* gene

ERD was originally described in Norwegian Elkhounds and first mapped for more than 10 years ago (Acland *et al*., 1999). The disease caused by an exonic short interspersed nuclear element (SINE) insertion of exon 4 in the gene STK38L. Although known to have neuronal cell function, this gene has not previously

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been associated with abnormal photoreceptor function, and being associated with such a disease in dogs. Goldstein and colleagues established this gene as a potential candidate for hereditary retinal degeneration in other species, including human (Goldstein *et al*., 2010).

4.1.5 Photoreceptor dysplasia (PD) of PDC gene

PD is a form of PRA in Miniature Schnauzers. This form is an autosomal recessive, early onset disorder as judged by histopathology, eletroretinography, and pedigree analysis. Zhang and colleagues identified a missense mutation in codon 82 (CGA to GGA) of PDC gene, that would create a non-conservative substitution which directly interacts with the βr-subunit of transducin (Zhang *et al*., 1998). Phosducin (PDC) is a regulatory cytosolic phosphoprotein for transducin. It modulates the transducin signaling a pathway by interacting with βr-subunit in the retinal phototransducin which is important for vision. There is a mutation – base test available for the disorder in this breed (Felix, 2005 and Petersen-Jones, 2005). On the other hand, Jeong and coworkers reported in 2008, that there is no correlation between PRA and the PDC genotype in Korean miniature schnauzer and these indicate that the commercial DNA test for the PDC mutation will not be used in this breed in South Korean (Jeong *et al*., 2008).

4.1.6 X-link progressive retinal atrophy 2 (XLPRA2) of *RPGR* gene

XLPRA2 in mixed-breed dogs is a mutation in the sex-linked retinitis pigmentosa GTPase regular gene (*RPGR*) causing a very severe form of PRA By 2 years, end-stage degeneration is present in all affected animals. Some carrier dogs often show progressive disease that results in advanced retinal degeneration by 5-6 years of age or older. The locus mutation is a two-nucleotide (delGA) deletion that results in a frameshift that significantly changes the predicted peptide sequence by leading to the replacement of many acidic glutamic acid residues with basic arginine residues resulting in the premature termination of the protein 71 amino acid downstream (Zhang *et al*., 2002).

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4.2 Late-onset PRA

4.2.1 Progressive rod-cone degeneration (PRCD) of *PRCD* gene

Progressive rod-cone degeneration (PRCD) is the most common form of late onset PRA (Acland *et al*., 1998; Andre *et al*., 2008; Petersen-Jones, 2005). This is typically diagnosed in dogs older than 3 years, although it may also be diagnosed in elderly dogs. This autosomal recessive photoreceptor degeneration has revealed that the point mutation of guanine to adenine substitution in the second codon of the exon1 of *PRCD* gene which codes a 54- amino acid protein of canine chromosome 9, a region orthologous to the telomeric end of human chromosome 17, was the cause of disease (Figure 6.). In human with autosomal recessive retinitis pigmentosa, this mutation was demonstrated to be caused by a G>A substitution and resulting in amino acid change from cysteine to tyrosine (TGC→TAC) (Zangerl *et al*., 2006). Expression study of this gene indicated the mainly expression in the retina with equal expression in the retinal pigment epithelium, photoreceptor, and ganglion cell layers (Zangerl *et al*., 2006). Affected dogs and human patients showed similar clinical signs of night blindness and eventually followed by total blindness because the degeneration usually began as a rod abnormality before the cone.

Diagnosis of PRCD is based on clinical signs, fundic examination, histopathology, electroretinography, and molecular genetic testing (Acland *et al*., 1998; Aguirre and Acland, 2006; Ofri, 2008). The disease affects at least 20 breeds (Table1), including popular breeds such as the toy and miniature Poodle, Labrador Retriever, and American and English Cocker Spaniels. The high prevalence of PRA in certain dog breeds, for example PRCD in Miniature Poodles was as high as 7% (Narfström and Petersen, 2007). Moreover, Dostal and coworkers reported the frequencies of the disease-causing *PRCD* mutation in difference of 699 dogs in Czech Republic. They found high frequencies of the mutant allele in Toy Poodle (0.45), Nova Scotia Duck Tolling Retrievers (0.44), English Cocker Spaniels (0.34), Portuguese Water dog (0.33) and Miniature Poodle (0.20) (Dostal *et al*., 2011).

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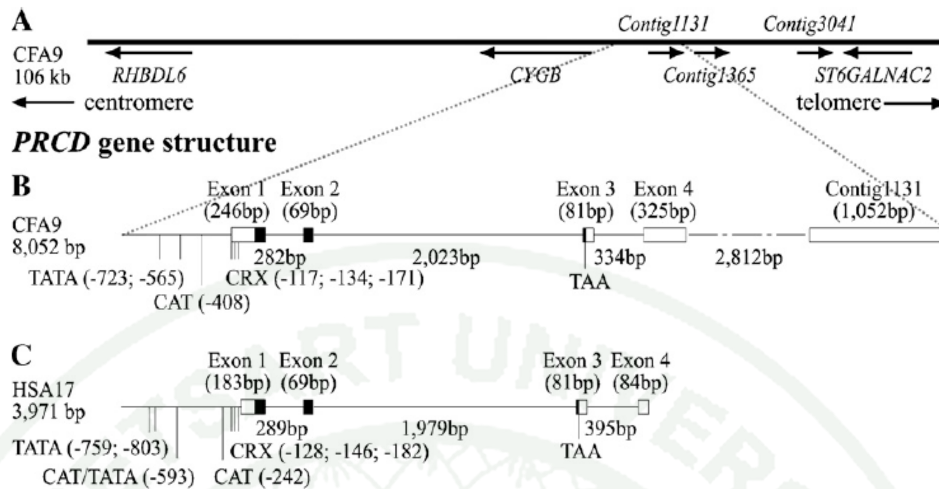

Figure 6 Display of the PRCD candidate region; (A), Linkage disequilibrium (LD) was used to reduce the candidate region to 106 kb. This interval harbors three canine retinal ESTs (contigs 1131, 1365, and 3041). PRCD was cloned within orthologous region in the (B) dog and (C) human (Modified from Zangerl *et al*., 2006).

4.2.2 Autosomal dominant progressive retinal atrophy (ADPRA) of *RHO* gene

ADPRA in English Mastiff dogs and Bull Mastiff were found to be inherited as an apparently autosomal dominant disease confirming by test mating. The disease progression is characterized by regions of initial focal photoreceptor degeneration surrounded by areas of structurally normal retina. A single nonsynonymous C→ G transversion at nucleotide 11 of *RHO* gene changes Thr-4 to Arg (T4R) and cosegregates with disease in the test pedigree (Kijas *et al*., 2002). Rhodopsin (RHO) is the G protein-coupled receptor involving the transduction cascade leading to night vision. *RHO* gene mutation causes human retinitis pigmentosa. This mutation, originally identified in the Mastiff, but it has not been identified in any other breeds to date (Kijas *et al*., 2003).

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4.2.3 X-link progressive retinal atrophy (XLPRA) of *RPGR* gene

XLPRA is an inherited blinding disorder caused by mutation in exon 15 (ORF15) of the *RPGR* (Retinitis Pigmentosa GTPase Regulator) gene (Zeiss *et al*., 1999). This disease is similar to human retinitis pigmentosa 3 (RP3). It is observed in 2 variations of XLPRA1 in Siberian Husky and Samoyed and XLPRA2 derived from mongrel dogs. The disease has been mapped to the short arm of the X chromosome (Figure 7). XLPRA1 is caused by a deletion of five nucleotides (GAGAA), leading to a frameshift and immediate premature stop codon; the truncated protein lacks 230 C-terminal amino acids, which causes a slight decrease in the isoelectric point (Zhang *et al*., 2002). While the XLPRA2 is caused by a 2-bp deletion (Figure 8) was reported. The X-linked retinal degeneration is characterized by initial degeneration of rod photoreceptors, followed by loss of cones and progressive atrophy of the inner retina. Carrier females display a phenotype consistent with random X-chromosome inactivation. Variation in genetic background may alter expression of the disease allele in affected animals. The abnormalities including ophthalmoscopic evidence of generalized retinal degeneration and ERG evidence of progressive photoreceptor dysfunction become apparent after 1 year of age, and progress to severe visual impairment by 2 to 3 years (Zeiss *et al*., 1999).

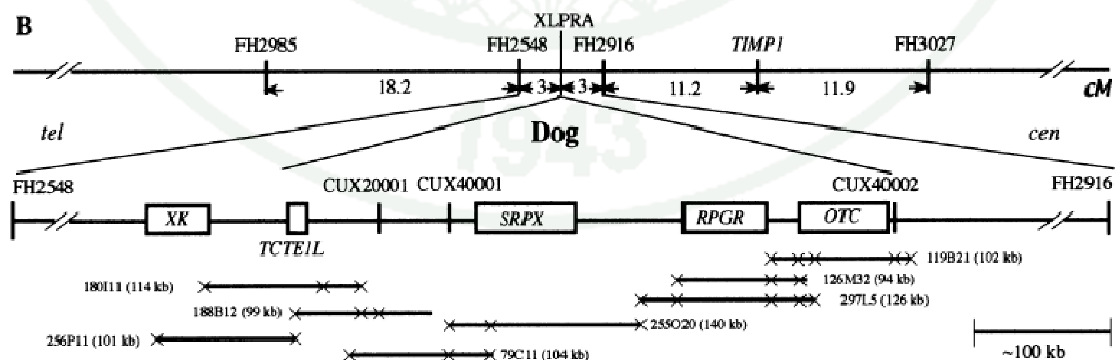

Figure 7 XLPRA and flanking region which mapped to the short arm of the X chromosome; the linkage map is taken from Zhang and colleague in 2002.

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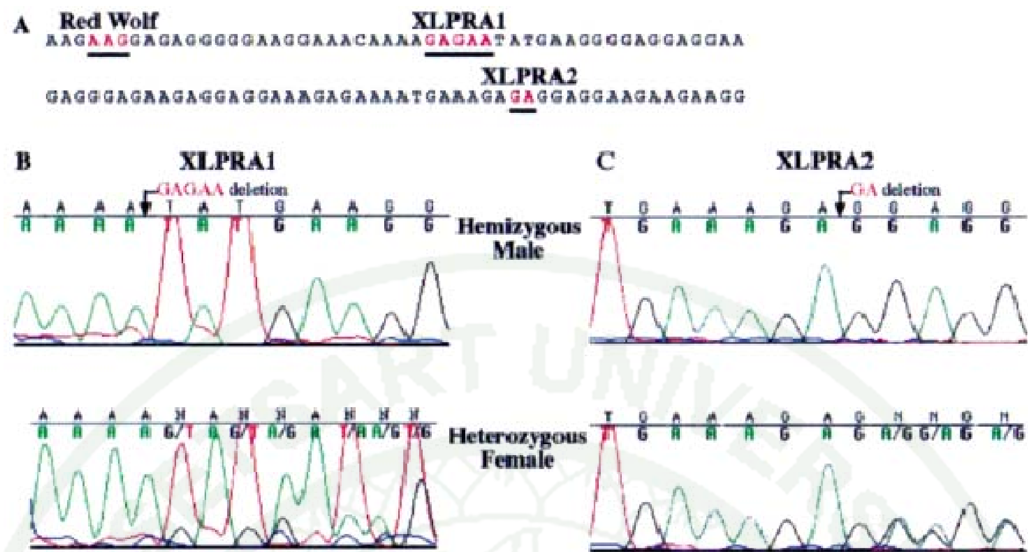

Figure 8 Mutation analyses of exon ORF15 in canids, (A) Wild-type sequence from the mutation region of canine exon ORF15. The three deletions are illustrated in the top lines. The electropherograms show the deletions for affected and carrier dogs with XLPRA1 (B), and XLPRA2 (C). Not an additional 3-bp deletion is observed in red wolf, not causing a change in the protein sequence other than the removal of one amino acid.

4.2.4 X-link progressive retinal atrophy 3 (XLPRA3)

A different form of late-onset, X-linked PRA has been described in the Border collie. This condition is genetically distinct from both XLPRA1 and XLPRA2, although as yet the causal mutation has not been identified. Vilboux and collaegues suggested that this PRA segregating in Border Collie was a new XLPRA called XLPRA 3 and proposed it as a potential model for the homologous human disease as X-linked retinitis pigmentosa (Vilboux *et al*., 2008).

4.2.5 PRA in Schapendoes *CCDC66* gene

PRA in Schapendoes dogs is late onset by 2-5 years. Lippmann and colleagues reported the genome-wide scan of five PRA-informative pedigrees which

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was assigned to canine chromosome 20 between microsatellite markers FH3358 and TL336MS (distance 5.6 Mb) (Lippmann *et al*., 2007). Then, Dekomien et al, described a PRA locus comprising the newly identified gene coiled-coil domain containing 66 (CCDC66) on the same chromosome by linkage analysis (Figure 9). Mutation screening of this gene revealed a 1-bp insertion in exon 6 leading to a stop codon as the cause of disease. The affected dog's retina lacks of CCDC66 protein which was detected mainly in the inner segments of photoreceptors in mouse, dog, and human by immunohistochemical analysis (Dekomien *et al*., 2010).

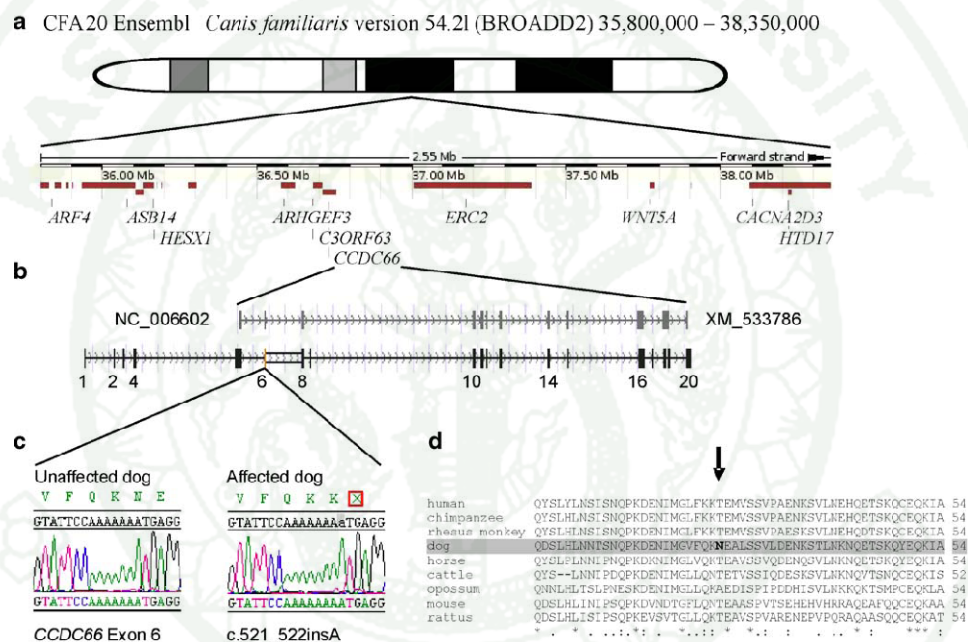

Figure 9 The CFA 20 candidate region for PRA in Schapendoes dog; (a) Physical map of the investigated candidate genes in the PRA critical region comprising 4.2 Mb of DNA, (b) exon/intron structure of the newly identified CCDC66 gene (database entry XM_533786) in addition to database entry herein exons 1-4, 6, 7, and 19 are demonstrated as well as additional 3' –part of exon 20 identified by cDNA investigation, (c) Chromatograms of part of CCDC66 exon 6 exhibit the wild-type sequence and an A insertion in homozygous state leading immediately to a stop codon in affected dogs, (d) comparison of the protein sequences of exon 6 of the CCDC66 gene in eight different mammalian species (Modified from Dekomien *et al*., 2010).

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5. Mutation finding of PRA in dogs

Several methods can be used to identify the causative genes and mutation, depending on the transmission mode, the ability to collect families of unrelated cases and controls, the pathophysiology of the disease and the knowledge of candidate genes. Nowadays, two predominated approaches in the search for mutations involved in retinal disease in dogs are the functional candidate gene approach and linkage analysis (Felix, 2005)

5.1 Functional candidate gene approach

The candidate gene approach is being used in an attempt to identify the mutation responsible for PRA in a number of dog breeds. A mutation test identifies the specific DNA abnormality which might involve an altered DNA sequence, a substitution in the sequence, or a deletion of DNA. This test is possible once the precise DNA defect for a specific disease is known for the breed being tested. Because different breeds can have different mutations that cause the same clinical disease, the specific cause must be discovered for each breed. To date, 11 mutations causing canine PRA have been discovered (Table 1) (Dekomien *et al*., 2002; KuKeKova *et al*., 2009; Petersen-Jones and Entz, 2002; Goldstein *et al*., 2010; Narfström and Petersen Jones, 2007; Zhang *et al*., 2002; Zangerl *et al*., 2006; Kijas *et al*., 2003; Zhang *et al*., 2008; Vilboux *et al*., 2008; Dekomien *et al*., 2010) . For candidate genes approach, the mutation is pursued in genes that have been implicated by their known normal activity or by their distribution among cells or tissues (Gould *et al.,* 1997). A previous report reviewed an evaluation of 23 candidate genes of canine retinal diseases from 377 published results. This report revealed that most of the results (66%) excluded the presence of a mutation in a gene or its coding region, while only 3.4% of the results identified the mutation causing the disease (Aguirre-Hernandez and Sargan, 2005).

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5.2 Linkage analysis

Linkage analysis is used when a disease gene and its mutations are not known. It is sometimes possible to develop a genetic test using DNA sequence markers closely linked to the gene. The linkage procedure consists of three steps. First, the segregation of genetic markers and the disease are analyzed in families with affected individuals to identify those markers that cosegregate with the disease. This indicates the region where the disease locus locates. Second, fine mapping with additional markers in the region of interest is performed to increase the resolution. Finally, candidate genes within the region of interest, chosen by their role, structure, or transcript distribution, are analyzed (Gould *et al*., 1997; Hyun *et al*., 2003). The results of linkage analysis on canine PRA are encouraging to extend its application to individuals in general population. Five linkage analysis on retinal diseases revealed three identified mutations and two mapped disease loci. However, these mapping studies have relied on dog research colonies (Aguirre-Hernandez and Sargan, 2005).

6. Thai native dogs

Thai Ridgeback and Thai Bangkaew have been recognized by the Kennel Club of Thailand as the national dogs of Thailand (KCT, 2003). In 1993, the Federation Cynologique Internationale (FCI) registered Thai Ridgeback as a pure-breed dog and Thai Bangkaew was registered similarly in 2011 (FCI, 2013). Their classifications are in a medium-sized, Spitz and primitive type dogs. The Ridgeback is a muscular, medium-sized with a wedge-shaped head, triangular-shaped prick ears, and a velvet short coat with solid colors of blue, black, red or fawn. It has been used mainly for hunting in the eastern part of Thailand and also used to escort farmer's carts as a watch dog. The Thai Ridgeback is one of only three breeds that have a ridge of hair that runs along its back in the opposite direction to the rest of the coat. The others are Rhodesian Ridgeback and the Vietnamese Phu Quoc Ridgeback. The dorsal hair ridge in these dogs is caused by a dominant mutation which also predisposes to the congenital developmental disorder called dermoid sinus (Hillbertz *et al*., 2007). Thai Bangkaew is an old breed that originated from the Bangkaew village in the Bang-

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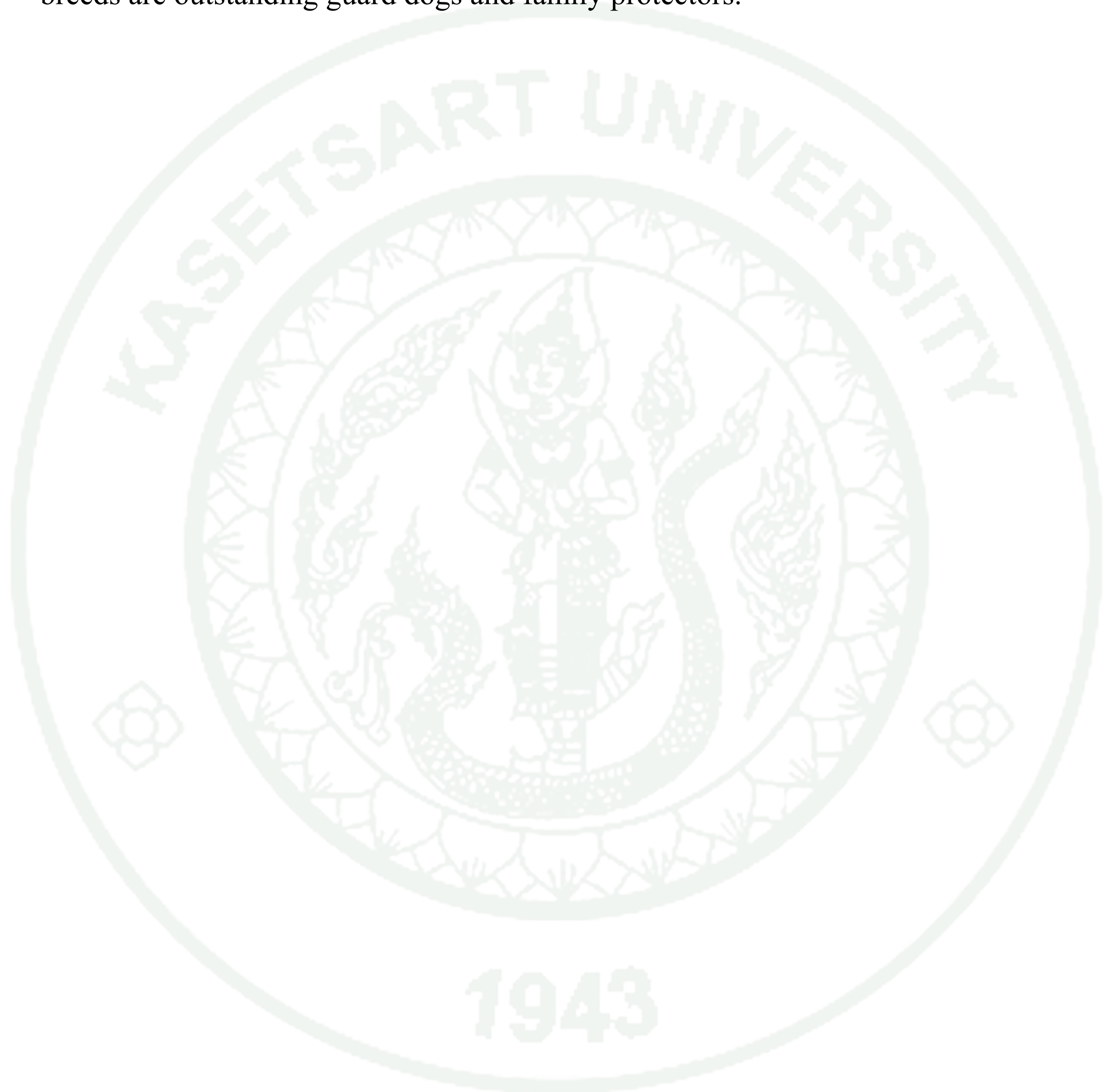
rakam district of Phitsanulok province in Thailand. These dogs are bred widely in the province and become famous nationwide. Characteristics of Thai Bangkaew are a pluming tail and double-coated which forms a ruff around the neck and shoulders with a wide variety of color patterns: red, gray, brown and black. Both Thai dog breeds are outstanding guard dogs and family protectors.



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MATERIALS AND METHODS

1. Establish the ERG normal parameters

1.1 Animal selection for determine the ERG normal parameters

Both eyes of 28 ophthalmoscopically normal, healthy dogs from the four breeds: 9 Poodle, 6 Labrador Retriever, 8 Thai Ridgeback and 5 Thai Bangkaew, were recruited for the study. The mean ± SD age was 4.64±1.56 years (ranging from 2 to 8 years) as shown in Table 1. There was no significant difference among the average ages of each breed. All dogs were housed and food was managed variously by their owners. All dogs were considered to be in good health with no history of visual deficit or ocular abnormality. Vision was confirmed by perfect scotopic/photopic obstacle test and positive menace response. Dazzle and pupillary light reflexes were tested in all dogs. The anterior segment was considered normal from ocular examination with a portable slit lamp biomicroscope (SL-15, Kowa Optimed, Japan). Intraocular pressure was measured using a tonometer (TonoVet, iCare, Finland). Fundus examination was investigated using a binocular indirect ophthalmoscope (Vantage, Keeler Ltd, UK) in a dark room after electroretinographic examination.

Table 2 The number and average age of dogs in the study.

Breed	Number	Average age (year)	Standard error
Poodle	9	4.67 (2-8)	0.55
Labrador Retriever	6	4.83 (3-8)	0.67
Thai Bangkaew	5	4.80 (2-7)	0.80
Thai Ridgeback	8	4.80 (3-7)	0.58
Total	28	4.64	0.29

* No significant difference (P=0.650) in average ages of each breed.

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1.2 Anesthetic protocol

All dogs underwent a physical examination, as well as a complete blood cell count and a standard biochemistry profile. Food and water were withheld for 10 hours before the test. Dogs were kept in a quiet, dimly lit room for 1 hour before performing the ERG procedure. Prior to anesthesia, the pupils of both eyes were maximally dilated by applying one drop of 1% tropicamide (Midriacyl, Alcon, Belgium) twice with a 15 minute interval between the drops. The pupil size was periodically evaluated to ensure full dilatation, especially at the beginning and at the end of the ERG recording. Animals were premedicated with intravenous 0.3 mg/kg diazepam (GPO, Thailand). Induction of anesthesia was performed with intravenous 4 mg/kg propofol (Anepol®, Hana Pharm, Korea). The dogs were endotracheally intubated and inhalation was maintained with 2.0% isoflurane (Aerrane®, Baxter Healthcare, Puerto Rico). Anesthesia was induced under ambient light. The depth of anesthesia was kept constant during the procedure. Proper oxygenation and ventilation of the anesthetized dog were maintained throughout the ERG recording via intubation. The body temperature was monitored and maintained constant at 100.5-102.5^{o} F.

1.3 ERG Recording

Each anesthetized dogs was positioned in sternal recumbency on a wooden table and the head was placed on a towel (Figure 11) Topical anesthetic eye drops (0.5% tetracaine hydrochloride ophthalmic solution, Alcon, Belgium) were applied. Both eyelids were opened with a lid speculum and a conjunctival stay suture was applied to obtain proper globe position. The ERG equipment was shown in Figure 10. The corneal electrode (ERG-jet®, Fabrinal SA, Switzerland) was positioned with artificial tear solution (Methocel® 2%, OmniVision, Germany) applied between the corneal surface and the contact lens. The skin needle electrodes were used as the reference and ground electrodes. The reference electrode was placed approximately 3 cm caudal to the lateral canthi and the ground electrode was placed over the external occipital protuberance (Figure 12). All electrodes were connected to

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a preamplifier and the signals were amplified with a bandpass filter between 0.3 and 300 HZ of the mini-Ganzfeld Handheld Multi-species ElectroRetinoGraph (HMsERG, Xenotec, USA). Placement of the electrodes was performed under light room condition. The mini-Ganzfeld was positioned as close to the eye as possible without touching the dog.

Before the ERG result was recorded, the impedance was measured and baseline tests were performed for evaluation of noise levels in the environment and the proper contact between electrodes and tissues. Lights in the examining room were turned off at the commencement of the first session of the scotopic ERG procedure (dark adaptation). The ERG results were recorded automatically using the standard protocol, Dog Diagnostic, which was a part of the software of the HMsERG unit and was recommended by the European College of Veterinary Ophthalmologists (Ekesten *et al*., 2013).

According to the guidelines for clinical ERG usage in dogs, the retinal signals were obtained in scotopic and photopic conditions for 33.81 minutes in total. For scotopic ERG procedures, three different responses were recorded: scotopic low intensity responses using 0.01 cd.s/m^2 of light stimuli during the 20 min of dark adaptation, an average of 10 flashes with an interval of 2 sec every 4 min for evaluation of rod function, which was designated Rod1, Rod2, Rod3, Rod4 and Rod5; scotopic standard intensity responses (Std R&C) using 3 cd.s/m^2 of light stimuli ;and scotopic higher intensity responses (Hi-int R&C) using 10 cd.s/m^2 of light stimuli for stimulation of both rods and cones. The photopic ERG procedure consisted of two different responses: a photopic single flash response (Cone) using 3 cd.s/m^2 of light after 10 minutes of light adaptation with 30 cd.s/m^2 of background light for evaluation of cone and a photopic 30 Hz flicker response (Flicker) using 3 cd.s/m^2 for cone evaluation in the light adapted state. Intensity of light and time for each ERG session were concluded in Table 3. The ERG recordings in each dog were performed in the right eye followed by the left eye.

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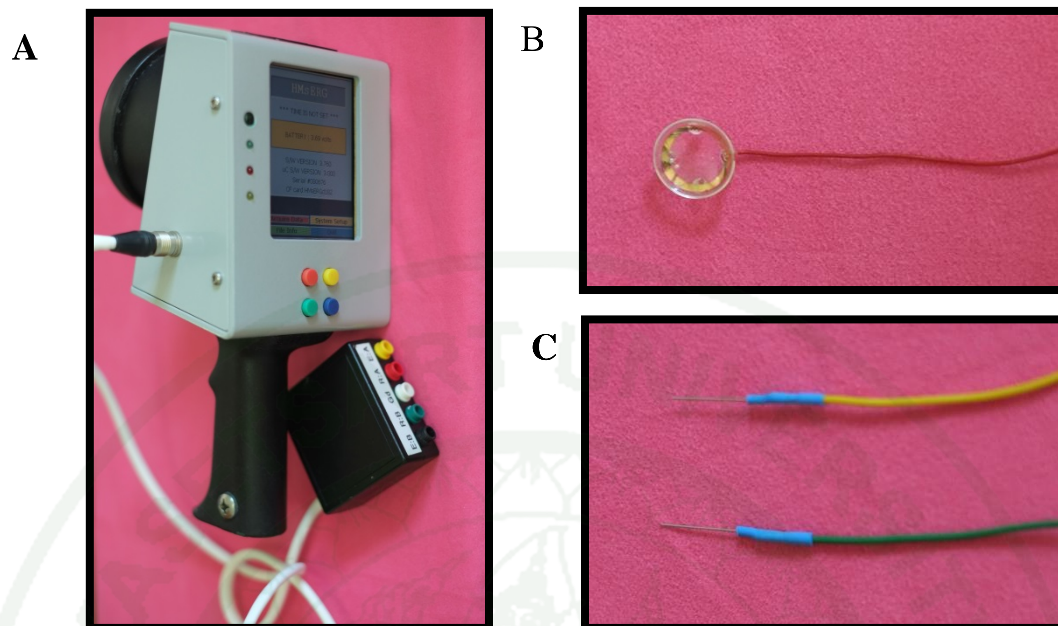

Figure 10 Photograph showing the ERG equipment; (A) Mini-Ganzfeld HMsERG unit, (B) The corneal or active electrode (ERG-jet®), (C) The skin needle electrodes which used as a reference and ground electrodes.

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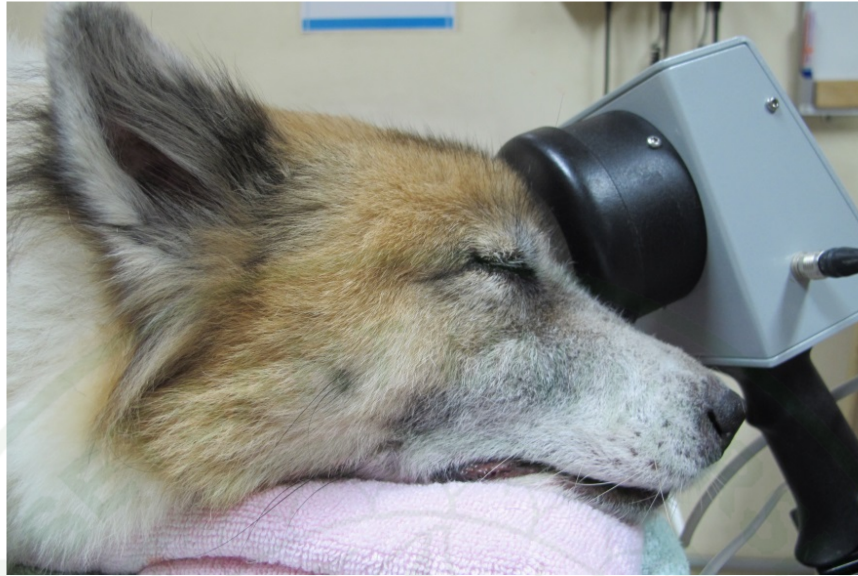

Figure 11 Photograph of ERG procedure setup using the HMsERG with a 5-year-old, Thai Bangkaew dog.

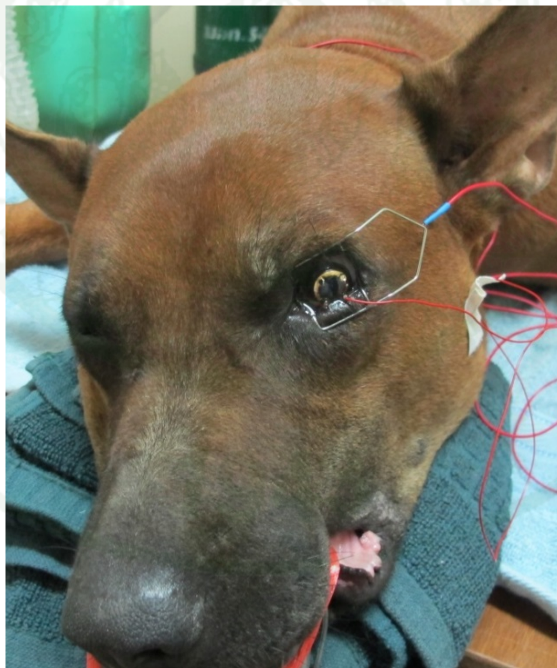

Figure 12 Photograph of electrodes placement with a 5-year-old, Thai Ridgeback dog.

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Table 3 Intensity of light and time that stimulate the retinal-function for each ERG session.

ERG test sessions	Light intensity ($mcd.s/m^2$)	Time (second)
1th Dark adaptation cycle		240
2nd Rod response	10	20
3rd Dark adaptation cycle		240
4th Rod response	10	20
5th Dark adaptation cycle		240
6th Rod response	10	20
7th Dark adaptation cycle		240
8th Rod response	10	20
9th Dark adaptation cycle		240
10th Rod response	10	20
11th Delay		2
12th Standard rod and cone response	3,000	40
13th Delay		30
14th High-intensity rod and cone	10,000	80
15th-1 Light adaptation cycle	3,000	600
15th -2 Cone response	3,000	16
16th Delay		2
17th Cone flicker response	3,000	4.1

1.4 ERG analysis

ERG waveforms were analyzed by measuring a-wave and b-wave amplitudes and implicit times as well as the b/a ratio calculation. The a-wave amplitude was measured from the baseline to the trough of the first negative peak and the b-wave amplitude was measured from the trough of the a-wave to the peak of the positive wave. The a- and b-wave implicit times were calculated from the onset of the light stimulus to the peak of their waves. For the scotopic low intensity responses and

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photopic 30 Hz flicker responses, only the b-wave amplitude and implicit time were measured. All reference ERG values were statistically defined as the median value and range between the 5th and 95th percentiles (lower and upper limits). To compare the mean amplitudes, implicit times and b/a ratios among the four dog breeds, statistical analysis was performed using NCSS 8 (www.ncss.com) by setting the statistical significance at a P value of less than or equal to 0.05. One-way ANOVA was used for statistical testing with the Bonferroni test as a post-hoc test of the ERG parameters which showed a normal distribution and same variance data. In addition, Kruskal-Wallis tests together with Dunn's test were used for analysis when the data was not normally distributed and/or the variances were not equal.

2. Confirm phenotypic and genotypic of responsible gene mutation of PRA in Poodle

2.1 Animal selection

Ten normal dogs and ten dogs previously diagnosed with PRA based on ophthalmic examination and a history of visual impairment in dim light were included for characterization of *PRCD* gene mutation.

2.2 Ophthalmic examination and vision test

Vision of 10 normal and 10 PRCD-suspected dogs was examined by a photopic/scotopic obstacle test. Complete ophthalmic examination was performed including menace response, dazzle reflex and pupillary light reflex (Figure 13). The anterior segment was examined using a portable slit lamp biomicroscope (SL-15, Kowa Optimed, Japan). Intraocular pressure was measured using a tonometer (TonoVet, iCare, Finland). Fundus examination was investigated using a binocular indirect ophthalmoscope (Vantage, Keeler Ltd, UK) in a dark room after electroretinographic examination.

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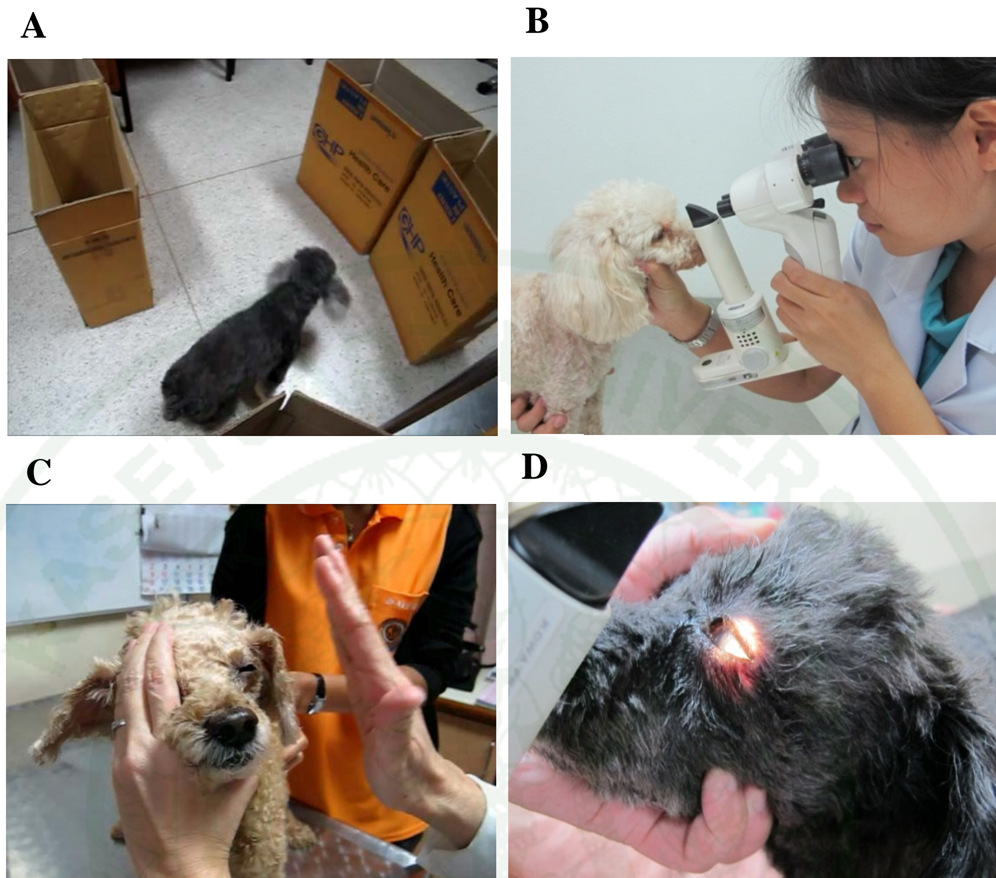

Figure 13 Illustration of ophthalmic examination; obstacle test (A), slit lamp biomicroscope (B), menace response (C), dazzle reflex and pupillary light reflex (D).

2.3 Histopathological examination

Only the left eye of a PRCD-suspected dog was enucleated with the owner permission. The eye was fixed in Bouin's solution overnight. The tissue was washed 6-8 hours in cold running tap water and dehydrated in 70% ethanol. The eye was subsequently embedded into paraffin blocks and sectioned into 4-µm thickness on a semi-automatic microtome at the level of the optic nerve and stained with hematoxylin and eosin (H&E) stain. The tissue sections were microscopically examined with bright-light microscopy.

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2.4 DNA testing

2.4.1 Blood Sample Collection

One millitre of whole blood samples were collected from 10 normal and 10 suspected Poodle dogs in a sterile tube containing EDTA (Ethylenediaminete-traacetic acid) as an anticoagulant. These blood samples were used for DNA extraction immediately or stored at 4°C for a few days before used.

2.4.2 DNA extraction

Genomic DNA was extracted from whole blood in EDTA as anticoagulant by performing to standard Phenol/Chloroform protocol which applied from Sambrook and Russel (2001), see appendix B.

2.4.3 Amplification of PRCD gene fragment

All DNA samples were amplified using PCR technique. The 346 bp DNA fragment of exon 1 of the *PRCD* locus was amplified using published primers sequence (Gentilini *et al*., 2009), forward (5'AGCCTCCTAATCCAGTGG3') and reverse (5'GTGCTCTGATGGGAAACC3'). Amplification conditions were 94^{0}C (2 min), followed by 45 cycles at 94 ^{0}C (15 sec), 55 ^{0}C (20 sec), 68 ^{0}C (20 sec), and final extension at 72 ^{0}C (10 min). Agarose gel (1.5%) electrophoresis and the gels screened under UV light were used to confirm the PCR products.

2.4.4 Identification of PRCD-PRA disease status

The PCR products were digested with restriction enzyme in specific DNA sequence (GT/AC) using *RsaI* endonuclease (FastDigest, Thermo Fisher Scientific, USA). 2% agarose gel electrophoresis of digested solution was performed to verify the disease status. To confirm the specific gene amplification, the amplified PCR product of 10 affected Poodle dogs and 10 normal Poodle dogs were purified

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with PCR-clean up kit following the manufacturer's directions as showed in Appendix B, and directly sequenced using a standard protocol. All sequenced DNA fragments were analyzed with the published sequence of *PRCD* gene (EMBL Accession number: DQ390331.1)

3. Analyze the allele frequencies of PRA in Poodle and Labrador Retriever

50 Poodles and 58 Labrador Retrievers at the age between 6 months to 12 years were randomly selected from dogs presented at Kasetsart University Veterinary Teaching Hospital with an unknown history. Analysis of allele frequency was performed using the same technique of DNA extraction and PCR-RFLP. The allele frequency was determined from the genotype as homozygous normal, heterozygous and homozygous mutant.

4. Identify phenotypic characterization and candidate gene mutations of PRA in Thai Bangkaew

4.1 Animal selection

Six unrelated normal Thai Bangkaew dogs and six affected Thai Bangkaew dogs were recruited for this study. Most affected dogs presented for examination because the owner suspected poor vision. These dogs were diagnosed compatible with bilateral retinal degeneration by the Ophthalmologic clinic of Kasetsart University Veterinary Teaching Hospital and Mahidol University Veterinary Teaching Hospital.

4.2 Clinical examination and Electroretinography

The ophthalmological examination of all Thai Bangkaew dogs included standard ocular reflexes, slit lamp biomicroscopy, applanation tonometry and indirect ophthalmoscopy.

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The electroretinography were performed in 5 affected dogs and 5 normal dogs with the owners' consent. The protocols used in this study were the short protocal called QuickRetCheck and the complete protocol called Dog Diagnostic. The Dog Diagnostic protocol was described in the first part of this study. QuickRetCheck protocol, a part of the software of the HMsERG unit was used in only one retinal degeneration Thai Bangkaew dog. Light stimuli from this protocol were generated by white LEDs in Mini-Ganzfeld flash Dome of which its intensities were shown in Table 4. Low light intensity stimuli were generated to evaluate rod function. A standard light intensity stimulation was generated to evaluate both rod and cone function. All steps were performed in scotopic test condition. The entire time for ERG protocol in one eye took 18 seconds.

Table 4 ERG test sessions: intensity of light and time for each ERG session of the short (QuickRetCheck) protocol of HMsERG (Modified from a user manual for HMsERG instrument, 2006).

Session	**ERG Test Sessions**	**Flash Intensity (mcd.s/m^2)**	**Number of Flashes**	**Time required (second)**	**Elapsed Time (second)**
1	Low light intensity	10	4	8	8
2	Standard light intensity	3,000	1	0	8
3	High light intensity	10,000	1	10	18

4.3 Candidate gene mutation testing

DNA of all dogs was extracted from whole blood leukocytes in EDTA according to standard Phenol/Chloroform protocol which applied from Sambrook and Russel (2001) as described in appendix B. Mutation screening in the six suspected and six normal Thai Bangkaew dogs was carried out in respective candidate genes by DNA sequencing of amplified products. Six loci from four genes which base on phenotypic of disease onset were chosen for analysis. Primers were designed to

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ensure sequencing coverage of all possible exons (Table 5) using Primer Analysis Software (Oligo® Version 7.57, Molecular Biology Insights, Inc).

Amplification of six loci was performed using PCR technique of thermal cycle. DNA fragments were amplified in a 20 µl reactions mix which contained 50 ng of genomic DNA, 150 mM $MgCl_2$, 250 µM of each dNTP, 50 pmoles forward and reward primers each, 0.5 Unit AmpliTaq polymerase (Invitrogen company) in 10X magnesium free PCR buffer. Optimal conditions for each primer pair were established by adjusting of DNA concentration, $MgCl_2$ concentration and annealing temperature. The PCR condition of cycle number, denaturation, annealing and extension for each primer pair were showed in Table 6.

PCR products were visualized under ultraviolet light with EtBr in 1.5% agarose gels. PCR products were either directly sequenced using forward or reverse primer according to standard procedures. Then, the sequencing results were analyzed by comparison with the mRNA sequence of the *PRCD* gene (Progressive rod-cone degeneration; Accession number: DQ390331.1), *CCDC66* gene (Coiled-coil domain-containing protein 66; Accession number: NC_006602), *RPGR* gene (Retinitis pigmentosa GTPase regulator; Accession number: NC_000023), *RHO* gene (NC_006602.3).

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Table 5 Candidate genes, chromosomal location, primers sequences and PCR product sizes deigned for genetic tests in affected Thai Bangkaew dogs.

Gene	Location	Primer name	Forward and Reverse (5' to 3')	Size (bp.)
PRCD	CFA9	PRCDE1F	AGCAGGAACCTCAGGATGGG	326
		PRCDE1R	TGCTCTGATGGGAAACCTCTCT	
PRCD	CFA9	PRCDE2F	GTCCAGAGAGGTTTCCCATCAGA	329
		PRCDE2R	GCTCGGGTGCACTCATGTACC	
PRCD	CFA9	PRCDE3F	TTCCACAGTCACAAATTCCAGC	320
		PRCDE3R	CCTTGCAGAGCCTGCCTTG	
RPGE	CFAX	XLPRAF	GAAAGTAAGCATGGTCTCTATCCACC	239
		XLPRAR	CAAGTTCCTCAGCCATATCCTCTGG	
RHO	CFA20	RHOF	GCAGCACTCTTGGGACTGAG	244
		RHOR	TGTAGTTGAGAGGTGTACGC	
CCDC66	CFA20	CCDCF	AAGTATGTAGCAGTATGCCAA	318
		CCDCR	AGCTCTTAGTCTTCAGAGTATT	

Table 6 PCR condition of each primer.

Primer	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycle number
PRCDE1	94°C for 3 min	94 °C for 30 sec	60 °C for 30 sec	72 °C for 30 sec	72°C for 10 min	39
PRCDE2	94°C for 3 min	94 °C for 30 sec	58 °C for 30 sec	72 °C for 30 sec	94°C for 10 min	39
PRCDE3	94°C for 3 min	94 °C for 30 sec	58 °C for 30 sec	72 °C for 30 sec	94°C for 10 min	39
XLPRA	94°C for 3 min	94 °C for 30 sec	60 °C for 30 sec	72 °C for 30 sec	94°C for 10 min	34
RHO	94°C for 3 min	94 °C for 30 sec	58 °C for 30 sec	72 °C for 30 sec	94°C for 10 min	34
CCDC	94°C for 3 min	94 °C for 30 sec	52 °C for 30 sec	72 °C for 30 sec	94°C for 10 min	34

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RESULTS AND DISCUSSION

Results

1. Determine the ERG normal parameters

The ophthalmic examination and blood test results revealed normal results in all dogs. The normal fundus pictures of 4 dog breeds were shown in Figure 14. The ERG outputs were successfully recorded from the 56 eyes of the 28 dogs using a portable ERG machine. The impedance of the active and reference electrodes was less than 5 kΩ in each eyes of all dogs. The anesthetic maintenance with isoflurane inhalation provided good immobilization and sufficient duration for light stimulation and recording.

Typical normal ERG waveforms recorded from the right eye of a 5-year-old Thai Ridgeback dog was shown in Figure 15. The a-wave amplitude was undetectable in scotopic low intensity responses in all dogs as usual. The b-wave amplitudes of the scotopic low intensity response steadily increased after 4, 8, 12, 16 and 20 minutes of light stimulation (Figure 15. A-E). The ERG wave forms of the scotopic standard and high intensity responses were similar (Figure 15. F-G) with the ERG a- and b- waves being clearly identified. The small a- and b-waves were detectable in the photopic single flash response while only the b-wave was detectable in the photopic flicker response.

The median and limits of normality using the 5th and 95th percentiles of all recordings are summarized in Tables 7 and 8 according to the guidelines for clinical electroretinography in dog (Ekesten *et al*., 2013). As well as the means and standard error of amplitudes, implicit times and b/a ratios obtained from the four dog breeds are shown in Table 9 and 10 respectively.

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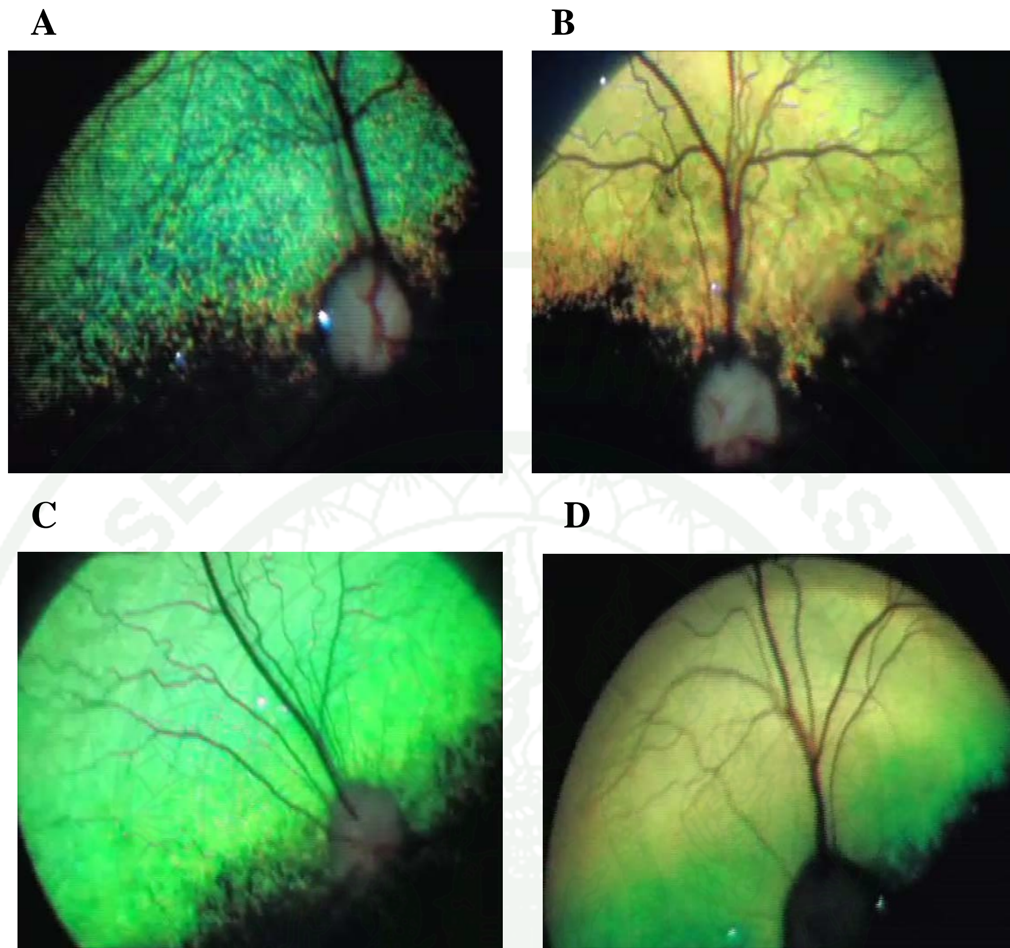

Figure 14 Normal fundus in 4 dog breeds; Poodle (A), Labrador retriever (B), Thai Bangkaew (C) and Thai Ridgeback (D).

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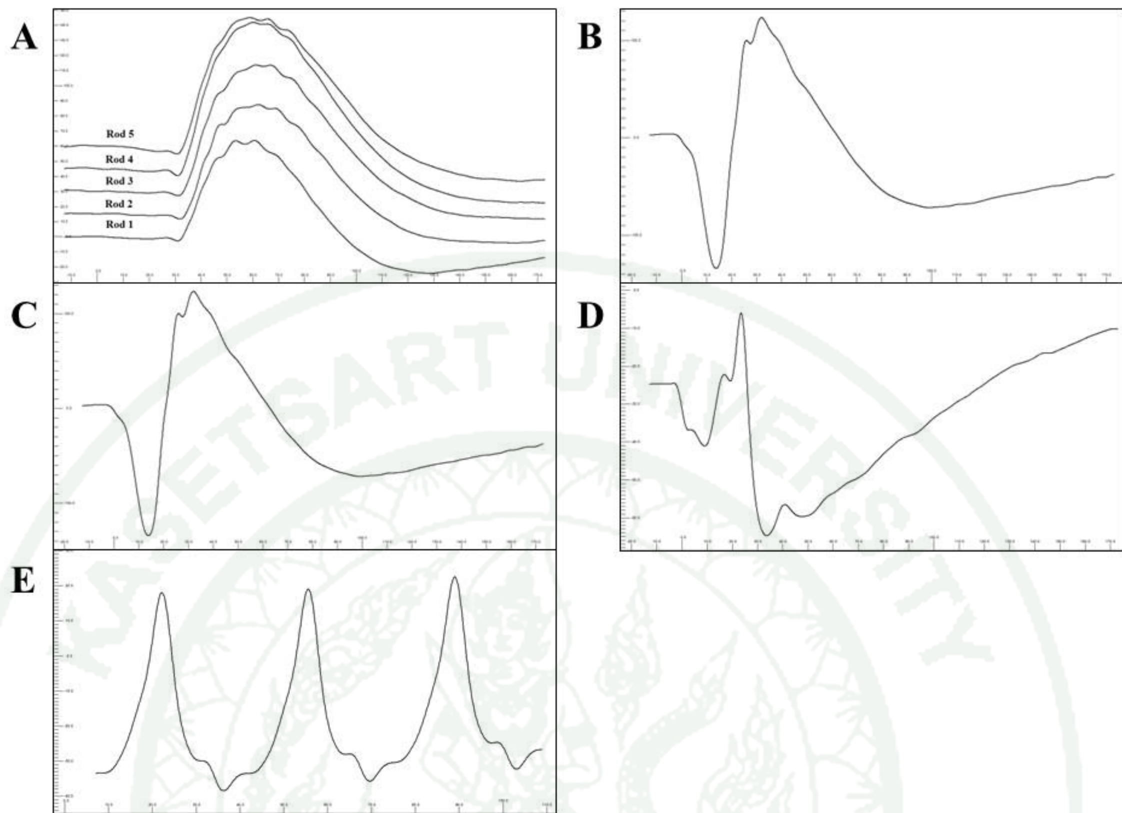

Figure 15 Typical normal ERG waveforms recorded from the right eye of a 5-year-old Thai Ridgeback dog. Scotopic low intensity responses (A) which were elicited 4, 8, 12, 16, 20 min in dark condition (designated Rod1, Rod2, Rod3, Rod4, and Rod5), Scotopic standard intensity response; Std R&C (B), and higher intensity responses; Hi-int R&C (C). Photopic single flash responses; Cone (D) and photopic flicker responses; Flicker (E) were shown. Vertical axis is in microvolts (μV) and horizontal axis is in milliseconds (ms).

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Table 7 Summary of ERG amplitudes and b/a ratios recorded from four dog breeds; data presented as the median (microvolts) and range of the 5th and 95th percentiles.

Retinal function	Labrador Retriever	Poodle	Thai Bangkaew	Thai Ridgeback
Rod 1 (b-wave)	38.10 (17.45~60.32)	41.45 (22.28~96.76)	67.00 (41.47~80.56)	72.45 (33.98~143.4)
Rod 2 (b-wave)	47.10 (24.16~71.37)	58.65 (30.21~108.31)	82.10 (44.50~100.36)	82.05 (56.05~154.28)
Rod 3 (b-wave)	51.60 (25.59~81.73)	84.10 (31.16~140.51)	95.2 (50.80~113.06)	88.60 (55.61~166.50)
Rod 4 (b-wave)	54.75 (27.47~88.93)	77.30 (35.37~143.57)	100.3 (63.1~125.42)	93.75 (67.58~176.13)
Rod 5 (b-wave)	55.70 (35.75~92.50)	94.80 (38.48~163.59)	108.5 (66.80~118.42)	103.80 (67.85~196.95)
Std R&C (a-wave)	81.10 (57.00~143.70)	114.90 (83.92~164.58)	94.8 (67.98~131.50)	119.10 (70.38-171.10)
Std R&C (b-wave)	151.20 (83.25~216.95)	209.25 (144.84~390.41)	178.6 (138.06-238.04)	214.85 (126.10~303.18)
b/a ratio	1.68 (1.28~2.66)	2.18 (1.03~3.05)	1.84 (1.59~2.08)	1.79 (1.35~2.71)
Hi-int R&C (a-wave)	110.40 (73.45~195.15)	173.20 (101.15~230.34)	168.30 (108.62~190.26)	152.20 (103.05~229.35)
Hi-int R&C (b-wave)	164.20 (95.70~256.35)	241.80 (163.82~382.89)	219.90 (171.44~262.06)	235.15 (103.05~229.35)
b/a ratio	1.37 (1.05~2.07)	1.65 (0.91~2.18)	1.37 (1.12~1.66)	1.45 (1.27~2.18)
Cone (a-wave)	8.60 (5.09~17.10)	13.45 (7.73~23.43)	10.30 (9.44~13.72)	10.90 (6.07~16.71)
Cone (b-wave)	18.30 (11.75~34.99)	41.30 (28.29~55.90)	31.90 (18.78~38.84)	32.20 (18.72~46.19)
b/a ratio	2.05 (1.26~4.41)	2.87 (2.10~4.47)	2.86 (1.95~3.59)	2.78 (2.27~3.85)
Flicker (b-wave)	49.15 (33.02~79.37)	68.80 (47.98~98.22)	55.40 (32.94~66.30)	51.80 (36.55~77.85)

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Table 8 ERG implicit times in four dog breeds; data presented as the median (milliseconds) and range of the 5th and 95th percentiles.

Retinal function	Labrador Retriever	Poodle	Thai Bangkaew	Thai Ridgeback
Rod 1 (b-wave)	54.15 (41.47~69.73)	49.65 (46.72~57.41)	68.80 (62.36~76.64)	58.00 (47.40~68.15)
Rod 2 (b-wave)	55.70 (41.86~71.75)	57.50 (46.59~66.75)	69.00 (64.56~79.40)	62.70 (53.73~76.65)
Rod 3 (b-wave)	54.45 (45.14~70.29)	56.80 (49.09~72.85)	70.10 (64.74~82.12)	63.50 (49.66~77.03)
Rod 4 (b-wave)	54.35 (43.76~68.57)	59.15 (49.56~72.84)	72.80 (63.08~80.24)	61.30 (50.90~77.18)
Rod 5 (b-wave)	54.10 (47.40~67.85)	57.35 (49.99~78.95)	71.90 (65.40~79.88)	60.85 (50.90~75.78)
Std R&C (a-wave)	12.40 (11.35~14.85)	13.80 (11.00~16.82)	15.90 (12.90~18.64)	12.25 (10.88~14.00)
Std R&C (b-wave)	25.20 (21.70~29.60)	27.15 (25.56~33.93)	35.80 (33.58~50.54)	29.30 (22.75~64.63)
Hi-int R&C (a-wave)	10.20 (9.85~11.55)	10.65 (9.34~12.71)	13.50 (10.60~15.32)	10.40 (9.23~11.58)
Hi-int R&C (b-wave)	24.30 (21.95~32.60)	27.20 (25.17~42.33)	39.20 (29.40~51.86)	32.00 (22.23~59.10)
Cone (a-wave)	9.35 (8.18~10.23)	9.90 (8.26~10.83)	10.90 (10.18~12.16)	9.20 (7.54~10.96)
Cone (b-wave)	21.40 (16.56~24.81)	24.55 (23.12~25.79)	24.00 (22.72~24.50)	24.40 (18.17~25.76)
Flicker (b-wave)	27.45 (26.20~29.19)	27.5 (26.14~29.02)	27.90 (26.44~29.40)	26.80 (25.98-29.18)

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Table 9 Summary of ERG amplitude and b/a ratio recorded from four dog breeds; data presented as the mean (microvolts) and standard error.

Retinal function	Labrador Retriever	Poodle	Thai Bangkaew	Thai Ridgeback
Rod 1 (b-wave)	38.39 ± 7.99	49.21 ± 6.52	63.89 ± 7.99	80.18 ± 6.92
Rod 2 (b-wave)	46.70 ± 8.88	59.99 ± 7.25	80.41 ± 8.88	93.96 ± 7.69
Rod 3 (b-wave)	53.68 ± 10.37	78.41 ± 8.47	104.43 ± 10.83	98.98 ± 9.28
Rod 4 (b-wave)	56.43 ± 10.79	87.22 ± 8.81	110.54 ± 11.27	104.14 ± 9.34
Rod 5 (b-wave)	60.76 ± 10.87	96.12 ± 8.49	96.108 ± 10.4	114.62 ± 9.01
Std R&C (a-wave)	88.99 ± 10.22	120.84 ± 7.99	100.32 ± 9.79	117.76 ± 8.47
Std R&C (b-wave)	151.00 ± 18.96	231.33 ± 14.82	170.63 ± 18.15	215.67 ± 15.72
b/a ratio	1.76 ± 0.15	2.00 ± 0.11	1.75 ± 0.14	1.89 ± 0.12
Hi-int R&C (a-wave)	118.95 ± 14.01	164.7 ± 10.94	170.91 ± 14.01	154.63 ± 11.61
Hi-int R&C (b-wave)	167.53 ± 19.01	253.26 ± 14.86	223.16 ± 18.2	236.37 ± 15.76
b/a ratio	1.46 ± 0.11	1.61 ± 0.14	1.34 ± 0.11	1.55 ± 0.27
Cone (a-wave)	9.68 ± 1.24	14.62 ± 1.02	10.64 ± 1.3	10.89 ± 1.11
Cone (b-wave)	20.82 ± 2.91	42.11 ± 2.38	30.21 ± 3.04	32.08 ± 2.61
b/a ratio	2.35 ± 0.23	3.09 ± 0.19	2.81 ± 0.24	2.98 ± 0.21
Flicker (b-wave)	52.00 ± 4.70	69.14 ± 3.95	53.15 ± 4.91	55.03 ± 4.07

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Table 10 Summary of ERG implicit times recorded from four dog breeds; data presented as the mean (milliseconds) and standard error.

Retinal function	Labrador Retriever	Poodle	Thai Bangkaew	Thai Ridgeback
Rod 1 (b-wave)	54.92 ± 1.91	51.12 ± 1.56	69.74 ± 1.91	57.27 ± 1.65
Rod 2 (b-wave)	57.01 ± 2.32	56.44 ± 1.89	70.73 ± 2.32	62.97 ± 2.01
Rod 3 (b-wave)	56.94 ± 2.32	57.66 ± 1.89	71.14 ± 2.42	62.6 ± 2.07
Rod 4 (b-wave)	56.63 ± 2.20	59.46 ± 1.81	71.75 ± 2.31	62.35 ± 1.92
Rod 5 (b-wave)	56.21 ± 2.52	60.27 ± 1.97	73.02 ± 2.42	63.24 ± 2.09
Std R&C (a-wave)	12.72 ± 0.55	13.85 ± 0.43	15.58 ± 0.53	12.32 ± 0.45
Std R&C (b-wave)	25.08 ± 2.46	28.31 ±1.92	40.29 ± 2.35	33.18 ± 2.04
Hi-int R&C (a-wave)	10.46 ± 0.39	10.93 ± 0.30	12.59 ± 0.37	10.49 ± 0.32
Hi-int R&C (b-wave)	26.48 ± 2.61	30.04 ± 2.04	41.91 ± 2.50	33.75 ± 2.16
Cone (a-wave)	9.25 ± 0.30	9.71 ± 0.24	11.32 ± 0.31	9.25 ± 0.27
Cone (b-wave)	21.34 ± 0.59	24.51 ± 0.48	23.63 ± 0.62	23.5 ± 0.53
Flicker (b-wave)	27.49 ± 0.32	27.49 ± 0.27	27.92 ± 0.34	27.18 ± 0.28

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2. Comparison of normal ERG traces

Traces of average ERG parameters of each dog breed were compared (Figure 16-24). Means and standard error (SE) of amplitudes, implicit times and b/a ratios obtained from the four dog breeds were summarized in Figure 25, 26 and 27. For the amplitudes, there were significant differences in all parameters: Rod1(P=0.002), Rod2 (P<0.001), Rod3 (P=0.006), Rod4 (P=0.001), Rod5 (P=0.002), a-wave of Std R&C (P=0.024), b-wave of Std R&C (P=0.014), a-wave of Hi-int R&C (P=0.029), b-wave of Hi-int R&C (P=0.01), a-wave of Cone (P=0.023), b-wave of Cone (P<0.001) and Flicker (P=0.011). For the implicit time, significant difference was observed in all parameters except for Flicker (P=0.061). Significant differences of implicit times were observed for Rod1(P<0.001), Rod2 (P<0.001), Rod3 (P<0.001), Rod4 (P<0.001), Rod5 (P<0.001), a-wave of Std R&C (P=0.003), b-wave of Std R&C (P<0.001), a-wave of Hi-int R&C (P=0.004), b-wave of Hi-int R&C (P=0.002), a-wave of Cone (P<0.001), and b-wave of Cone (P=004).

In all Rod responses, Thai Ridgeback had higher b-wave amplitudes than Labrador Retriever. Poodle had higher b-wave amplitudes than Labrador Retriever in all responses except for Rod responses. There was no significant difference among amplitudes in all responses between Thai Ridgeback and Thai Bangkaew. Among all breeds, Thai Bangkaew had a more prolonged implicit time of b-waves of Rod1, Rod 3, Rod4 and Std R&C waves, including for a-waves of Hi-int R&C and Cone than in other breeds (Figure 26). However there was no significant difference in the b/a ratio of Std R&C (0.655) and Hi-int R&C (0.467) among the four breeds. Only the b/a ratio of the Cone response had a significant difference (P=0.035) among dog breeds (Figure 27).

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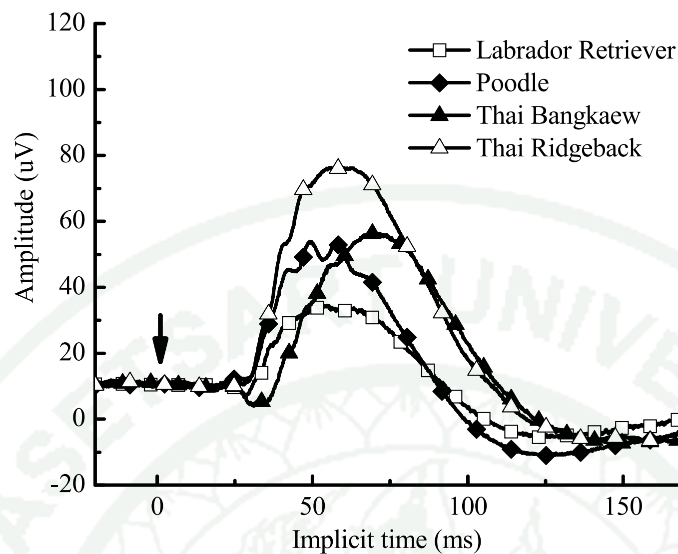

Figure 16 Comparison of normal ERG traces among 4 dog breeds. Scotopic low intensity responses were obtained using 0.01 cd.s/m^2 of light stimulation at 4 min after dark adaption (Rod1).

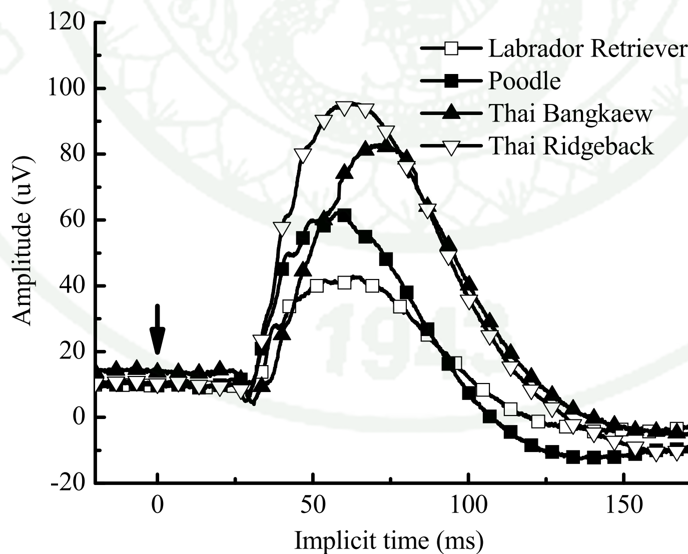

Figure 17 Comparison of normal ERG traces among 4 dog breeds. Scotopic low intensity responses were obtained using 0.01 cd.s/m^2 of light stimulation at 8 min after dark adaption (Rod2).

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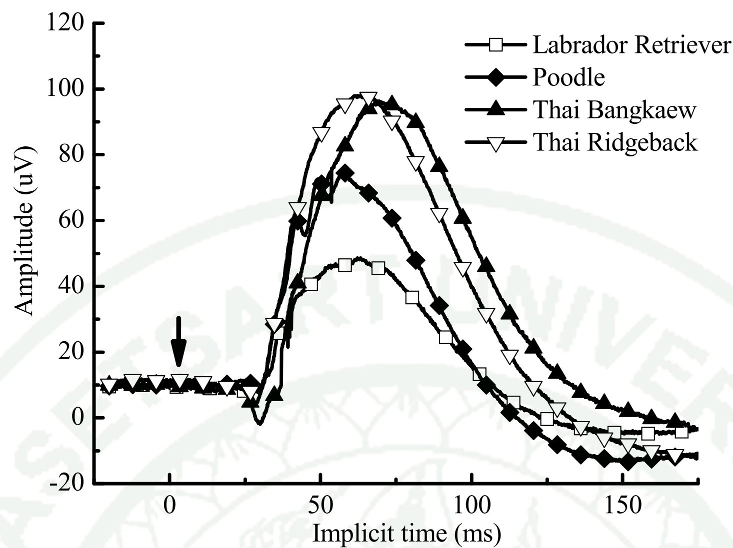

Figure 18 Comparison of normal ERG traces among 4 dog breeds. Scotopic low intensity responses were obtained using 0.01 cd.s/m^2 of light stimulation at 12 min after dark adaption (Rod3).

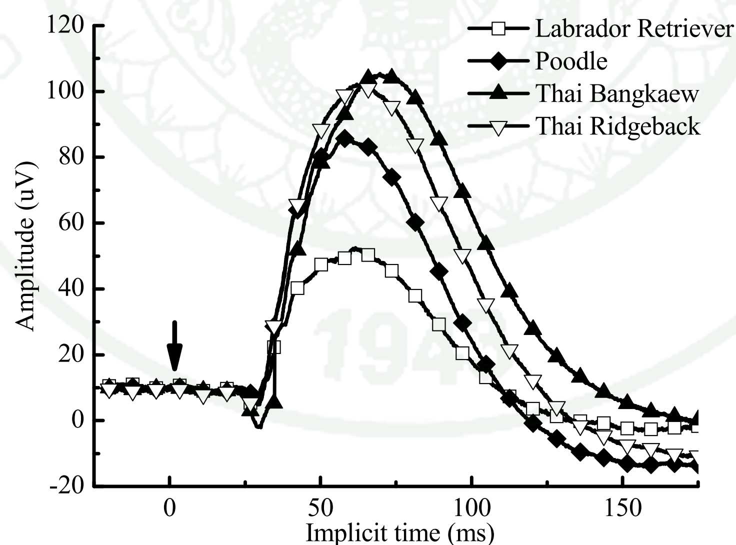

Figure 19 Comparison of normal ERG traces among 4 dog breeds. Scotopic low intensity responses were obtained using 0.01 cd.s/m^2 of light stimulation at 16 min after dark adaption (Rod4).

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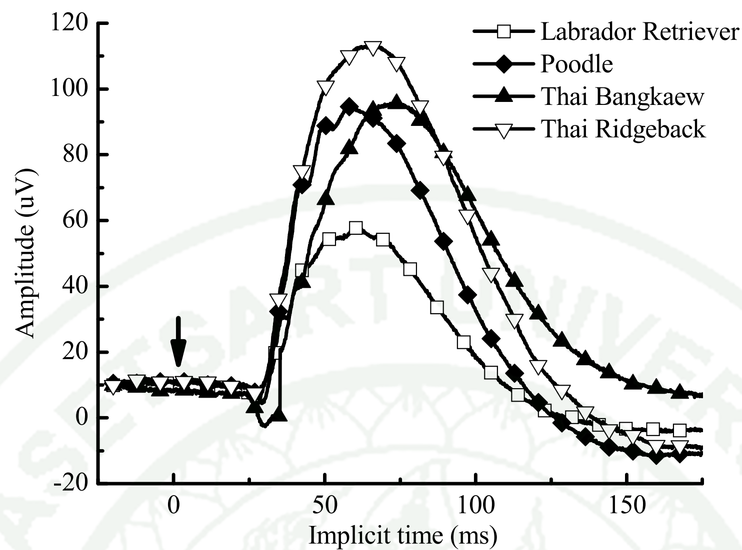

Figure 20 Comparison of normal ERG traces among 4 dog breeds. Scotopic low intensity responses were obtained using 0.01 cd.s/m^2 of light stimulation at 20 min after dark adaption (Rod5).

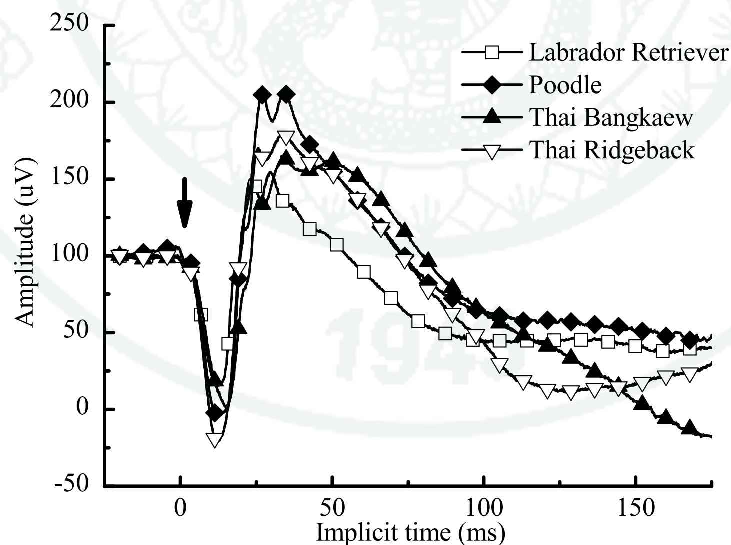

Figure 21 Comparison of normal ERG traces among 4 dog breeds. Scotopic standard intensity responses were obtained using 3 cd.s/m^2 of light stimulation (Std R&C).

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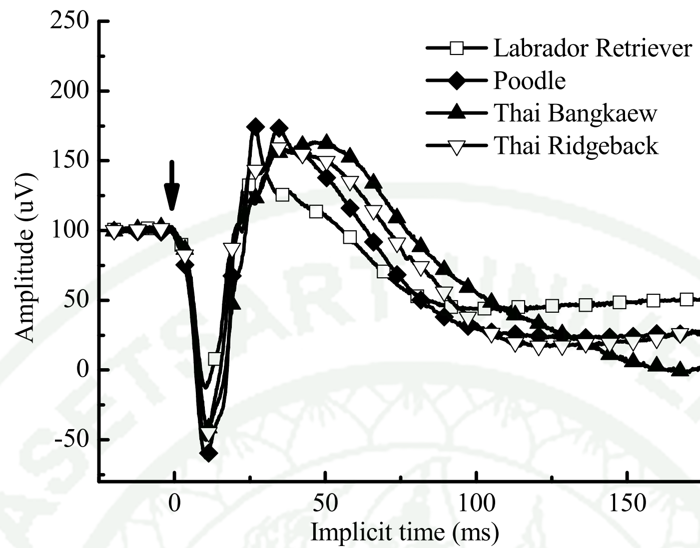

Figure 22 Comparison of normal ERG traces among 4 dog breeds. Scotopic higher intensity responses were obtained using 10 cd.s/m^2 of light stimulation (Hi-int R&C).

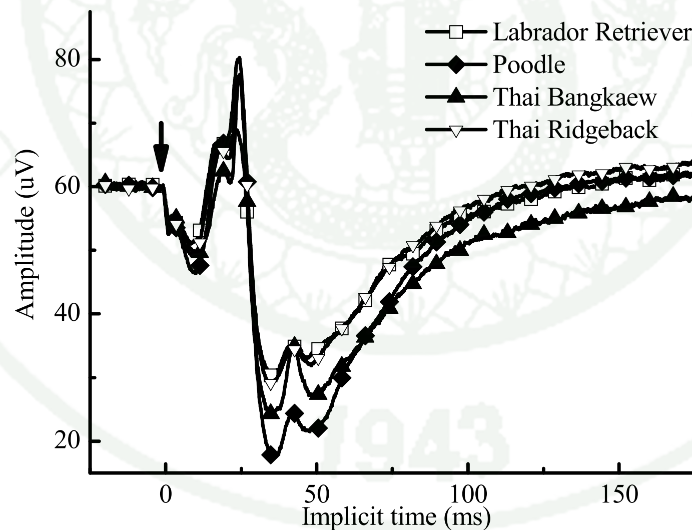

Figure 23 Comparison of normal ERG traces among 4 dog breeds. Photopic single flash responses were obtained using 3 cd.s/m^2 of light stimulation after 10 minutes of light adaptation with 30 cd.s/m^2 of background light (Cone).

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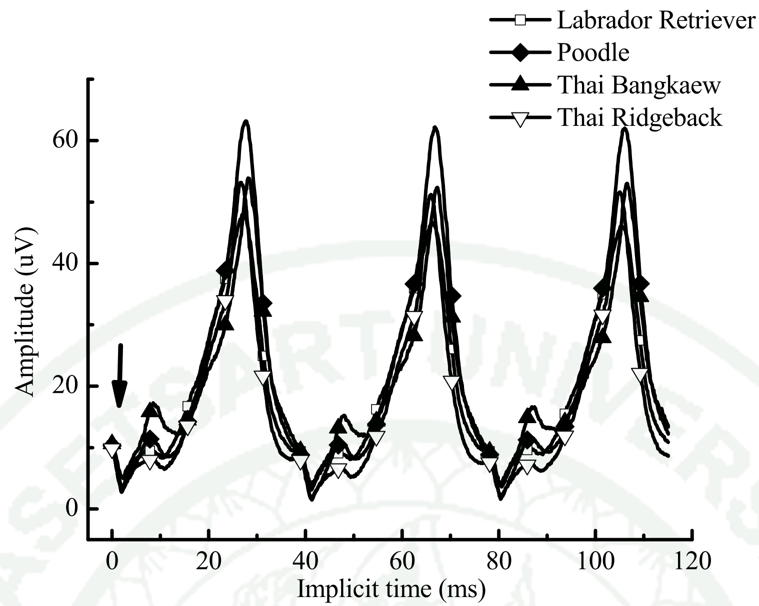

Figure 24 Comparison of normal ERG traces among 4 dog breeds. Photopic 30 Hz flicker responses were obtained using 3 cd.s/m^2 of light stimulation after 10 minutes of light adaptation (Flicker).

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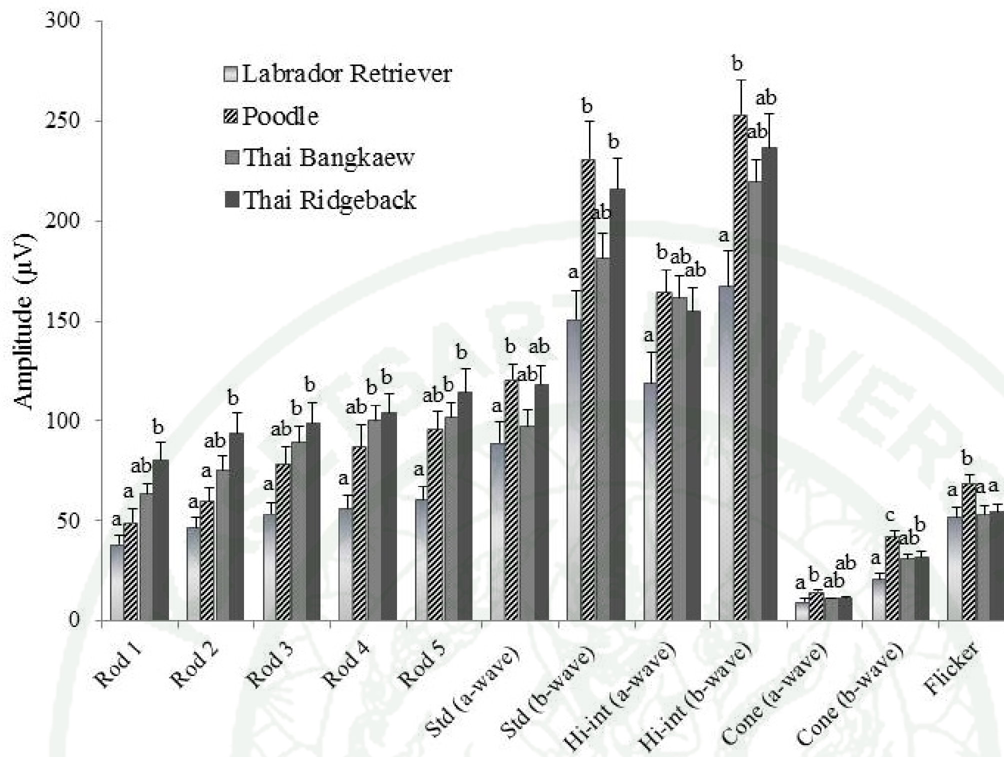

Figure 25 Mean and SE of amplitudes recorded in 4 dog breeds: a, b, and c indicate significant differences among dog breeds. ERG amplitudes of all responses were significantly different among dog breed (P<0.05). Rod1-Rod5 are the b-wave of scotopic low intensity responses, Std and Hi-int are scotopic standard and higher intensity responses, respectively.

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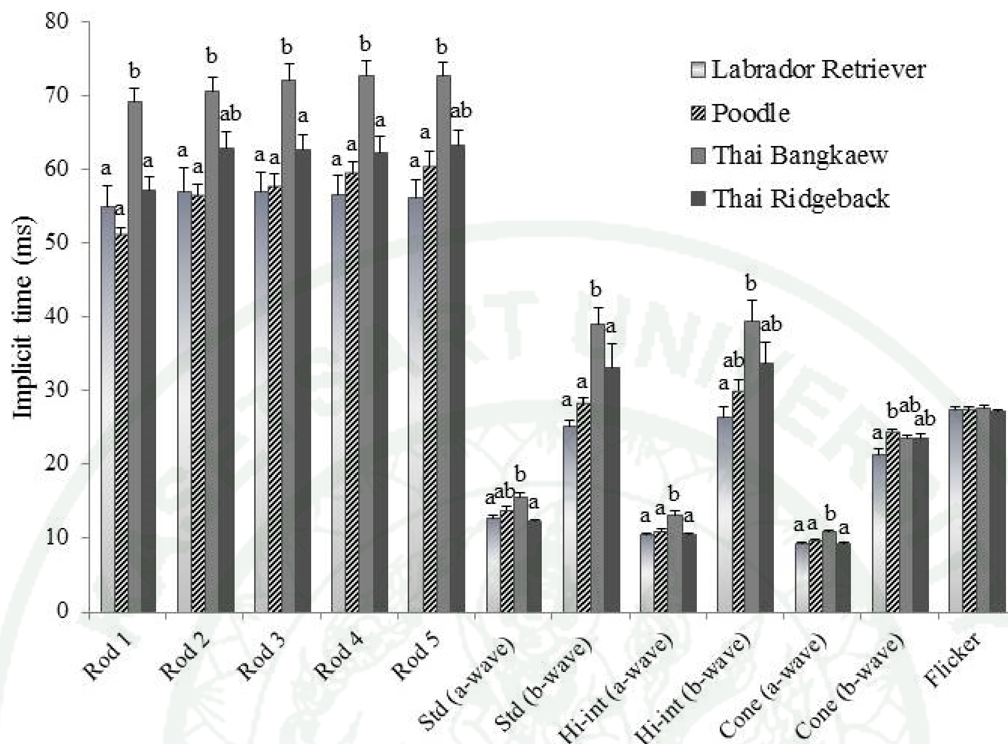

Figure 26 Mean and SE of implicit time recorded in 4 dog breeds: a, b, and c indicate significant differences among dog breeds (P<0.05). Implicit times of all responses are significantly different among dog breeds except for Flicker (P=0.610). Rod1-Rod5 are the b-wave of scotopic low intensity responses, Std and Hi-int are scotopic standard and higher intensity responses, respectively.

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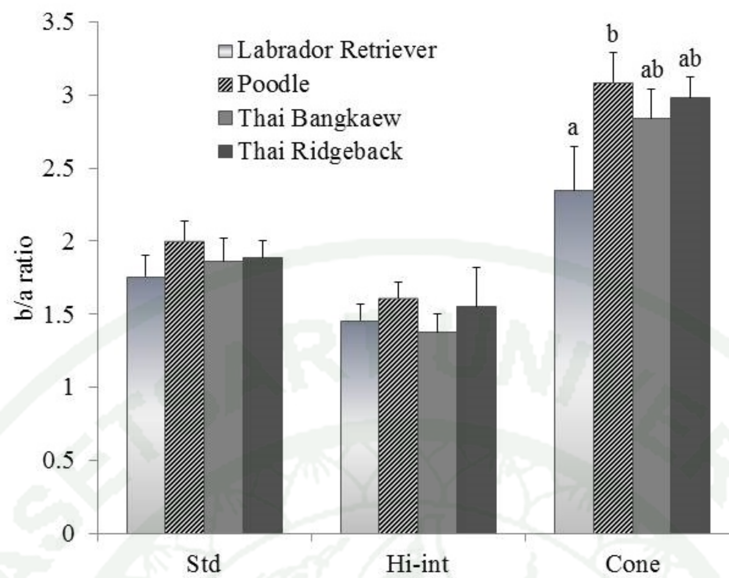

Figure 27 Mean and SE of b/a ratio recorded in 4 dog breeds: a, b, and c indicate significant differences among dog breeds. There was no statistical difference in b/a ratio of scotopic standard (Std) (P=0.476) and higher intensity (Hi-int) (P=0.214) responses. A significant difference (P=0.035) was observed for b/a ratio of the photopic single flash response (Cone).

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3. Confirm phenotypic of responsible gene mutation of PRA in Poodle

3.1 Clinical Characterization of PRCD-suspected Poodles

All PRCD-suspected dogs were brought for ocular examination due to visual deficit. Both sexes were presented equally. Age onset of visual impairment noticing by the owners was 7.9 ± 2.18 (5-11) years (Table 11). Only one dog had completely loss of vision at first visit (Dog1). This dog had a history of progressive loss of vision starting at night time. The other affected dogs remained partial vision, at least in the photopic condition. The mean age for all dogs at first examination was 8.9±2.33 (6-13) years. Seven suspected dogs (Dog 1, 2, 3, 6, 7, 9 and 10) had visual problems for greater than or equal to 1 year before being brought for diagnosis. Only 3 affected dogs (Dog 4, 5 and 8) were promptly brought for ocular examination after the owner noticing their dogs' visual problem. Fundic appearance of all PRCD-suspected dogs included with tapetal hyperreflectivity, retinal vessel attenuation, and optic disc atrophy. Seven PRCD-suspected dogs had cataract at the incipient or immature stage at the study day. Five dogs (Dog 4, 5, 7, 9 and 10) had incipient cataract at the first presentation and showed immature stage at the study.

Fundic appearance of all PRCD-suspected dogs included with tapetal hyperreflectivity, retinal vessel attenuation, and optic disc atrophy including cataract formation in some PRCD-suspected dogs. The fundus pictures of Dog 1, 2, 6 and 8 were shown in Figure 28, 29, 30 and 31 respectively, as well as photograph of cataract formation of Dog 5 was shown in Figure 32.

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Table 11 Sex, age onset noticed by the owners, age at first presentation, age at the study and results of obstacle test at the study of PRCD-suspected Poodles.

Dog No.	**Sex**	**Age onset (year)**	**Age at first presentation (years)**	**Age at the study (years)**	**Obstacle test at the study**		**Cataract stage at the study**
					Scotopic	Photopic	
1	M	7	8	8	Neg	Neg	-
2	M	11	13	15	Neg	Poor	Incipient
3	M	9	10	10	Poor	Pos	Incipient
4	M	11	11	13	Poor	Pos	Immature
5	M	6	6	7	Neg	Poor	Immature
6	F	5	6	6	Neg	Poor	-
7	F	6	8	8	Neg	Poor	Immature
8	F	7	7	9	Neg	Poor	-
9	F	10	11	13	Neg	Poor	Immature
10	F	7	9	9	Poor	Pos	Immature
Mean±SD		7.9±2.18	8.9±2.33	9.8±2.94			

M = male

F = female

Neg = negative

Pos = positive

\- = no cataract formation

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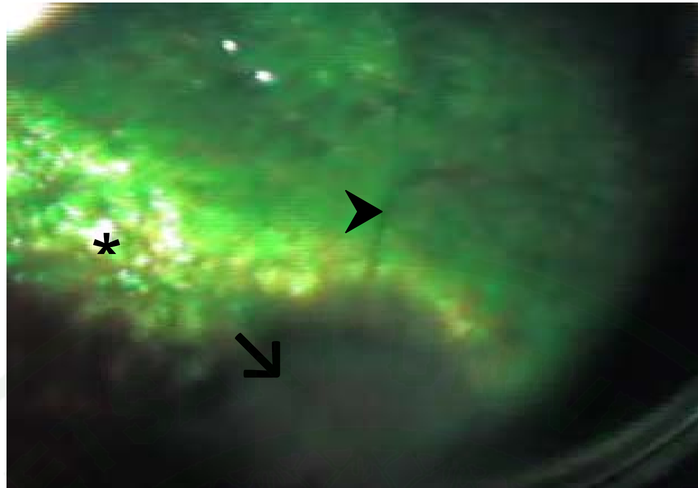

Figure 28 A fundus picture from an eight-year-old Poodle which has clinical signs and genetic test correspond with PRCD (Dog1) with retinal thinning causing mark tapetal hyperreflection (*) and retinal vessel attenuation (arrow head). The optic disc was appearing pale (arrow). The dog had completely loss of vision at first visit.

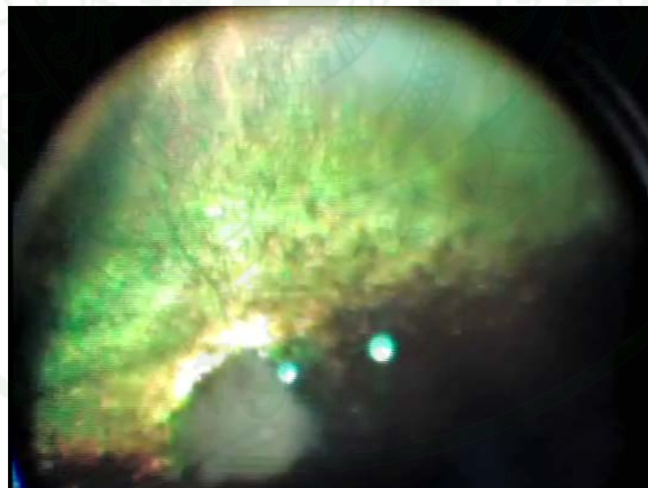

Figure 29 A fundus picture from a fifteen-year-old Poodle which has clinical signs and genetic test correspond with PRCD (Dog 2) with marked retinal degeneration.

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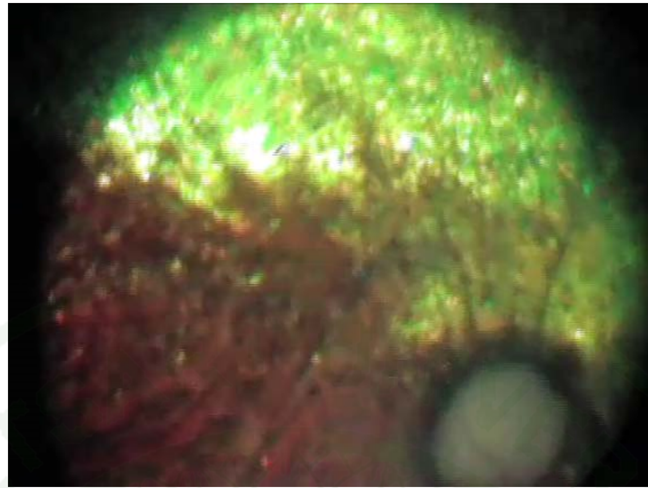

Figure 30 A fundus picture from a six-year-old Poodle which has clinical signs and genetic test correspond with PRCD (Dog 6) with retinal degeneration. The dog had no cataract formation and still remained poor vision in day light.

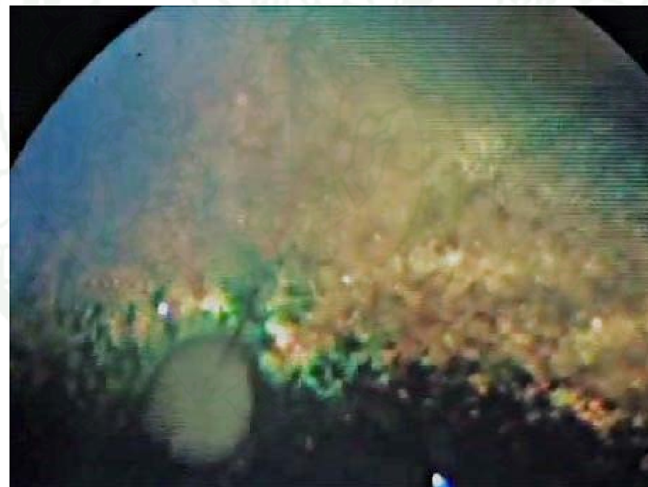

Figure 31 A fundus picture from a nine-year-old Poodle which has clinical signs and genetic test correspond with PRCD (Dog 8) with tapetal hyperreflection, retinal vessel attenuation and pale of optic disc while the dog was still visual in bright light condition, but had no vision in dim light.

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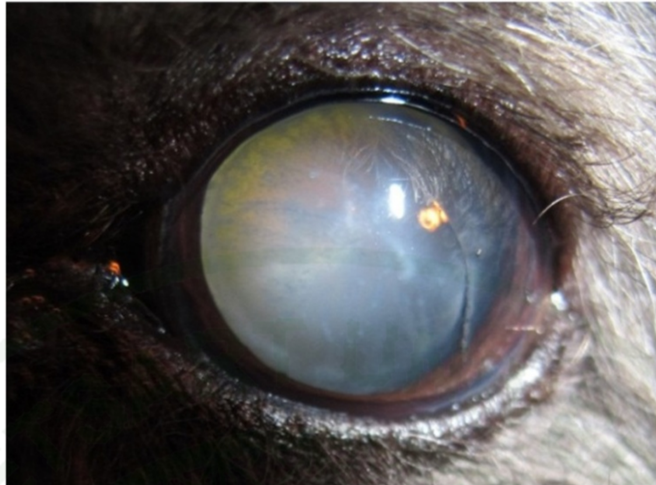

Figure 32 Immature cataract in the right eye of a 7-year-old Poodle which has clinical signs and genetic test correspond with PRCD (Dog 5). This cataract formation was common with the advance stage of the disease.

3.2 Electroretinography

ERG record consists of 9 sessions of photoreceptor stimulation (Figure 33). ERGs of Dog 7 (8-year-old) and Dog 3 (10 year-old) showed a mark reduction of a- and b-wave amplitudes in all stimulation compared to ERG recordings in an 8-year-old normal Poodle. In the session of low light intensity stimulation (Figure 33 a-e), ERGs of Dog 3 showed extremely small in the amplitude and delay in the implicit time of b-wave while ERG waveform of Dog 7 was not recordable. Attenuated waveforms of both PRCD-suspected dogs were recorded when stimulated with high light intensity and flicker flash after 10 minutes of light adaptation. ERGs from Dog 5, 6, 9 were similar to Dog 7 while Dog 4 still had attenuated ERG waveform for rod response as Dog 3. Waveforms in Dog 1 revealed flat lines of all ERG recordings. (data not shown).

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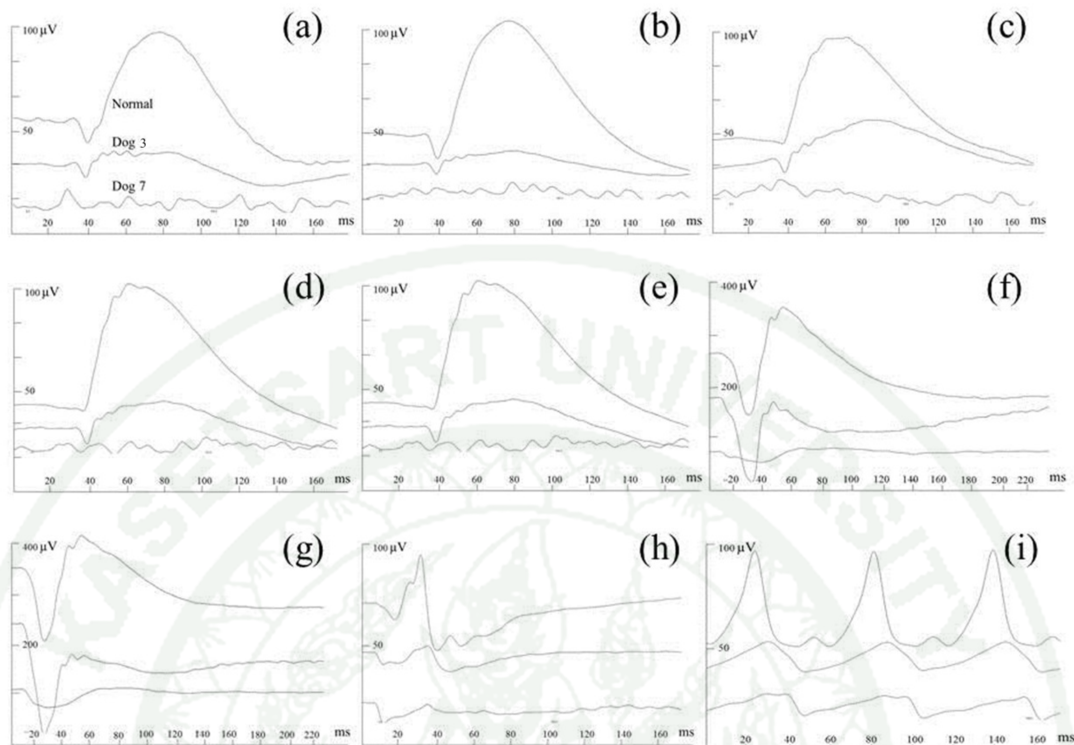

Figure 33 ERG comparison graphs of normal and dogs which have clinical signs and genetic test correspond with PRA using the Dog Diagnostic protocol of HMsERG. The upper was 8-year-old normal Poodle, the middle and lower graphs were PRCD-suspected dogs, Dog 3 and 7 respectively. (a-e: Rod 1-5, f: Std R&C, g: Hi-int R&C, h: Cone, i: Flicker. Note the affected dogs showed profoundly low a- and b- wave amplitudes.

3.3 Histopathological examination

An enucleated eye from a PRCD dog (Dog 1) was microscopically examined. Under hematoxylin & eosin stain, the major lesions were confined to the retina. Thinning of the retina was profound, and characterized by collapse of the nuclear and photoreceptor layers, with loss of ganglion cell layer where few ganglion cells remained. The complete loss of the photoreceptor nuclei were noted (Figure 34).

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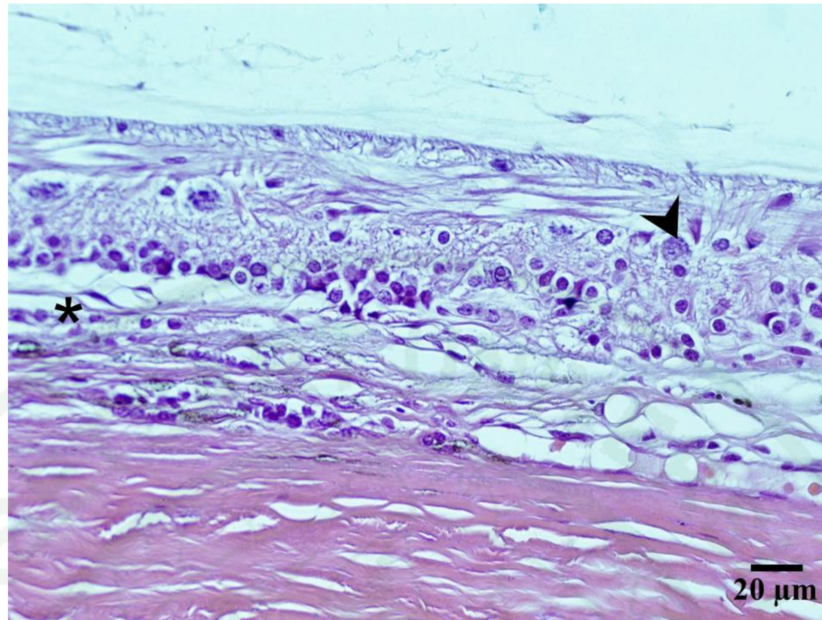

Figure 34 Retina adjacent optic nerve of an 8-year-old Poodle which has clinical signs and genetic test correspond with PRCD (Dog 1) showed retinal degeneration characterized by thinning of retina, collapse of the nuclear and photoreceptor layers (*), presence of gliosis, and few remaining ganglions (arrow head). H & E, 40x.

3.4 Genetic testing

To identify the result, PCR product (346 bp) of wild type allele (TGC) was not digested by restriction endonuclease while mutant allele (TAC) was digested into two bands (219 bp and 127 bp) when separated in agarose gel electrophoresis. All patterns of allele (346 bp, 219 bp and 127 bp) were found in the carrier (Figure 35-36). The direct sequencing of PCR product of affected dogs exactly correlated with PCR-RFLP result (Figure 37). All PRCD-suspected dogs showed a fragment DNA sequences containing the *PRCD* gene. This gene had a homozygous mutation that caused amino acid change from cysteine to tyrosine (TGC→ TAC) in the second codon of *PRCD* gene. While the normal dog showed wild type allele of TGC on the same locus. This result confirmed that all 10 PRCD-suspected dogs were affected.

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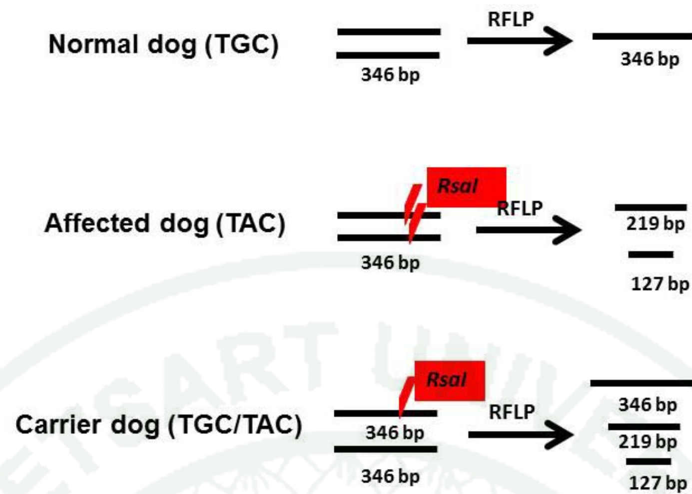

Figure 35 Genetic testing using PCR-RFLP technique, the 346 bp DNA fragment of normal dog was not digested by RsaI restriction endonuclease. While the mutant alleles (TAC) of affected dog were digested into two bands of 219 bp and 127 bp. As well as, all patterns of allele; 346 bp, 219 bp and 127 bp were found in the carrier dog.

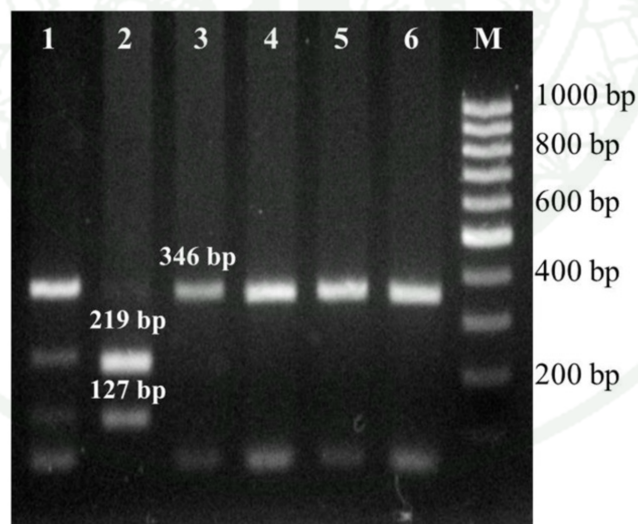

Figure 36 The PCR products after digested with *RsaI* restriction enzyme that separated in 2% agarose gel electrophoresis. The pattern of DNA fragments showed the PRCD-PRA status; lane 1 and 2 were carrier and affected dog (Dog 1) respectively, lane 3, 4, 5 and 6 were normal dogs.

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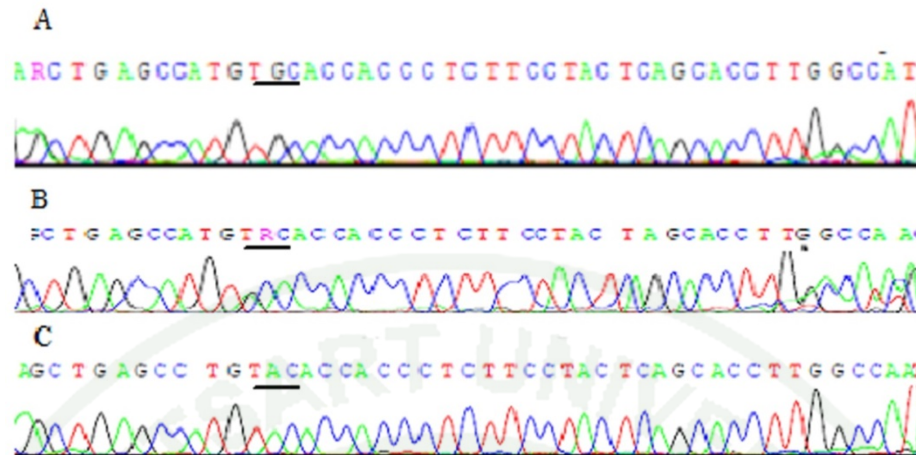

Figure 37 Direct sequencing of PCR product spanning the site of exon 1 of the *PRCD* locus; A. Normal, B. Carrier and C. Affected (Dog 1). For the carrier, the DNA sequence of “R” is the coding of Guanine (G) and Adenine (A).

4. Analyze the allele frequencies of PRA in Poodle and Labrador retriever

Prevalence of affected and carrier poodle dogs were 4% (2/50) and 12% (6/50) respectively. The allele frequency of normal and mutant allele was 0.9 and 0.1 respectively. For Labrador retriever dogs, a prevalence of carrier was 3.45% (2/58) and none was diagnosed as affected dog by genetic testing. The allele frequencies of normal and mutant alleles in Labrador retriever were 0.98 and 0.02 respectively**.**

5. Identify phenotypic characterization and the responsible gene mutations of PRA in Thai Bangkaew

5.1 General examination

All Thai Bangkaew dogs underwent a history taking and physical examination. Additionally, a complete blood cell count and standard biochemistry profile such as BUN, creatinine, SGPT, ALK, glucose, cholesterol, triglyceride,

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protein, albumin and thyroid hormone were examined. Six suspected dogs were designated TB1-TB6. Among these, only one dog was female (TB2) (Table 12).

Dog TB1 was a male, 7 years old at the examination day. The dog was examined by veterinarian after suspicion of visual impairment for 2 months. The dog had a history of toxic hepatitis at 2 years of age, but responded well to treatment and declared healthy. The dog had gain weight and had hypothyroidism. The others blood profiles examination showed normal results except for mild increasing of triglyceride. The diet consisted of commercial dog food together with homemade food including meat and chicken.

Dog TB2 was a female, 8 years old at the examination day. The owner estimated the visual impairment for 1 year without an observation of different in night and day time. The dog was referred to ophthalmology clinic during treatment of cholecystitis with gall stone and hepatopathy. The treatment of these diseases lasted for 3 months and the dog responded well. Environmental history of this dog was not available.

Dog TB3 was a male, 10 years and 3 months old at the examination day. He was brought for ocular examination because of visual impairment as hesitation when going up and down stairs for 3 months. The owner reported the dog had never been ill. General examination including blood test revealed normal result except for ocular problem.

Dog TB4 was a male, 6 years old. This dog was during chronic renal failure treatment for a year and the respond to treatment was not well. The dog had visual impairment due to hesitatation to walk in his familiar house for 6-7 months with. Blood examination at the date of the study revealed chronic renal failure. Finally, the dog died at next 5 months.

Dog TB5 was a male, 11 years old. It was more than 7 months that the owner had observed for his visual impairment. The dog was sick with pneumonia for

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3 months when he was 9 years old and improved well after treatment. At the times of ocular examination the dog had normal result for blood profile tests and showed healthy.

Dog TB6 was a male, 11 years and 2 months old. The owner observed that the dog had visual problem for approximately 1 or 2 months. Because the dog had joint disease for more than 6 months, the owner could not indicate the exact time of the ocular disease onset. The dog was diagnosed with hypothyroidism and treated with hormone supplement for more than 1 year. The result of others profile of blood examination showed normal including cholesterol and triglyceride.

5.2 Ocular examination

Six suspected Thai Bangkaew dogs were recruited for complete ocular examination. The age onset of visual impairment observed by owners was 8.5 ± 2.07 (mean ± SD) years with range of minimum to maximum of 6-11 years. Ocular reflex examination including menace response, dazzle reflex and PLR was performed. All dogs had no menace response and dazzle reflex except for one dog still had delayed dazzle response (TB4). For direct and indirect PLR testing, all dog had positive response for light stimulation by constriction of the pupil with different speed response. Dog TB1 and TB5 showed normal PLR while the others showed slowly response but complete pupil constriction except for TB2 had incomplete response (Table 12).

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Table 12 Sex, age and ocular examination in the six retinal degeneration Thai Bangkaew dogs.

Dog	Name	Sex	Age		Ocular reflex		
			Onset	At exam	Menace response	Dazzle	PLR
TB1	Kulpol	M	7y	7y	Neg	Neg	Delayed complete
TB2	Spotty	F	7y	8y	Neg	Neg	Delayed incomplete
TB3	Keawnguan	M	10y	10y 3m	Neg	Neg	Delayed complete
TB4	Dingding	M	6y	6y	Neg	Slow positive	Delayed complete
TB5	Muyong	M	10y	10y	Neg	Neg	Delayed complete
TB6	Thong	M	11y 1m	11y 2m	Neg	Neg	Delayed complete

M = male
F = female
Neg = negative
PLR = pupillary light reflex

Two dogs had negative response for scotopic and photopic obstacle test (TB1 and TB2) while TB3, TB4 and TB6 showed negative response for scotopic but positive with poor response for photopic obstacle test. We could not examine the obstacle test in TB6 because of his hip problem and degenerative joint disease.

ERG was performed on five suspected dogs based on the owner consents and the subject condition. TB1 and TB2 showed flat line graph which were complete loss of rod and cone photoreceptor function. TB3 and TB6 revealed nonrecordable graph

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in rod function with low response of cone while TB5 showed low response of rod and cone (Table 13).

Fundus examined of all dog showed bilateral degeneration with tapetal hyperreflextivity, retinal vessel attenuation and optic disc atrophy with different degree of degeneration. Fundus photographs of the six all dogs were shown in Figure 38 and the comparison between ERG in suspected and normal dogs was shown in Figure 39-42.

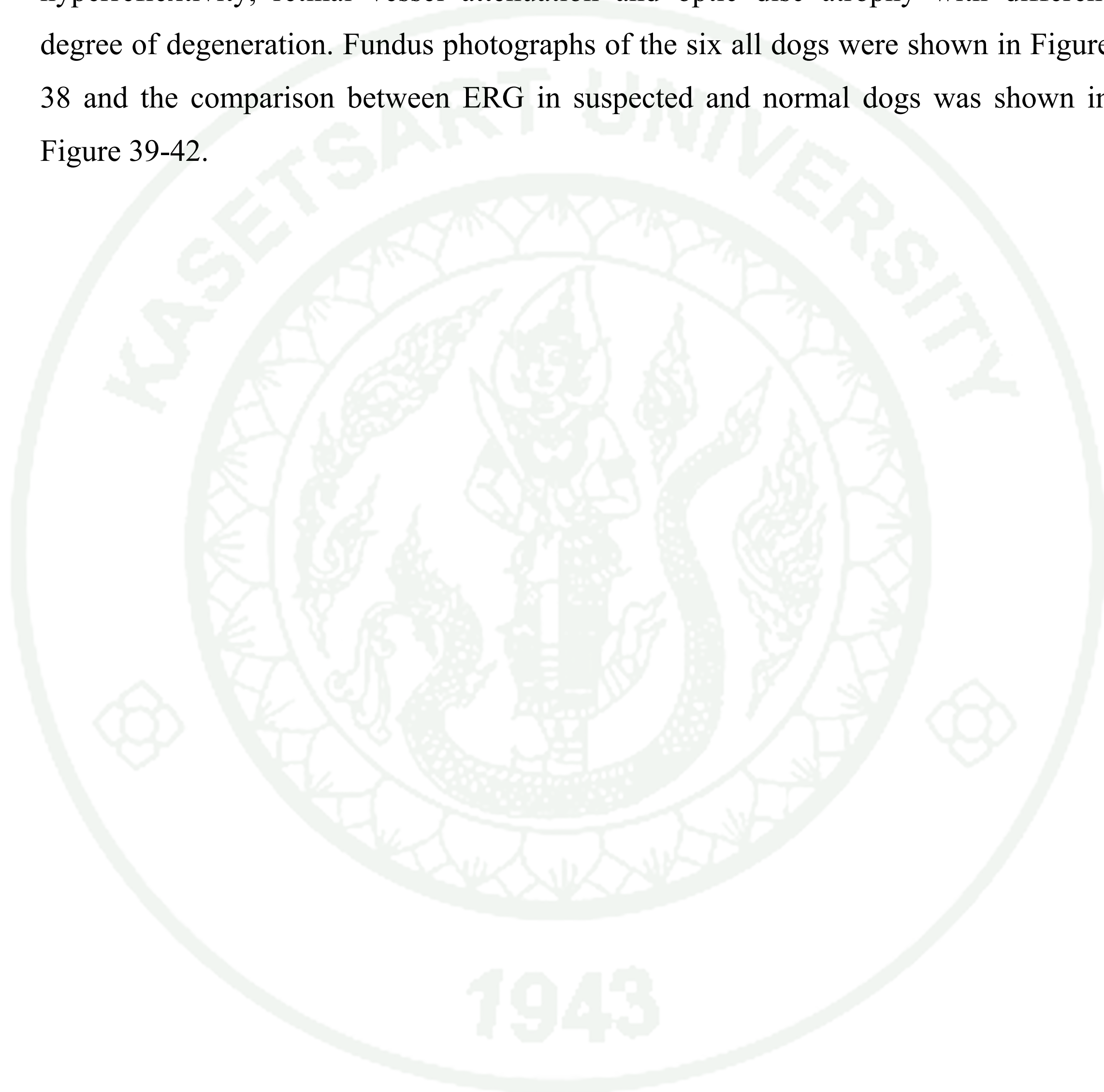

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Table 13 Obstacle test, ERG and fundus examination in the six suspected Thai Bangkaew dogs.

Dog	Obstacle test		ERG	Fundus examination of both eyes
	Scotopic	Photopic		
TB1	Neg	Neg	Flat graph (Short protocol)	Generalized tapetal hyperreflectivity, retinal vessel attenuation and pale optic disc
TB2	Neg	Neg	Flat graph (Complete protocol)	Mild tapetal hyperreflectivity, vessel attenuation and pale optic disc
TB3	Neg	Poor	Complete loss of rod function with remain low response of cone (Complete protocol)	Retinal vessel attenuation, optic disc slightly pale with normal tapetal reflectivity
TB4	Neg	Poor	NA	Generalized tapetal hyperreflectivity, retinal vessel attenuation and optic disc atrophy
TB5	Neg	Poor	Low rod and cone response (Complete protocol)	Generalized tapetal hyperreflectivity, vessel attenuation, pink optic disc
TB6	NA	NA	Complete loss of rod with low function of cone response (Complete protocol)	Mild tapetal hyperreflectivity, mild vessel attenuation, pale optic disc

NA = not available, Neg: negative

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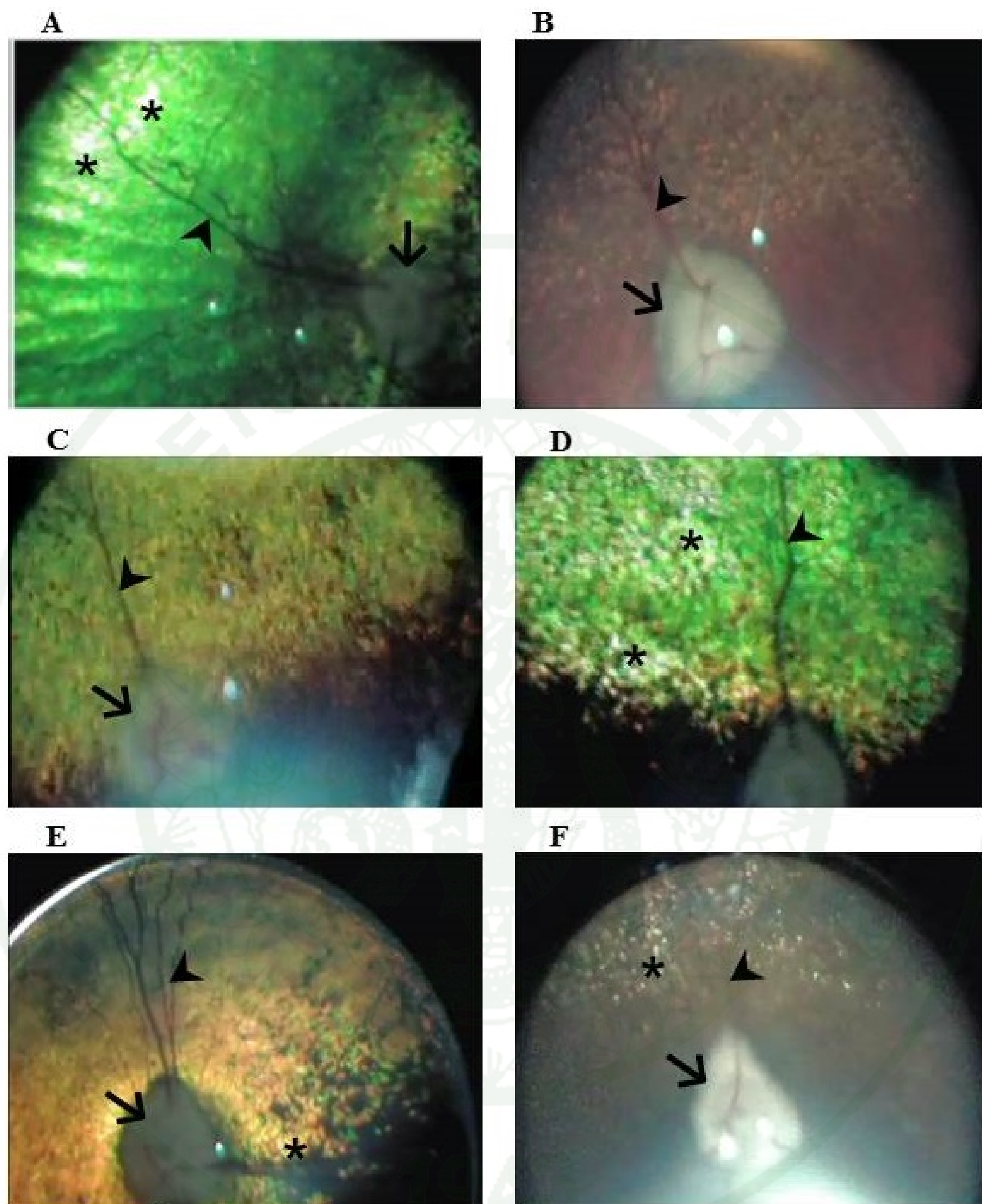

Figure 38 Fundus photographs of retinal degeneration Thai Bangkaew dogs; A: TB1, B: TB2, C: TB3, D: TB4, E: TB5, F: TB6. Fundus abnormalities observed were tapetal hyperreflectivity (*), vascular attenuation (arrow head) and pale optic disc (arrow).

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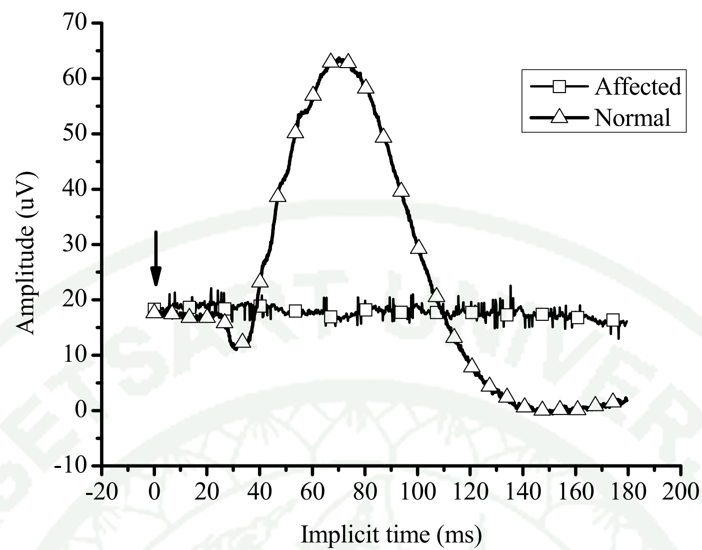

Figure 39 Rod responses ERG from a retinal degeneration dog (TB3) and a normal Thai Bangkaew dog. A flat graph was observed in the retinal degeneration dog.

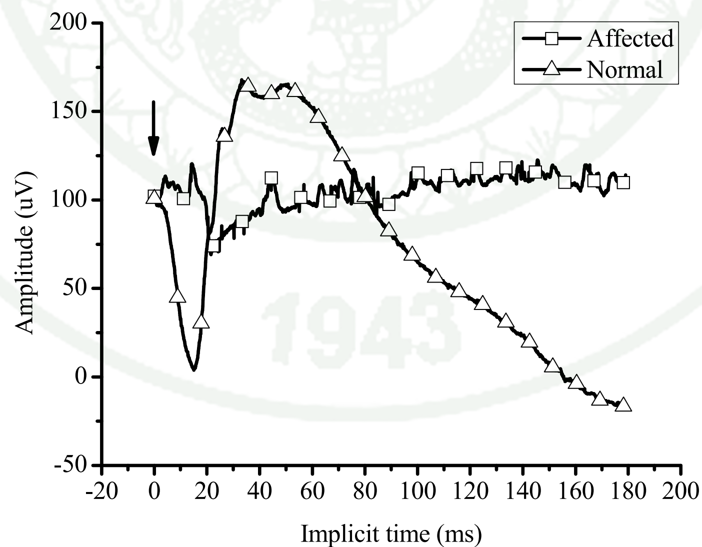

Figure 40 Std R&C responses ERG from a retinal degeneration dog (TB3) and a normal Thai Bangkaew dogs. The retinal degeneration dog showed low a- and b- wave amplitudes and prolong implicit times.

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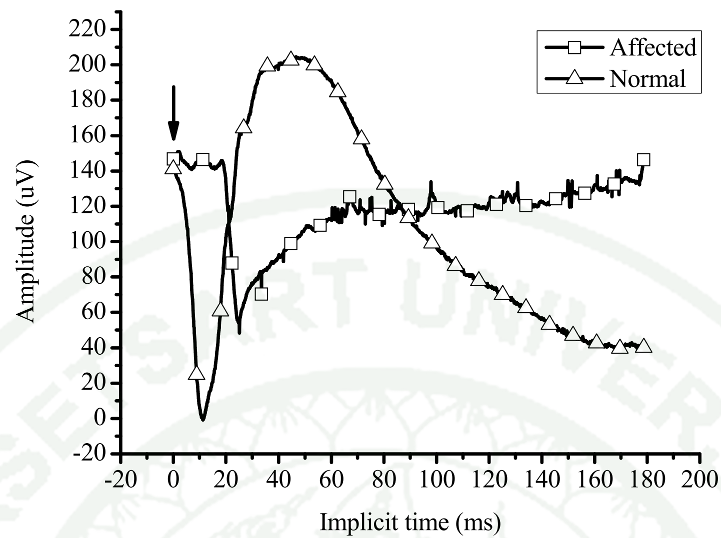

Figure 41 Hi-int R&C responses ERG from a retinal degeneration dog (TB3) and a normal Thai Bangkaew dog. The retinal degeneration dog showed low a- and b- wave amplitudes and prolong implicit times.

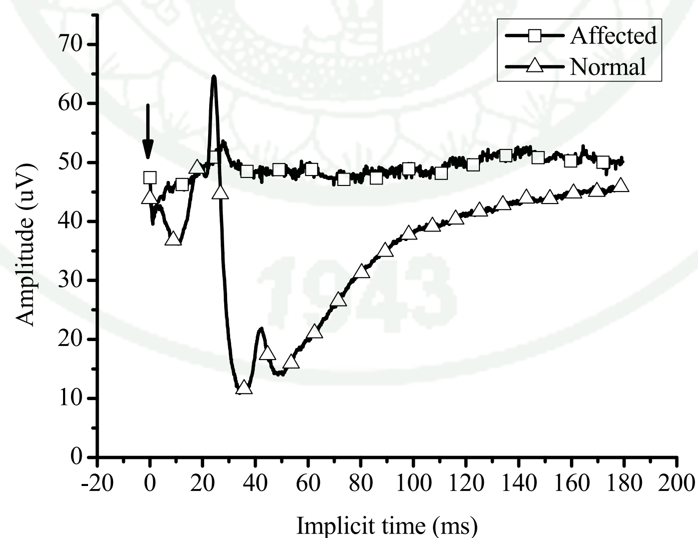

Figure 42 Photopic Cone responses ERG from a retinal degeneration dog (TB3) and a normal Thai Bangkaew dog. Noted: undetectable a-wave amplitudes and low b-wave amplitudes in the retinal degeneration dog.

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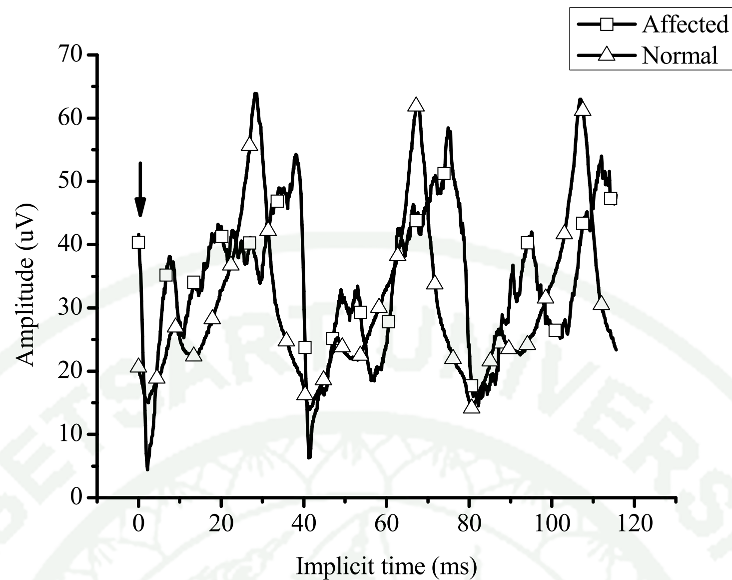

Figure 43 Flicker response ERG from a retinal degeneration dog (TB3) and a normal Thai Bangkaew dog. Note: an abnormal wave forms with many noises and prolong implicit times in the retinal degeneration dog.

5.3 Analysis of candidate gene

Six candidate loci from four genes of suspected and normal dogs were amplified by specific primers. The DNA fragments from each PCR products were visualize via agarose gel electrophoresis. These fragments had estimated size as 326 bp for PRCDE1, 329 bp for PRCDE2, 320 bp for PRCDE3, 239 bp for XLPRA, 244 bp for RHO and 318 bp for CCDC primer. The alignment identities with available published gene sequence were 99-100% identities. There was no sequence variant of coding region in exon 1-3 of PRCD gene when compared between the sequences amplified from normal dogs. All retinal degeneration dogs showed homozygous for the normal allele of PRCD, RPGR, RHO and CCDC66 gene with no base changes in critical size of gene mutations (Figure 44-48).

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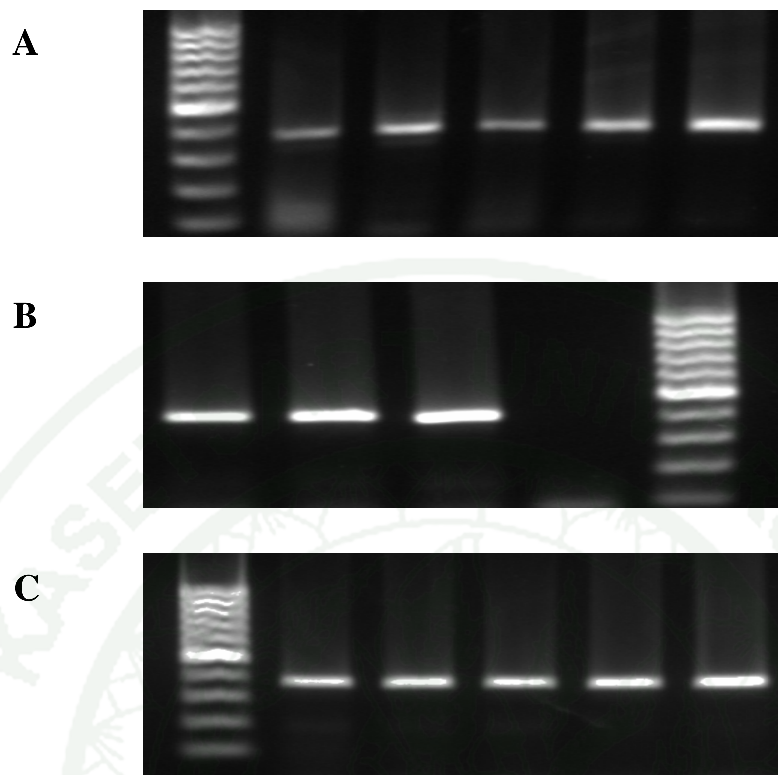

Figure 44 Agarose gel showing 326 bp of PRCDE1(A), 329 bp of PRCDE2 (B) and 320 bp of PRCDE3 (C), Marker = 1 Kb DNA Ladder (Invitrogen®).

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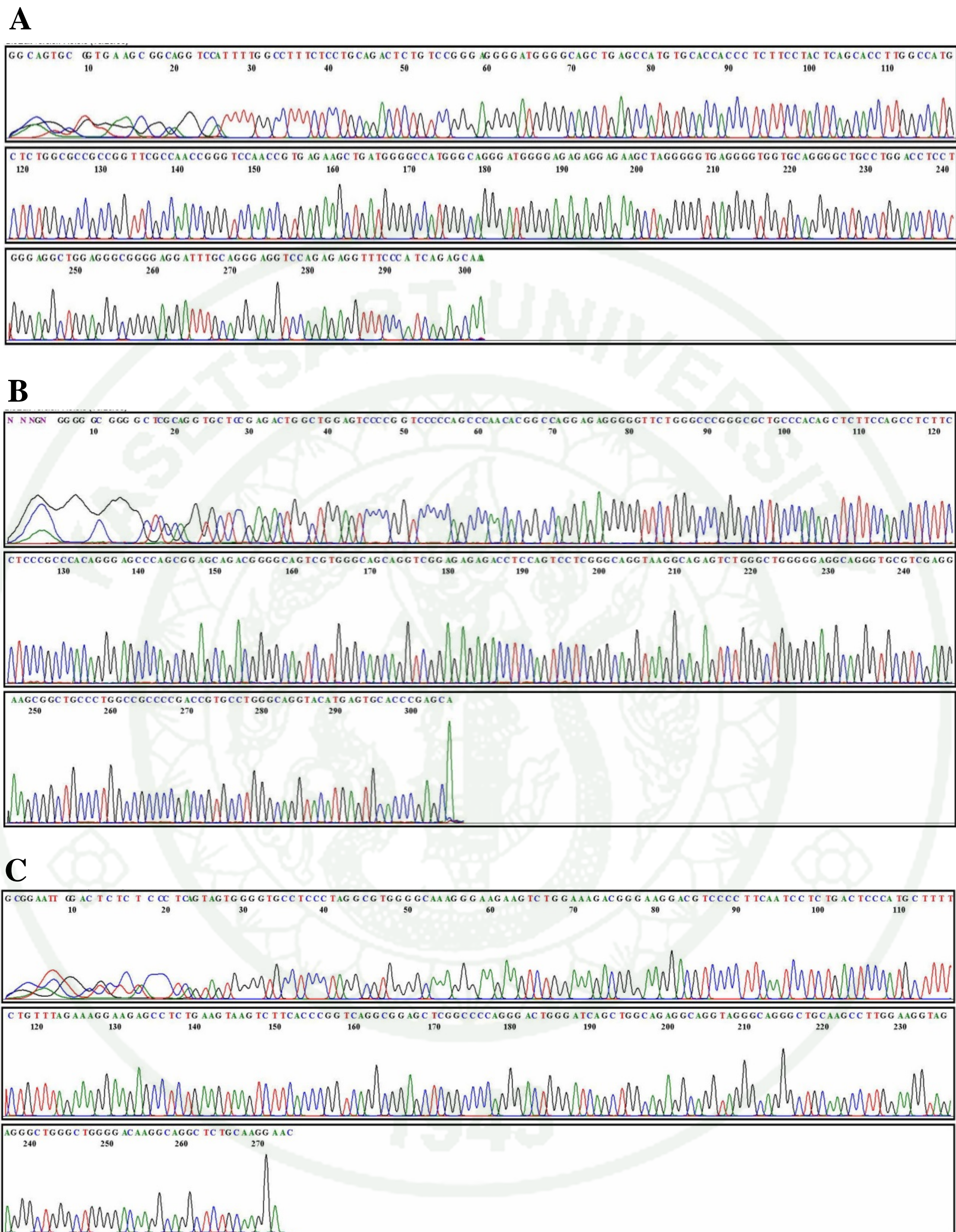

Figure 45 Direct sequencing of PCR product from coding region of PRCD gene; exon 1 (A), exon 2 (B) and exon 3 (C). All of DNA sequence traces from a retinal degeneration dog show no sequence variance when compared to normal sequences.

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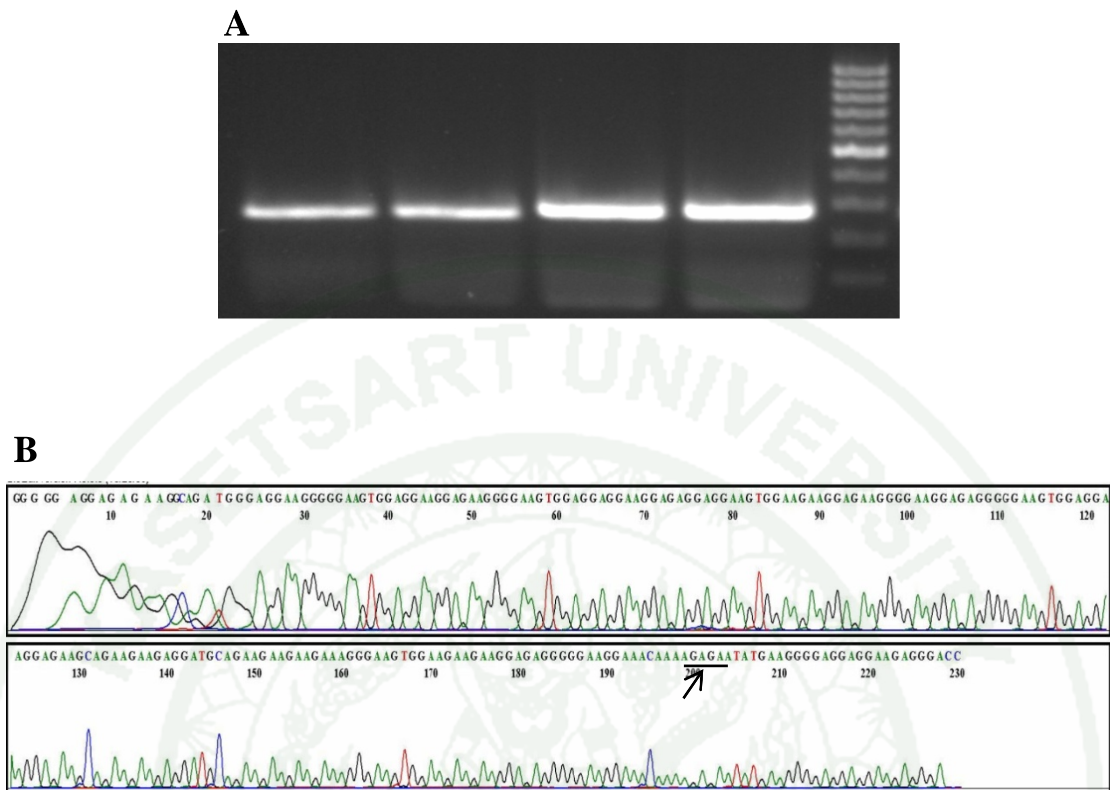

Figure 46 Agarose gel showing 239 bp from RPGR gene, Marker = 1 Kb DNA Ladder (Invitrogen®) (A). XLPRA sequence analysis from a retinal degeneration dog show homozygous allele at the mutation site of RPGR gene (arrow) (B).

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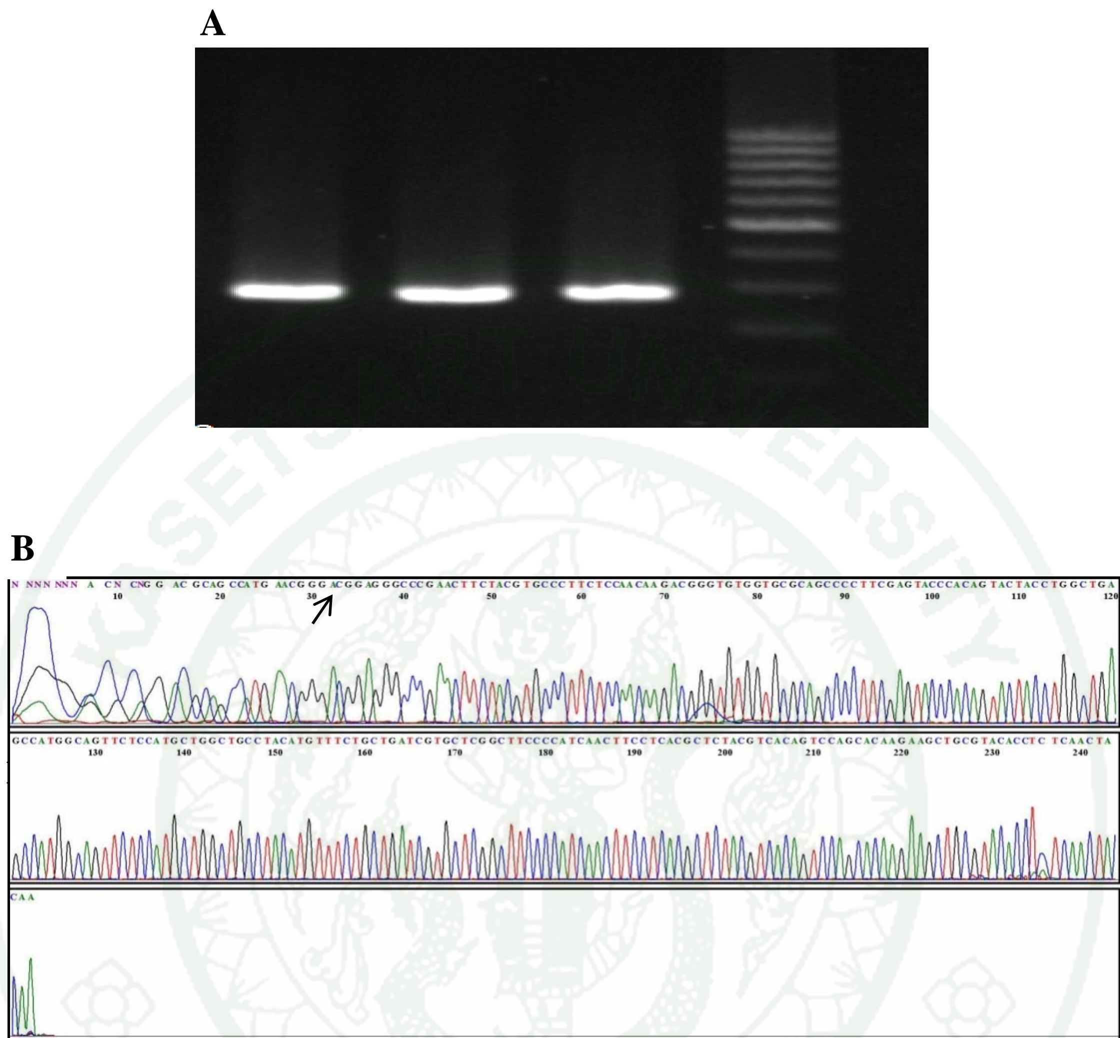

Figure 47 Agarose gel showing 244 bp from RHO gene, Marker = 1 Kb DNA Ladder (Invitrogen®) (A). RHO sequence analysis from a retinal degeneration dog show homozygous allele at the mutation site of RHO gene (arrow) (B).

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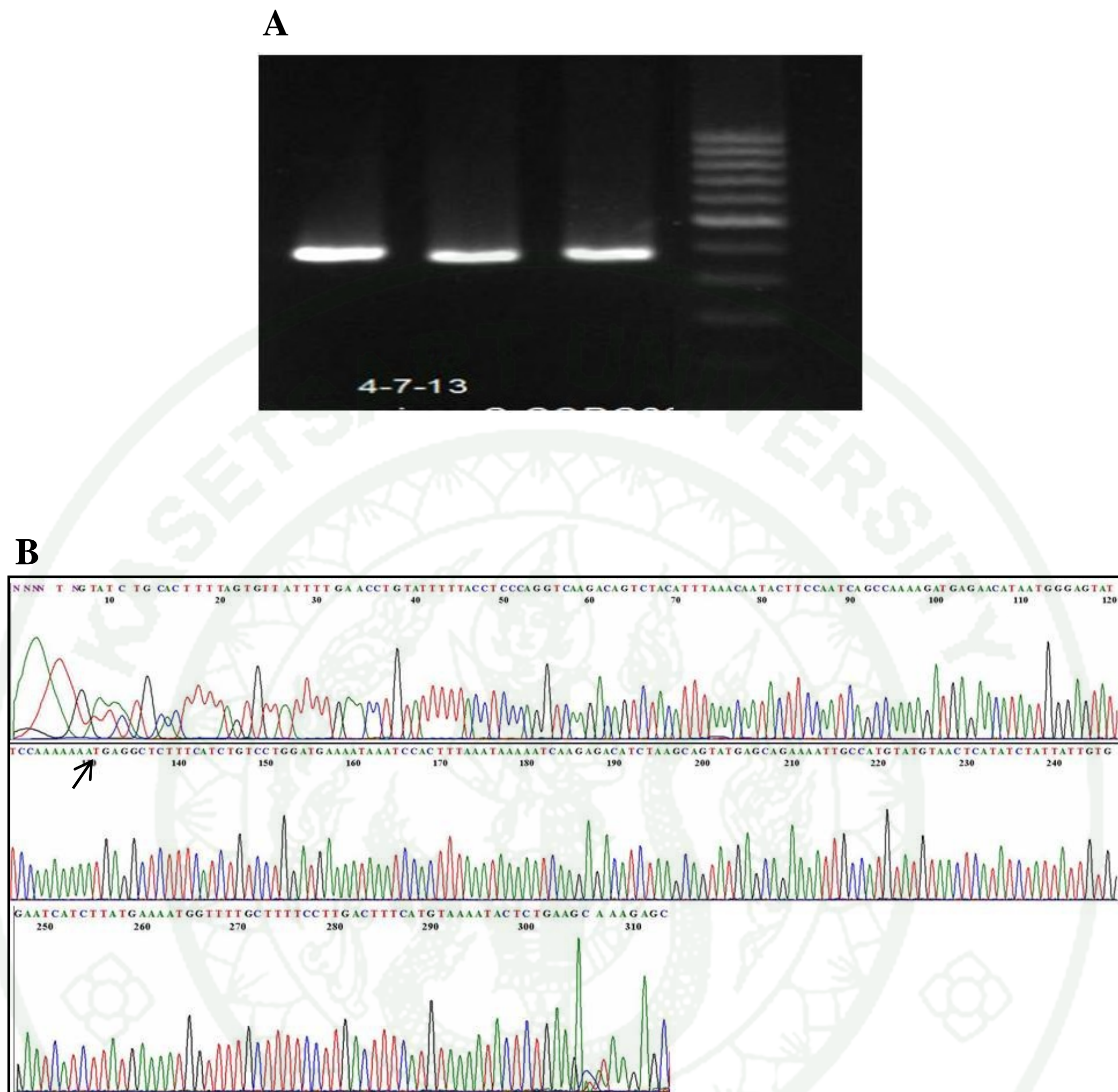

Figure 48 Agarose gel showing 318 bp from CCDC66 gene, Marker = 1 Kb DNA Ladder (Invitrogen®) (A). CCDC sequence analysis from a retinal degeneration dog show homozygous allele at the mutation site of CCDC66 gene (arrow) (B).

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Discussion

1. Normal ERG parameters and comparison among dog breeds

This study demonstrated normal ERG parameters in four dog breeds and supported an earlier study of different ERG parameters in different breeds (Itoh *et al*., 2013). In the present study, the dog age, examination environment, anesthetic protocol and equipment factors were standardized as far as possible leaving the breed-related factor as the main variable. According to the study of the origin of the major ERG wave, the a-wave reflects activity in the photoreceptors and the b-wave originates from post-synaptic neurons called bipolar cells which are associated with signal transmission in the distal retina (Ekesten, 2007; Kofuji *et al*., 2000). The differences in the physiological process in the retina of each dog breed may play a role for different ERG parameters. The actual cause of differences in the ERG recordings for each breed has not been fully understood. It was speculated that the resistance and voltage of the ERG signal vary widely due to the large variation in skull conformation (Ekesten *et al*., 2013).

Thai Bangkaew dogs had significantly more prolonged ERG implicit time than the other breeds in many ERG parameters. In general, the prolonged implicit time of the b- wave concurrent with the low amplitude in dogs indicated retinal degeneration (Hertil *et al*., 2010). However, in this study, all Thai Bangkaew dogs had normal ocular appearance and vision, and the a- and b-waves amplitudes of the Thai Bangkaew sample were not considered low when compared to the other breeds. Therefore, the results of the ERG recordings were the actual reference parameters in Thai Bangkaew.

In puppies, the eyes open at 10-15 days after birth. No ERG can be recorded until the animal is two weeks old. However, at this age, an ERG result could be recorded only as a small negative wave which would not exceed 5 µV. From 5-8 weeks old, the differentiation of photoreceptor segments and other retinal layers develop (Gum *et al*., 1984). A previous report showed that the ERG variables in

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Beagle differed by age and assumed that the ERG changes could be detectable in older individuals (more than 6 years). These might be caused by ocular media alterations which decrease the effective intensity of the stimulus, decreased photoreceptor density and decrease bipolar or Müller cell (Itoh *et al*., 2013). The age of subjects recruited for the present study was from 2 to 8 years old (4.64±1.56 years) which was the period following the complete development of the retina and also was the age range of the late-onset form of progressive retinal degeneration.

The Dog Diagnostic Protocol is the standard ERG procedure in veterinary medicine (Narfström, 2002). This protocol was modified from the standard ERG protocol used in humans. It is a complex, diagnostic protocol providing complete information about photoreceptor function separately, which was appropriate for detecting subtle changes in rod and cone function especially in hereditary retinal degenerations (Ekesten *et al*., 2013).

General anesthesia using both various anesthetic agents and protocols affects ERG recordings. Diazepam is in the benzodiazepine group which results in modulation of gamma-aminobutyric acid (GABA) found in the retina of all vertebrates (Elder, 1992). Previous studies showed that diazepam altered retinal function solely by affecting the amacrine-ganglion cell interaction and had the potential to alter visual field sensitivity after long-term use but neither affected photopic nor scotopic ERG results in humans (Robbins and Ikeda, 1989). Propofol has been shown to have little effect on GABA, which is similar to other anesthetic agents but the effect on the retina was significant when used as a continuous infusion. Increasing the propofol infusion rate increases the b-wave amplitude, while a decrease in the rate produces a parallel decreased in the b-wave amplitude in normal Beagle (Kommonen *et al*., 2007). However, in the current study, propofol was used only for induction before maintenance with isoflurane. Isoflurane has been demonstrated to decrease the amplitude of the a- and b-waves compared to tiletamine-zolazepam in dark-adapted dogs (Lin *et al*., 2009) while halothane and sevoflurane modulately decreased b-wave amplitudes and markedly depressed the scotopic threshold response in normal Beagles (Yanase and Ogawa, 1997). In addition, isoflurane anesthesia

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resulted in slightly short implicit times at the higher flash intensity stimulation and often caused the eyelids to partially close and significant eye movement in rats (Nair *et al*., 2011). These responses could result in the movement of the corneal electrodes creating a large impedance and a reduced ERG signal. Therefore, an eyelid speculum and stay-sutures are recommended when isoflurane is used. Moreover, the depth of anesthesia is very important for ERG recording. When the dog was not sufficiently relaxed, background electrophysiologic activity from excessive muscle activity would distort normal ERG waveforms (Komaromy *et al*., 2002). Therefore, the anesthetic depth should be kept constant at the appropriate level to obtain proper results.

2. Confirm phenotypic and genotypic of responsible gene mutation of PRA in Poodle

The present study showed a variable onset (5-11 years) of PRCD in affected Poodles based on clinical observation. The range of the age onset was surprisingly much greater than previously reported, 3-5 years old (Petersen-Jones, 1998). Indeed, the entire age onset investigated in this study obtained from observation by the owners when their dogs had suffered from the progressing disease. Since the loss of vision only in dim light might be difficult to detect by the owners and the loss of vision in PRCD Poodles was slowly progressed, disease onset reported by the present study was possible much later than the actual onset. However visual impairment in dim light which was an early sign of the disease in Dog 3, 4, 10 was first recognized by the owners and confirmed by an experienced veterinarian at the age of 9, 11 , 7 years respectively. These results implied that much later age onset could be expected in clinical practice than the previous report.

Our observations revealed longer remained vision than the previous report which stated that complete blindness in Poodles usually occurred between 5-6 years (Acland *et al*., 1990). Only one dog (Dog 1) in the present study had completely loss of vision. Dog 3, 4 and 10 (10, 13 and 9 years of age respectively) remained normal vision in bright light and had visual impairment in dim light at study. The previous report was studied on dogs which most were maintained in the same laboratory

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animal facility. Variable of phenotypic severity between this study and the previous report might be determined by genetic and environmental modifiers (Aguirre and Acland, 2006). However, the rate of progression was believed to be faster in young affected dogs than in disease that developed later in life (Narfström and Petersen-Jones, 2007).

ERG could be used to determine the progression of the diseases. Our ERG results corresponded well to stages of the disease. ERG was widely used to detect PRA when cataract obscured fundus (Sandberg *et al*., 1986). However, for the electroretinographic diagnosis, normal parameters of ERG should be established. Because the results of recording can vary by many factors such as types of recording equipment, anesthetic protocols, environmental factors, intrinsic factors of breed, and age ranges of animals (Ofri, 2002).

In PRA, the retinal tissue slowly died and released toxic by-products of cell death that were absorbed by the lens, causing lens damage and cataract development (Babizhayev and Deyev, 1989). Cataract formation in the present study was observed in quite a high number (7/10 dogs) when compared to a previous reported which revealed the concurrent cataract in 44% of dogs with PRA. However, that report mainly studied in Miniature Schnauzer which was accounted for 50% of all PRA cases (Park *et al*., 2009). A study in Republic of Korea surveyed the age at cataract formation revealed the mean age onset of cataract in the small dogs breed at 8.3±3.9 years. These included the common presumed etiologies of breed predisposition (53.9%), aging (23.2%) and PRA (7.6%), (Park *et al*., 2009). However, the age onset in affected Poodles in this study was quite late and Poodle is a breed predisposed to cataract formation. Therefore the cataract formation in these Poodles might result from aging, hereditary, disease condition, or a combination of any causes.

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3. Analyze the allele frequencies of PRA in Poodle and Labrador retriever

This study is the first description of the disease-associated mutation *PRCD* gene in Poodle and Labrador Retriever in Thailand. The mutation allele frequency of Poodle (0.1) and Labrador retriever (0.02) corresponds to the mutation allele of Miniature Poodle (0.2) and Labrador retriever (0.07) which was reported in Czech Republic gene mutation in 17 domestic dog breeds (Dostal *et al*., 2011). There were 20 dogs breed affected with PRCD mutation which was reported by Zangerl and colleague (Zangerl *et al*., 2006). Among 20 breeds, Poodle and Labrador Retriever are the popular breed in Thailand. Thus, they were recruited for our study. Moreover, the study on frequencies of the disease-causing *PRCD* mutation in difference of 699 dogs in Czech Republic reported by Dostal and coworkers revealed high frequency in English Cocker Spaniels (0.34), Nova Scotia Duck Tolling Retrievers (0.44), and Miniature Poodles (0.20). In contrast, no disease alleles were observed in English Springer Spaniels, Welsh Springer Spaniels, Flat Coated Retrievers, Golden Retrievers, Poodle Standards and Australian Cattle Dogs (Dostal *et al*., 2011).

4. Identify phenotypic characterization and the responsible gene mutations of PRA in Thai Bangkaew

Thai dog breed is outstanding guard dogs and family protectors. Adult dogs which go blind may cause an inability to perform outstanding guards and become burden to their owners. Thai Bangkaew dog has never historically been screened for hereditary eye diseases before. The fact that dogs perform reasonably well for such a long time after visual progressively impairment may explain why investigation of an underlying cause of blindness in this breed has never been performed earlier. The age at 6-11 years in Thai Bangkaew that showed signs of blindness with retinal degeneration indicates that the disease should be a late-onset disorder. A history of visual deficit in dim light ahead of bright light was not clear in all dogs. Therefore it could not be concluded that progressive retinal degeneration was the cause of this problem.

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All dogs in the present study had an absence of menace response with an intact PLR. In fact, the PLR can present in dogs with PRA because of intrinsic melanopsin-mediated PLR activity in retinal ganglion cells which was induced by high light-intensity. However in low light intensities, dogs with retinal degeneration should have a profoundly diminished rod-cone –mediated PLR (Semo *et al*., 2003; Grozdanic *et al*., 2007). In this study, both high and low light intensities were used to examine the pupillary response in all retinal degeneration dogs. Therefore, the intact PLRs were recorded in different forms such as delayed incomplete and delayed complete. For trustworthy diagnosis of retinal degeneration, more objective testing techniques, such as the use of the ERG together with opthalmoscopic examination must also be evaluated.

Electroretinogram was performed in 5 dogs. Among these ERG recordings, two dogs (TB3 and TB6) showed partial loss of retinal function starting at the photoreceptor level, affecting the rod system much more than the cone system. These results corresponded to previous report of a very abnormal ERG indicating abnormal outer retinal function in accordance with PRA described in several other dog breeds (Petersen-Jones, 1998; Sussadee *et al*., 2014). While one dog (TB 5) showed partial loss of rod and cone function as well as two dogs (TB1 and TB2) showed complete loss of outer retinal function. According to ERG results and fundus appearances which were tapetal hyperreflectivity, retinal vessel attenuation and optic disc atrophy, we can conclude that these six Thai Bangkaew dogs had the problem of retinal degeneration.

The cause of retinal degeneration in these six Thai Bangkeaw dogs was further investigated. The pedigree analysis and dog breeding colonies of these dogs were not available. Thus, the functional candidate gene approach was applied to identify the mutation responsible for these retinal degeneration dogs. According to the age onset of retinopathy was late. Therefore, we decided to select the published gene that mainly involved of late onset PRA in dogs as candidate genes. These genes include *PRCD*, *RHO*, *RPGR* and *CCDC66*. The PRA forms causing these gene mutations were progressive rod-cone degeneration in multiple 20 breeds (Zangerl *et al*., 2006),

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autosomal dominant PRA in English Mastiff and Bullmastiff (Kijas *et al*., 2003), X-linked PRA in Siberian Husky and Samoyed (Zhang *et al*., 2002; Zeiss *et al*., 1999) and unclassified PRA form in Schapendoes dog (Lippmann *et al*., 2007; Dekomien *et al*., 2010). Mutation screening in these genes revealed no causative sequence deviations. Therefore, these candidate genes were excluded as a cause of retinal degeneration in these six Thai Bangkaew dogs. Although causative mutations have been identified in several breeds of dogs with autosomal recessive transmitted PRA, a large number of additional canine breeds suffer from PRA forms for which DNA tests are not yet available (Petersen-John, 2005). Until now, unknown genetic causes of retinal degeneration were reported in Swedish Jämthund dogs (Hertil *et al*., 2010), Tibetan Spaniel dog (Bjerkas and Narfström, 1994), Miniature Schnauzer dogs of South Korea (Jeong *et al*., 2013) and Shetland Sheepdog (Karlstam *et al*., 2011).

Although, the present data support clinical findings of PRA, some suspected Thai Bangkaew dogs had visual problem together with other systemic diseases such as hypothyroidism (TB1 and TB6) and chronic renal failure (TB4) at the study day. Hypothyroidism in dog may lead to lipid dystrophy, lipid deposition in the aqeous, and lipemia retinalis with retinal bleeding and detachment (Aroch *et al*., 2008; Panciera, 2001). Although hypothyroidism affected to retinal developing in juvenile and adult rats and controlled cone opsin expression in retina of adult rodents (Amal *et al*., 2010; Glaschke *et al*., 2011), there was no report this disease as a primary cause of retinal degeneration in adult dog. Chronic renal failure can be associated with systemic hypertension which may be reflected in retinal hemorrhages, arterial tortuosity or detached retinas (Rogertson *et al*., 2006; Ofri, 2008). However, a renal disease occurring in the studied Thai Bangkaew dog did not show the ophthalmic signs as the previous reports. Therefore, it was also excluded from the primary cause of retinal degeneration.

Except for genetic defects, there were several causes of generalized retinal degeneration that has been reported in dog. These include specific retinopathies such

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as atrophy secondary to glaucoma, sudden acquired retinal degeneration (SARD), and uveodermatologic syndrome (Ofri, 2008).

Retinal degeneration can occur following an advanced glaucoma with increased tapetal reflectivity, attenuation or complete loss of retinal vessel, atrophy of the pigment epithelium in the nontapetal fundus, and optic atrophy (Miller, 2008). Although these findings are similar to retinal degeneration in dogs which observed in this study, glaucoma was less likely to be the cause of retinal degeneration in these dogs due to the uncapped optic disc and lack of other signs of glaucoma.

Sudden acquired retinal degeneration is a disease of unknown etiology. It is a sudden onset disease affected in middle aged to older dogs due to numerous apoptotic nuclei in the outer layer of retinal (Miller *et al*., 1998). The typical presentations are acute blindness with dilated and nonresponsive pupils. The fundus looks normal initially then it appears degeneration over the next few months. The ERG response extinguished is usually observed in affected dogs (Ofri 2008, Montgomery *et al*., 2008). A history of polyuria, polydipsia, weight gain, and lethargy is typical. A significant percentage of patients have a blood profile suggestive of hyperadrenocorticism due to lymphopenia, elevated alkaline phosphatase and hypercholesterolemia (Montgomery *et al*., 2008). Although the onset of blindness in these six retinal degeneration dogs was not clear, the fundic appearances were compatible with the advance stage of SARD. However, all dogs remained having PLR and ERG indicated partial photoreceptor function remained in two out of five dogs at the study day. In addition, none of these dogs had clinical signs and blood profiles correlated with SARD. Therefore, SARD was ruled out as a cause of retinal degeneration in these six Thai Bangkaew dogs.

Uveodermatologic syndrome is an immune-mediated disease which attack against the melanocytes of affected dog’s skin and uvea. It also characterized by chronic and recurrent bilateral uveitis and dermal depigmentation in dogs. This disease has been described in several dog breeds including Samoyed, Old English Sheepdog, Siberian husky, Saint Bernard, Akita, Irish setter, Chow Chow, Shetland

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Sheepdog, Golden Retriever, Dachshund and Australian Shepherd (Aroch *et al*., 2008; Carter *et al*., 2005; Herrera and Duchene, 1998). Secondary ocular lesions of this disease such as cataract, posterior sysnechia, glaucoma, bullous retinal detachment, retinal and optic nerve atrophy, and acute blindness, are common. Dermatologic lesions such as poliosis, vitiligo, and ulceration are usually occurring concurrently with ocular signs in uveodermatologic syndrome. The progressive depigmentation of the retinal pigment epithelium in the non tapetum, tapetal hyperreflectivity, and attenuation of retinal vessels could be present in both uveodermatologic syndrome and PRA (Aroch *et al*., 2008). However, none of these dogs had uveitis and dermatologic lesions; therefore this disease was excluded as a cause of retinal degeneration in these six Thai Bangkaew dogs

The ophthalmoscopic findings which were observed in the six Thai Bangkaew dogs as well as the ERG results corresponded to retinal degeneration. The etiology of retinopathy in these dogs, however need further investigation whether it affected from unknown causal gene mutation or from other systemic diseases. Enucleation of an affected eye would possibly provide more information on pathogenesis of this retinal degeneration form in Thai Bangkaew dogs. Moreover, analysis of pedigrees of affected dogs must be performed for analysis of the mode of inheritance.

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CONCLUSION

Normal ERG parameters obtained with an HMsERG in four common dog breeds in Thailand were identified. Significant differences in the ERG parameters were described. All processes including anesthetic protocols were safe and appropriate for long periods of ERG protocol recording. The ERG protocol, anesthetic agents, and equipment used in this study resulted in desirable ERG waves which were very helpful for providing an accurate assessment of the remaining retinal function in case of progressive retinal degeneration in specific dog breeds.

This study revealed a correspondence of the phenotypic characterization observed from obstacle test, ophthalmoscopy, and electroretinography in progressive retinal degeneration to genotypic characterization in Poodle dogs in Thailand. Clinical observation revealed that the age onset and remained vision was longer than expected. Genetic defect of PRCD-PRA of Poodles in Thailand was consistent with the previous report. It is for the first time that the molecular testing has been applied to this disease in Thailand. Relatively high prevalence of the PRCD-carrier in Poodles (12%) and Labrador Retriever (3.45%) in Thailand indicated the beneficial of the genetic testing to identify PRCD-PRA status before breeding to avoid the transmission of affected allele to offspring. As well as, these findings were useful for genotype-phenotype comparisons among different affected dog breeds.

The affected Thai Bangkaew dogs had the ophthalmoscopic abnormalities including ERG alterations which compatible with retinal degeneration. We can conclude that causative of this form of retinal degeneration in the Thai Bangkaew had a different genetic cause than *PRCD*, *RHO*, *RPGR* and *CCDC66* gene mutation. This form of this disease needs further investigation of it etiology and factors potentially affecting the retinal degeneration, such as more in formation of systemic disease conditions, microscopic examination of affected eyes, pedigree analysis and various environmental factors.

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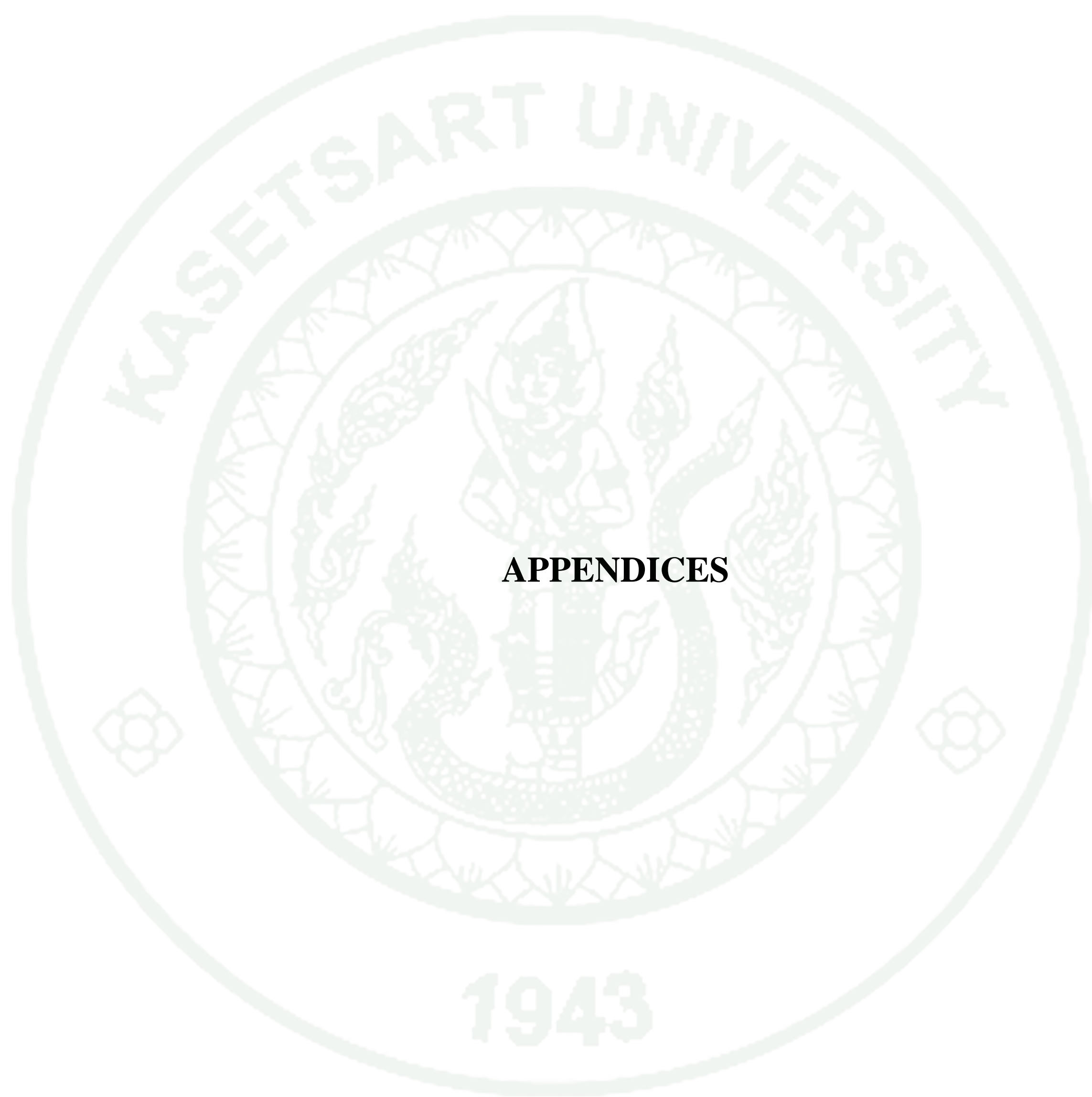
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APPENDICES

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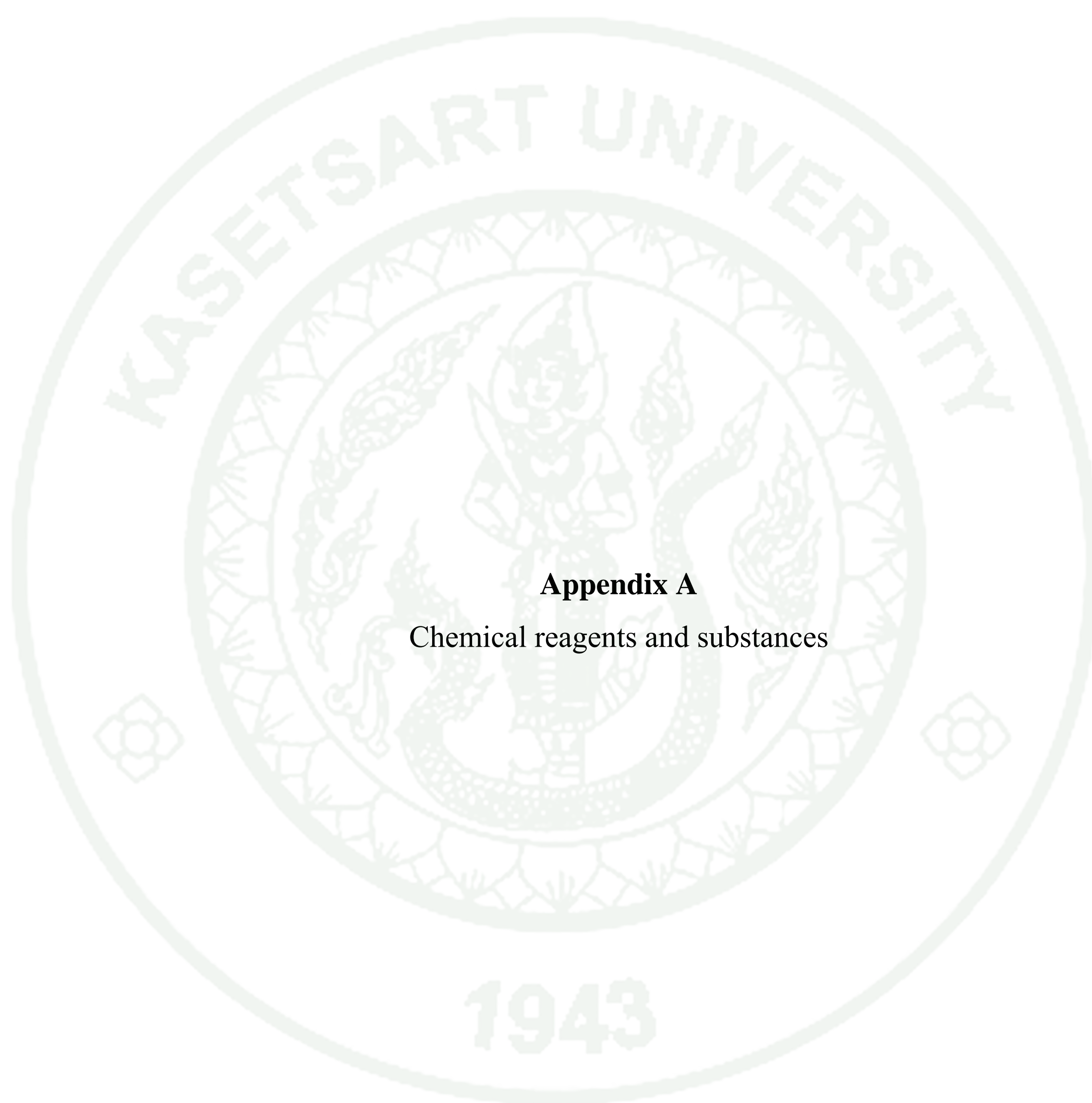

Appendix A

Chemical reagents and substances

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1. DNA extraction solution

Denaturing Solution (D-Solution)

0.75 M Sodium Citrate	17.6 ml
10% N-laurylsarcosine	26.4 ml
Guanidinine Thiocyanate	250 g
Add distilled water to	293 ml

Sodium Citrate pH 7

Sodium Citrate	5.88 g
Add distilled water to	20 ml

DNA phenol

Phenol	100 ml
Tris buffer	6.5 ml

TE Solution pH 8

Tris base	121.1 mg
EDTA	372.24 mg

Add distilled water to 500 ml

2. Gel Electrophoresis Solution

0.5 M EDTA (100 ml, pH 8.0)

80 ml autoclaved milli-Q H_2O

18.612 g EDTA (disodium, MW 372.24)

5 ml 10 M NaOH required for EDTA to go into solution

Initial pH ~ 8.6

Adjust to final volume

Filter sterilze

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1 M Tris Base (500 ml, pH 8.0)

Autoclaved milli-Q H_2O	400 ml
Tris (base, MW 121.14)	60.57 g
Adjust to final volume	
Filter sterilize	

50X TA (1 litre) pH 8.3

Tris base	242.25 g
Glacial acetic acid	57.2 ml
Add ultra-pure water to final volume	1 liter

10X loading buffer / dye

20% glycerol

0.001% bromophenol blue

Add TE to final volume

5 mg/ml ethidium bromide (EtBr)

EtBr	500 mg
Add distilled water to	100 ml

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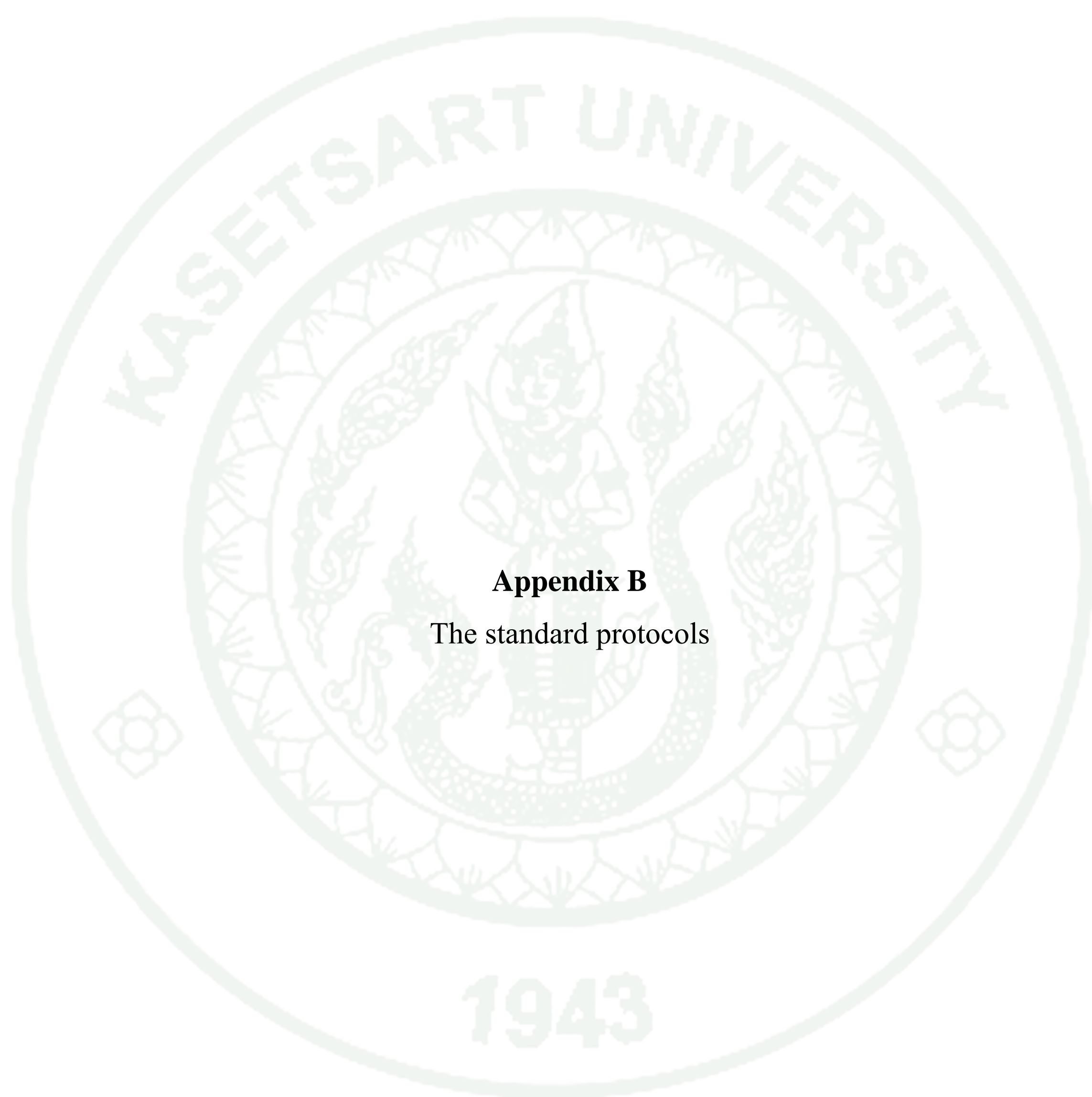

Appendix B

The standard protocols

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Protocol for DNA extraction, Sambrook and Russel (2001).

1. Transfer 100 µl of whole blood from each sample to 1.5 steriled Microcentrifuge tubes. Add 500 µl of denaturing solution (see Appendix B), 150 µl of DNA-phenol and 150 µl of Chloroform. Then the capped tubes were vortexed vigorously for 10 minutes for mix the contents. At this step, the color was changed from red to dark reddish brown.

2. The tubes were centrifuged at 13,000 rpm for 5 minutes at room temperature.

3. Transfer the supernatant to a fresh microcentrifuge tubes containing 150 µl of DNA-phenol and 150 µl of Chloroform. Mix the solution well by vortexing for 10 minutes. Then, the tubes were centrifuged for 5 minutes.

4. Carefully transfer the supernatant to the fresh tubes and add 1,000 µl of absolute ethanol. Invert the tubes several times and incubate for precipitate the DNA at -20°C for 2 hours or overnight.

5. Recover the precipitate of DNA by centrifuging the tubes at maximum speed for 10 minutes at room temperature.

6. Remove the supernatant by aspiration or carefully poured off and added 500 µl of 75% ethanol to the DNA pellet for wash the DNA pellet. Invert the tube several times and centrifuge the tube at 13,000 rpm for 5 minutes. Then the tubes were carefully poured off supernatant and were repeated this step for 2-3 time. DNA pellet may dislodge at this step, so carefully watched pellet and made sure it did not get poured into waste container.

7. Carefully remove the supernatant again by aspiration and allow the DNA pellet to dry in air for 2 hours.

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8. Redissolve the pellet of DNA in 50 µl of TE (PH 8). Let it sit at room temperature. DNA sample was diluted 1:20 to check OD using a Spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) for concentration and transferred to vial for storage before use. These DNA samples were kept at -20 ^{o}C.

Protocol for Fast Digestion (Thermo Scientific®, FastDigest RsaI)

Combine the following reaction components at room temperature in the order indicated:

Water, nuclease-free	17 µl
10X FastDigest Buffer	2 µl
DNA in PCR product	10 µl (~0.2 µg)
FastDigest enzyme	1 µl
Total volume	30 µl

Mix gently and spin down. Then incubate at 37^{0}C in a water thermostat for 10 min.

Protocol for agarose gel electrophoresis (Sambrook and Russel, 2001).

1. Prepare an agarose gel by combining the agarose with buffer in the Erlenmeyer flask and melt the agarose for 3 minutes by microwave oven. Check the gel temperature before pouring the gel onto plate.

2. Pour the gel onto a taped plate with casting combs in place. Allow 30-40 mins for solidification

3. Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1X TAE electrophoresis buffer to the reservoirs unit the buffer just covers the agarose gel.

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4. Add at least 2/3 volume of 1x agarose gel loading dye to each DNA sample or PCR product, mix and load into the wells. Electrophoresis the gell at 100 Volt until the required separation has been achieved.

5. Incubate the agarose gel in EtBr tank for 10-20 minutes.

6. Visualize the DNA fragments on a long wave UV light box.

Protocol for DNA extraction from agarose gels (E.Z.N.A.®, Gel Extraction Kit)

1. Perform agarose gel/ethidium bromide electrophoresis to fractionate DNA fragments. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

2. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 ml microcentrifuge tube. Assuming a density of 1 g/ml of gel, the volume of gel is derived as follow as: a gel slice of mass 0.3 g will have a volume of 0.3 ml. Add an equal volume of Binding Buffer. Incubate the mixture at 60^{0}C for 7 min. or until the gel has completely melted. Mix by shaking or vortexing the tube in increments of 2-3 minutes.

3. Place a HiBind DNA Mini Column in a provided 2 ml collection tube. Then, add the DNA/ agarose solution from step 2 to the Column and centrifuge at 10,000 x g for 1 min at room temperature. Discard the flow-through liquid and place the Column back into the same collection tube.

4. Add 300 µl of Binding Buffer into the Column. Centrifuge at 13,000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the Column back into the same collection tube.

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5. Add 700 µl of SPW Wash Buffer (diluted with absolute ethanol) to the Column. Centrifuge at 13,000 x g for 1 min at room temp to wash the Column. Discard the flow-through liquid and place the Column back into the same collection tube. Repeat this step with another 700 µl of Wash Buffer.

6. Centrifuge the empty Column for 2 min at maximal speed (≥ 13,000 x g) to dry the column matrix. Do not skip this step, it is critical for removal of ethanol from the DNA column.

7. Place the Column into a clean 1.5 ml microcentrifuge tube. Depending on desire concentration of the final product, add 30-50 µl Elution Buffer (10 mM Tris-HCl, PH 8.5) or water directly onto the column matrix and incubate at room temperature for 2 minutes. Centrifuge for 1 min at maximum speed (≥ 13,000 x g) to elute DNA.

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