# Research article

# **Open Access**

# Polymorphisms within the canine *MLPH* gene are associated with dilute coat color in dogs

Ute Philipp<sup>1</sup>, Henning Hamann<sup>1</sup>, Lars Mecklenburg<sup>2</sup>, Seiji Nishino<sup>3</sup>, Emmanuel Mignot<sup>3</sup>, Anne-Rose Günzel-Apel<sup>4</sup>, Sheila M Schmutz<sup>5</sup> and Tosso Leeb<sup>\*1</sup>

Address: <sup>1</sup>Institute of Animal Breeding and Genetics, University of Veterinary Medicine Hannover, Bünteweg 17p, 30559 Hannover, Germany, <sup>2</sup>Department of Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4467, USA, <sup>3</sup>Center of Narcolepsy Department of Psychiatry Stanford University School of Medicine, 701 Welch road B, Palo Alto CA 94304-5742, USA, <sup>4</sup>Institute for Reproductive Medicine, University of Veterinary Medicine Hannover, Bünteweg 15, 30559 Hannover, Germany and <sup>5</sup>Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5A8

Email: Ute Philipp - Ute.Philipp@tiho-hannover.de; Henning Hamann - Henning.Hamann@tiho-hannover.de; Lars Mecklenburg - LMecklenburg@cvm.tamu.edu; Seiji Nishino - nishino@stanford.edu; Emmanuel Mignot - mignot@leland.stanford.edu; Anne-Rose Günzel-Apel - Anne-Rose.Guenzel-Apel@tiho-hannover.de; Sheila M Schmutz - schmutz@sask.usask.ca; Tosso Leeb\* - Tosso.Leeb@tiho-hannover.de

\* Corresponding author

Published: 16 June 2005

BMC Genetics 2005, 6:34 doi:10.1186/1471-2156-6-34

This article is available from: http://www.biomedcentral.com/1471-2156/6/34

© 2005 Philipp et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<u>http://creativecommons.org/licenses/by/2.0</u>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

#### Abstract

**Background:** Pinschers and other dogs with coat color dilution show a characteristic pigmentation phenotype. The fur colors are a lighter shade, e.g. silvery grey (blue) instead of black and a sandy color (Isabella fawn) instead of red or brown. In some dogs the coat color dilution is sometimes accompanied by hair loss and recurrent skin inflammation, the so called color dilution alopecia (CDA) or black hair follicular dysplasia (BHFD). In humans and mice a comparable pigmentation phenotype without any documented hair loss is caused by mutations within the melanophilin gene (*MLPH*).

**Results:** We sequenced the canine *MLPH* gene and performed a mutation analysis of the *MLPH* exons in 6 Doberman Pinschers and 5 German Pinschers. A total of 48 sequence variations was identified within and between the breeds. Three families of dogs showed co-segregation for at least one polymorphism in an *MLPH* exon and the dilute phenotype. No single polymorphism was identified in the coding sequences or at splice sites that is likely to be causative for the dilute phenotype of all dogs examined. In 18 German Pinschers a mutation in exon 7 (R199H) was consistently associated with the dilute phenotype. However, as this mutation was present in homozygous state in four dogs of other breeds with wildtype pigmentation, it seems unlikely that this mutation is truly causative for coat color dilution. In Doberman Pinschers as well as in Large Munsterlanders with BHFD, a set of single nucleotide polymorphisms (SNPs) around exon 2 was identified that show a highly significant association to the dilute phenotype.

**Conclusion:** This study provides evidence that coat color dilution is caused by one or more mutations within or near the *MLPH* gene in several dog breeds. The data on polymorphisms that are strongly associated with the dilute phenotype will allow the genetic testing of Pinschers to facilitate the breeding of dogs with defined coat colors and to select against Large Munsterlanders carrying BHFD.

Received: 23 December 2004 Accepted: 16 June 2005

# Background

Coat color dilution leads to the so-called blue pigmentation phenotype in black-and-tan Pinschers (Doberman Pinschers, German Pinschers, Miniature Pinschers), characterized by a silver-blue shade of the black fur areas (Figure 1). Similarly, coat color dilution is responsible for the Isabella fawn phenotype in brown-and-tan or tan Pinschers. Color dilution in Pinschers is inherited as a Mendelian autosomal recessive trait. Although there are no severe impairments known, this pigmentation variation is of clinical relevance as Pinschers with coat color dilution show an increased prevalence of color dilution alopecia (CDA) also called Blue Doberman syndrome. CDA is characterized by a progressive loss of hair, which is sometimes accompanied by recurrent bacterial infections of the hair follicles (folliculitis). Melanosome clumping occurs within melanocytes of the epidermis and hair follicles, resulting in macromelanosomes in hair shafts that subsequently fracture when emerging from the skin. The exposed skin of CDA affected dogs is often dry and scaly as well as sensitive to sunburn or extreme cold [1]. Black hair follicular dysplasia (BHFD), a form of alopecia in various breeds where only the black coat areas are affected, is phenotypically similar to CDA [2-4].

In human and mouse, genes are already known which lead to phenotypically similar coat color variations. In mice the mutants dilute, ashen and leaden are well characterized [5-7]. These mutants correspond to the human Griscelli syndromes (GS) 1 to 3 [8-10]. Griscelli syndromes 1–3 as well as the above mentioned mouse mutants are all inherited as Mendelian autosomal recessive traits.

Mutations in three different genes, i.e. myosin Va (*MYO5A*), *RAB27A*, and melanophilin (*MLPH*), are responsible for these phenotypes. The proteins which are encoded by these genes are part of the melanosome transport complex. Therefore, in the melanocytes of affected individuals an accumulation of melanosomes around the nucleus is observed as well as large clumps of pigments in the hair shaft.

In the dilute mouse mutant the Myo5a gene is mutated [5], while mutations in the Rab27A gene lead to the ashen mouse mutant [6]. The phenotypes of these mutants are close to their human counterparts of GS1 or GS2 affected patients, respectively. Individuals carrying a mutation in one of these two genes usually develop severe neurological (GS1) or rather immunological disorders (GS2) in addition to their skin and hair color dilution [8,9]. One human case report describes that the deletion of the MYO5A gene exon F, which is only expressed in melanocytes, leads to hypopigmentation without further disorders [10]. In contrast to MYO5A and RAB27A mutations, which normally cause complex phenotypes, mutations in the MLPH gene are responsible for color dilution without any further impairment in human GS3 patients or leaden mice [7,10]. Therefore the MLPH gene seemed to be the most suitable candidate gene for coat color dilution in dogs and we report here the analysis of this gene in several dog breeds with an emphasis on Doberman Pinschers and German Pinschers.



#### Figure I

**Blue Doberman Pinscher und black-and-tan Doberman Pinscher**. Blue Doberman Pinscher (A) and black-and-tan Doberman Pinscher (B). Note the coat color differences between the two animals. The black and reddish fur parts of the black-and-tan Doberman Pinscher are changed to paler coloring in the blue dog. Classical genetics states that the blue dog is homozygous for the recessive dilute allele (d).

### Results

#### Characterization of the canine MLPH gene

A human *MLPH* cDNA probe was used to retrieve a canine genomic clone (RP81-203J24) from a Doberman Pinscher BAC library. A draft sequence of this 198 kb BAC clone was determined. In order to finish this draft sequence additional public whole genome shotgun sequences from a Boxer were used. The BAC clone contained the complete collagen type VI alpha 3 gene (*COL6A3*) as well as the exons 1 to 10 of the 16 exon *MLPH* gene. To obtain the missing 3'-end of the canine *MLPH* gene, Boxer whole genome shotgun sequences were assembled and joined to the sequence of the BAC clone resulting in one large contiguous sequence of 212,696 bp. Comparison of these sequences revealed a number of polymorphisms between Doberman Pinscher and Boxer DNA.

The canine MLPH gene spans approximately 48 kb of genomic sequence compared to 67 kb for the human MLPH gene. The genomic organization of the canine MLPH gene was inferred by comparison of the genomic dog sequence with an experimentally derived canine cDNA sequence (Figure 2). The genomic structure of the MLPH gene was not entirely conserved between human and dog. All but one of the 16 human MLPH exons could be identified in the canine MLPH sequence. No dog exon homologous to the human exon 9 could be identified; however, this exon is not used constitutively in all human transcripts. On the other hand, the canine MLPH gene contains a fifth exon of 39 bp that is not present in the human or murine MLPH genes. The MLPH gene has a very high GC-content of about 59.5%, which is significantly above the mammalian average of 41%. Consistent with the high GC-content a CpG island is located upstream of exon 1 in the dog sequence in addition to numerous CpG islands within the gene. A canonical polyadenylation signal AATAAA was identified approximately 3.1 kb downstream of the stop codon but 3'-RACE experiments indicated that in dog polyadenylation occurs only ~ 300 bp downstream of the stop codon following a sequence motif ATTGAA that weakly resembles the canonical polyadenylation signal.

The canine *MLPH* mRNA contains an open reading frame of 1746 nt encoding a protein of 581 amino acids. The canine MLPH protein was predicted to have a molecular weight of 62.7 kDa, a pI of 5.7, and shows 62% identity to the orthologous human protein (human MLPH isoform lacking the amino acids encoded by exon 9).

# Mutation analysis of the canine MLPH gene and association with dilute phenotype

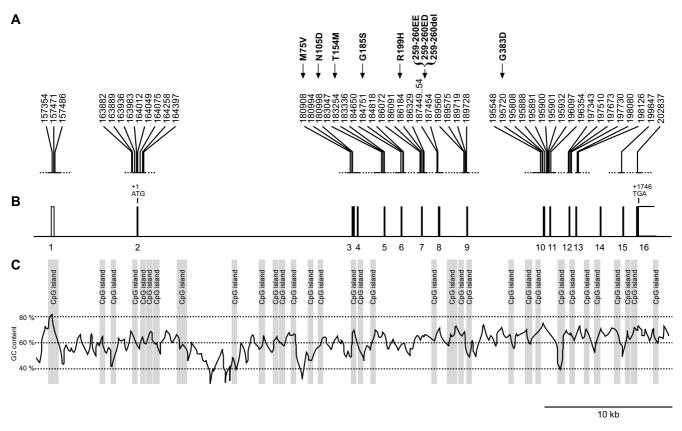
Comparative sequencing of the exons and adjacent sequences of 6 animals from one Doberman Pinscher and

5 from one German Pinscher family revealed 43 sequence differences within these closely related breeds and an additional five variations between the breeds (Table 1). Within the Doberman Pinscher family 39 polymorphisms were observed while only 7 sequence variations were found in the German Pinscher family members. Only 3 variations segregated in both families and none of them was in the coding sequence.

Most polymorphisms were SNPs (46), only two indel polymorphisms were observed. Of the 48 observed polymorphisms 33 were located in introns. The remaining 15 polymorphisms were in exons, of which 7 led to amino acid exchanges in the MLPH protein (Figure 3).

Although an indel in exon 8 was the most striking variation observed, dogs of normal color and homozygous for each of the possible variants were encountered at this polymorphism. A  $G \rightarrow A$  transition in exon 7 causing an exchange from arginine to histidine at position 199 of the MLPH protein showed perfect co-segregation with the dilute and wildtype phenotypes in the German Pinscher family (Figure 4). We therefore established a HhaI RFLP assay (Figure 5) and analyzed this mutation in 341 dogs. The histidine variant was homozygous in all dilute German Pinschers (18), Beagles (2), and Large Munsterlanders with BHFD (4). Although this mutation showed strong association with the dilute phenotype in German Pinschers, it must be noted, that we observed four dogs with wildtype color from other breeds (one Large Munsterlander and three Doberman Pinschers) that were also homozygous for the 199H allele. Therefore, the R199H mutation is a tightly linked marker for the d allele in German Pinschers but it seems unlikely that it represents a loss-of-function mutation that could cause dilute coat color. The allele distribution of the R199H mutation in Doberman Pinschers turned out to be very interesting. In samples collected form Doberman Pinschers in North America the 199H allele was very rare and not obviously associated with the dilute phenotype. However, in samples from European Doberman Pinschers we observed a strong albeit not perfect association of the 199H allele with the dilute phenotype (Table 2). The available genotyping data for the amino acid changing mutations are summarized in Table 3. These data show that homozygous animals with wildtype color exist for every single amino acid replacement that we found in dilute animals.

A set of eight SNPs around exon 2 showed perfect association with the dilute allele in all 140 Doberman Pinschers that were analyzed. From the available genotyping data four haplotypes could be reconstructed (Table 4). A single haplotype (termed haplotype 2) was associated with the d allele in all Doberman Pinschers as well as in one Beagle



Architecture of the canine MLPH gene. (A) The 48 polymorphisms that were identified in Doberman Pinschers and/or German Pinschers are indicated. PCR products spanning each of the *MLPH* exons with adjacent flanking sequences were sequenced. The eight SNPs around exon 2 show strong association with the dilute allele in Doberman Pinschers, however they are monomorphic in German Pinschers. The R199H mutation shows strong association with the dilute allele in German Pinschers and in some Doberman Pinschers of European origin. (B) Genomic organization of the canine *MLPH* gene. Exons are denoted as boxes. Solid boxes represent coding sequence while open boxes contain the untranslated regions. The genomic section corresponds to a 50 kb interval in the analyzed sequence of 212.696 bp (positions 156.001 – 206.000). (C) Illustration of the unusually high GC-content of the canine *MLPH* gene. The GC-content was calculated using a 300 bp window. CpG island criteria were: GC > 0.5, CpG<sub>obs</sub>/CpG<sub>exp</sub> > 0.6, and length > 200 bp.

family and the Large Munsterlander family segregating for BHFD. However, all German Pinschers under investigation were monomorphic around exon 2 and had the common haplotype 3. A PCR-RFLP assay was developed for the silent C/T SNP in exon 2 (position +106 in the *MLPH* cDNA sequence) since the presence of a T at this position was unique to haplotype 2.

While polymorphisms at the 5'-end of *MLPH* are tightly associated with dilute in American Doberman Pinschers and polymorphisms at the 3'-end are tightly associated with dilute in German Pinschers, a large group of dogs including European Doberman Pinschers, Large Munsterlanders and Beagles show strong association of dilute with markers across the entire *MLPH* gene. Detailed inspection

of the available dilute chromosomes across different breeds revealed that all dilute chromosomes belonged to three different *MLPH* marker haplotypes. Each of the three families shown in Figure 4 carries one of these dilute haplotypes. A detailed comparison of the three dilute haplotypes is given in Table 5. The three different dilute haplotypes do not share extended haplotype blocks within the coding region of the *MLPH* gene. However, they do share the first three marker alleles from the region around exon 1. Thus it is possible that a single ancestral founder mutation within the promoter of the *MLPH* gene followed by subsequent recombinations is responsible for the observed diversity of dilute haplotypes.

#### Table 1: Polymorphisms within the canine MLPH gene

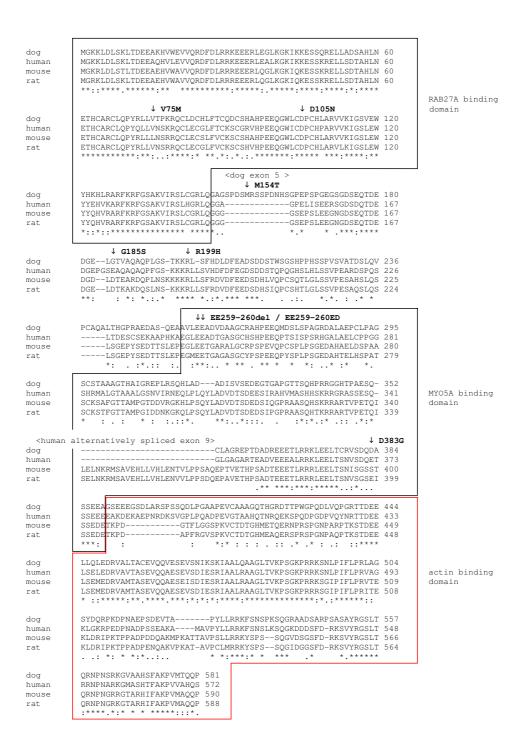
position <sup>1</sup>	cDNA position <sup>2</sup>	Doberman Pinscher	German Pinscher
157354 (exon 1)	-139 (5'-UTR)	G/T	G
57471 (exon I)	-22 (5'-UTR)	A/G	A/G <sup>3</sup>
57486 (intron I)		A/C <sup>3</sup>	Α
63882 (intron I)		C/T	т
63889 (intron I)		A/C	С
63936 (intron I)		A/G	А
63983 (intron I)		A/G	Α
64012 (intron I)		A/G	A
64049 (intron I)		A/G	А
64075 (intron I)		C/T	С
64258 (exon 2)	+106	C/T (silent)	С
64397 (intron 2)		A/G	А
80908 (exon 3)	+223	A/G ( <sup>75</sup> M/ <sup>75</sup> V)	A
80994 (exon 3)	+309	C/T (silent)	c
80998 (exon 3)	+313	A/G ( <sup>105</sup> N/ <sup>105</sup> D)	A
183047 (intron 4)		C/G	G
83254 (exon 5)	+461	C/T ( <sup>154</sup> T/ <sup>154</sup> M)	C/T <sup>3</sup> ( <sup>154</sup> T/ <sup>154</sup> M)
83336 (intron 5)		C/T	C/T <sup>3</sup>
84650 (intron 5)		СЛ	Т
84751 (exon 6)	+553	A/G ( <sup>185</sup> S/ <sup>185</sup> G)	G
	+333		
84818 (intron 6)		A/G	G T
86072 (intron 6)		C C	T
86091 (intron 6)			
86184 (exon 7)	+596	A/G <sup>3</sup> ( <sup>199</sup> H/ <sup>199</sup> R)	A/G ( <sup>199</sup> H/ <sup>199</sup> R)
86329 (intron 7)			G
8744954 (ex. 8) <sup>4</sup>	+775 – +780	indel GAGGAT +/- (indel <sup>259</sup> E <sup>260</sup> D)	indel GAGGAG +/-3 (indel <sup>259</sup> E <sup>260</sup> E
89560 (intron 8)		A/G	G
89575 (intron 8)		A	A/C
89719 (exon 9)	+1032	C/T (silent)	С
89728 (exon 9)	+1041	G	A/G (silent)
95548 (intron 9)		A/G	G
95720 (exon 10)	+1148	G ( <sup>383</sup> G)	A ( <sup>383</sup> D)
95808 (exon 10)	+1236	A (silent)	G (silent)
95888 (intron 10)		A/G	G
95891 (intron 10)		C/G	G
95900 (intron 10)		A/G	A/G
95901 (intron 10)		indel G +/-	indel G -
95932 (intron 10)		A/G	G
96097 (exon 11)	+1263	A/G (silent)	G
96354 (intron 11)		C/T	С
97343 (intron 11)		C/T	т
97510 (intron 11)		C/T	С
97673 (intron 12)		C/T	С
97730 (intron 12)		A/G	G
98080 (intron 12)		A	A/G
98126 (intron 12)		A/G	A/G
199847 (intron 13)		A/G	A
202837 (exon 16)	+1801 (3'-UTR)	С/Т	C/T

<sup>1</sup> Positions refer to the entire determined genomic sequence of 212.696 bp [EMBL:BN000728].  $^{2}$ +1 corresponds to the adenosine of the translation start codon in the *MLPH* cDNA.

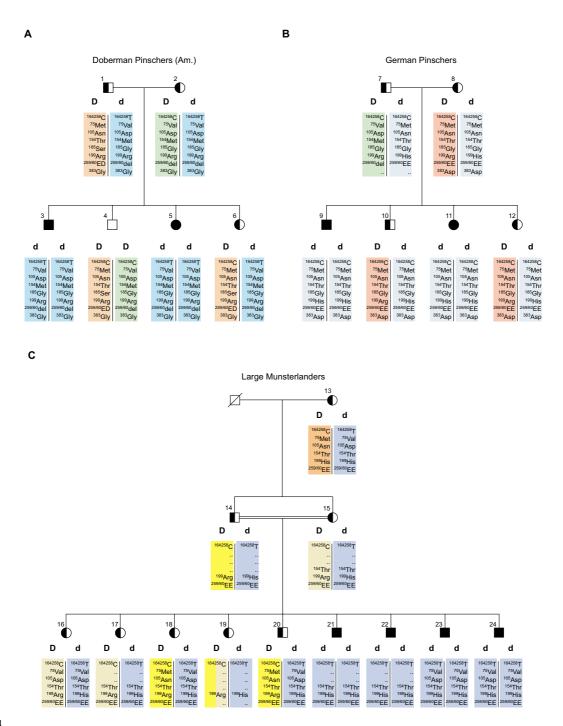
<sup>3</sup> These polymorphisms were not present in the 11 samples of the initial mutation analysis. They were identified in additionally sequenced Pinscher

samples.

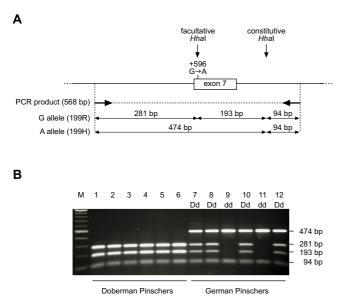
 $^4$  Note that there are three alleles at this site (GAGGAG, GAGGAT, : : : : : :).



Alignment of MLPH proteins from different species. The MLPH protein sequences were translated from nucleotide database accessions [EMBL:AJ920333] (dog), [Genbank:AK022207] (human), [Genbank:AF384098] (mouse), and [Genbank:BC081894] (rat), respectively. The three major predicted protein domains of MLPH are indicated in accordance with [24]. Note the 13 additional amino acids in the dog MLPH protein encoded by dog exon 5, which is not conserved in other species. Another big difference between the sequences is caused by the fact that dog is lacking a homologous exon to human exon 9. In human this exon is not used constitutively and for the alignment a protein isoform without the amino acids encoded by this alternative exon was used. Polymorphisms that affect the amino acid sequence of the dog MLPH protein are indicated with arrows. None of the observed protein polymorphisms has a segregation pattern in the investigated families that would be compatible with a causative mutation for dilute.



**Selected families and MLPH genotyping data**. (A) Doberman Pinscher family of American origin that was used in the initial mutation analysis. Dilute animals (dd) are indicated as solid black symbols. Animals I and 2 are obligate heterozygotes for dilute as they were black-and-tan with blue offspring. Animals 4 and 6 were classified DD and Dd based on their *MLPH* exon 2 genotypes. Genotypes for the seven polymorphic amino acid positions in the MLPH protein and the silent C/T SNP in exon 2 of the *MLPH* gene are shown. Three different marker haplotypes are color-coded. (B) Animals 8–12 of the depicted German Pinscher family were used for the initial mutation analysis. Animals 7 and 8 are obligate heterozygotes for dilute as they were black-and-tan with blue offspring. Animals 10 and 12 were classified as Dd based on their genotypes with respect to the R199H mutation. (C) Large Munsterlander family used in this study. Black symbols indicate dogs with dilute coat color and BHFD. Animals 14 and 15 are obligate heterozygotes for dilute and BHFD as they were normal with BHFD offspring. Classification of the animals 13 and 16–20, respectively, was done based on their *MLPH* exon 2 genotypes.



**Genotyping of the R199H mutation**. (A) Schematic diagram of the *Hha*l RFLP used for genotyping the R199H mutation. (B) Genotyping of the R199H mutation in Doberman Pinschers and German Pinschers. Numbers of the animals correspond to the numbers in Fig. 3A and 3B. Note that the Doberman Pinschers are homozygous for the presumed wildtype allele (<sup>199</sup>R) while in the studied German Pinscher family the R199H mutation cosegregates with the d allele.

In order to rule out potential splicing aberrations we isolated skin RNA from a heterozygous Large Munsterlander (#15 in Figure 4), a dilute Beagle and a Beagle with wildtype color. We amplified the coding part of the *MLPH* cDNA by RT-PCR. Agarose gel electrophoresis gave no evidence for splicing aberrations or transcriptional silencing because the bands of the normal and dilute dogs were of the same sizes and comparable intensities. Sequencing of the RT-PCR products confirmed the *MLPH* polymorphisms previously obtained by comparative sequencing of genomic PCR products.

# Discussion

Pinschers affected by coat color dilution have a phenotype comparable to the leaden mouse mutant ( $Mlph^{ln}$ ). Therefore analyzing the canine ortholog of the Mlph gene causing this mutant in mice seemed a logical approach to elucidate the molecular basis for coat color dilution in dogs. The assignment of the canine MLPH gene to CF25q24 is in accordance with the location of the human and murine orthologous genes and with the known synteny data of the integrated canine map [11,12]. The orientation of the MLPH and COL6A3 genes to each other is

also consistent with their orientation on the human map. The genomic structure of the *MLPH* gene is similar but not identical in dog, human, and mouse. Differences were observed with respect to the dog exon 5, which is lacking from other species and the human/mouse/rat exon 9 that could not be identified within the genomic dog sequence by sequence comparisons. All the experimental canine cDNA sequences obtained in this study lacked a corresponding sequence. As there are known splice variants in human lacking exon 9 (e.g. accession AK022207) it might be possible that this alternative explanation would be that the homology between the human and canine exon 9 is very low, so that it can not be identified by cross-species sequence comparison.

The Pinscher breeds are considered as closely related and sometimes Doberman Pinschers are still interbred with German Pinschers in order to modulate the size of the animals. In support of this, we observed 48 sequence polymorphisms within and between the two related Pinscher breeds, of which only five variations seemed to be breed specific. Taking into account the limited number of animals used in the mutation analysis it is quite likely that there are even less or no breed-specific polymorphisms at all in these breeds. Generally, German Pinscher sequences showed less variation than those of Doberman Pinschers. This had to be expected because the German Pinscher breed experienced a severe bottleneck after the second world war (7 founders in Germany, personal communication by breeders).

We identified a set of eight SNPs including a silent C to T change in exon 2, which are in linkage disequilibrium with the dilute phenotype in some breeds. In Doberman Pinschers, Large Munsterlanders, and in Beagles one haplotype co-segregated with the dilute phenotype.

The R199H mutation is in linkage disequilibrium with the dilute phenotype in German Pinschers. The R199H mutation also showed perfect association with dilute in the Beagle family and was strongly associated with the d allele in Doberman Pinschers from Europe but not from North America.

A Large Munsterlander family with pups affected with BHFD was included in this study. The phenotype of the BHFD affected animals is very similar to CDA affected Pinschers [2,3]. Histological analysis of skin biopsies of BHFD affected dogs showed the typical perinuclear clumping of melanosomes within melanocytes of the hair matrix, which is also observed in leaden mice and human GS3 patients. Since the same haplotype as in the dilute Doberman Pinschers cosegregated with BHFD in the

Breed and phenotype	No. of animals	dilute genotype <sup>1</sup>	Exon 2 <sup>2</sup>			Exon 7		
			CC <sup>3</sup>	СТ	TT	<sup>199</sup> R <sup>199</sup> R	<sup>199</sup> R <sup>199</sup> H	<sup>199</sup> H <sup>199</sup> H
Doberman Pinscher (all)	140		50 (36%)	69 (49%)	21 (15%)	82 (59%)	47 (34%)	11 (8%)
wildtype coat color	98	D.	50 (51%)	48 (49%)	-	62 (63%)	33 (34%)	3 (3%)
wildtype coat color	21	Dd	-	21 (100%)	-	9 (43%)	12 (57%)	-
dilute coat color	21	dd	-	-	21 (100%)	11 (52%)	2 (10%)	8 (38%)
Doberman Pinscher (American origin)	38		6 (16%)	21 (55%)	11 (29%)	35 (92%)	3 (8%)	-
wildtype coat color	19	D.	6 (32%)	13 (68%)	-	16 (84%)	3 (16%)	-
wildtype coat color	8	Dd	-	8 (100%)	-	8 (100%)	-	-
dilute coat color	П	dd	-	-	(100%)	11 (100%)	-	-
Doberman Pinscher (European origin)	102		44 (43%)	48 (47%)	10 (10%)			
wildtype coat color	79	D.	44 (56%)	35 (44%)	-	46 (58%)	30 (38%)	3 (4%)
wildtype coat color	13	Dd	-	13 (100%)	-	I (8%)	12 (92%)	-
dilute coat color	10	dd	-	-	10 (100%)	-	2 (20%)	8 (80%)
German Pinscher	143		143 (100%)	-	-	64 (45%)	61 (43%)	18 (13%)
wildtype coat color	117	D.	117 (100%)	-	-	64 (55%)	53 (45%)	-
wildtype coat color	8	Dd	8 (100%)	-	-	-	8 (100%)	-
dilute coat color	18	dd	18 (100%)	-	-	-	-	18 (100%)
Beagle	6		I	3	2	I	3	2
wildtype coat color	2	D.	I	I		I	I	
wildtype coat color	2	Dd		2			2	
dilute coat color	2	dd			2			2
Large Munsterlander	12		-	8	4	-	7	5
wildtype coat color	8	Dd	-	8	-	-	7	I
dilute coat color & BHFD	4	dd	-	-	4	-	-	4
Weimeraner (dilute coat color)	I	dd		I			I	
Am. Staffordshire (dilute col. & CDA)	I	dd		I			I	
Mountain Dogs	26	D.	25	I		25	I	
Breeds unspecified	12	D.	10	2		9	3	
total	341		229 (67%)	85 (25%)	27 (8%)	181 (53%)	124 (36%)	36 (11%)

#### Table 2: Genotype frequency of two MLPH polymorphisms in different breeds

<sup>1</sup> A dog with wildtype coat color has the dilute genotype DD or Dd. If the animal has never produced any dilute (dd) offspring, the genotype can not be deduced unambiguously from the phenotype and the genotype is then denoted "D.". Among the Pinschers with wildtype coat colors there were also obligate carriers of the dilute allele (Dd), as these animals had dilute (dd) progeny.

<sup>2</sup> This polymorphism corresponds to the silent C/T SNP in exon 2 at position 164258 in the reference sequence [EMBL:BN000728].

<sup>3</sup> The following associations were highly significant in Pinscher breeds under Fisher's Exact Test (p < 0.001): Exon 2 for all Doberman Pinschers, American Doberman Pinschers, and European Doberman Pinschers; exon 7 for European Doberman Pinschers and for German Pinschers. Non-Pinscher breeds were not evaluated statistically because of their small sample numbers.

Large Munsterlander family, this result supports the idea that CDA and BHFD are indeed the same disorder.

that there may be different mutations causing coat color dilution in dogs.

The data clearly imply that mutations in or near the *MLPH* gene are causing dilute coat color in dogs. The fact that the observed linkage disequilibrium between marker alleles and dilute is strongest around exon 2 in Doberman Pinschers and around exon 7 in German Pinschers suggests

The newly identified polymorphisms in exon 2 of the *MLPH* gene should be suitable DNA markers for coat color dilution in Doberman Pinschers and for the BHFD allele in Large Munsterlanders. In German Pinschers the exon 7 polymorphisms can be used as a diagnostic test for the dilute allele. For Beagles a larger sample should be

	Large Muns	Large Munsterlander		Pinschers	German Pinschers	
Polymorphism	wild type	dilute	wild type	dilute	wild type	dilute
Exon 3 (V75M)						
VV	-	-	2	5	-	-
VM	-	-	H	-	4	-
MM	-	-	4	-	21	9
Exon 3 (D105N)						
DD	-	-	2	5	-	-
DN	-	-	11	-	4	-
NN	-	-	4	-	21	9
Exon 5 (MI54T)						
MM	-	-	5	11	2	-
MT	-	-	22	2	7	-
TT	6	4	34	6	45	14
Exon 6 (G185S)						
GG	-	-	6	7	17	9
GS	-	-	5	-	-	-
SS	-	-	-	-	-	-
Exon 7 (R199H)						
RR	-	-	71	11	64	-
RH	7	-	45	2	61	-
НН	I	4	3	8	-	18
Exon 8 (259–260)						
del/del	-	-	I	2	-	-
del/ED	-	-	3	-	-	-
del/EE	-	-	-	-	Ι	-
ED/ED	-	-	-	-	-	-
ED/EE	-	-	-	-	-	-
EE/EE	7	4	-	-	3	2
Exon 10 (D383G)						
DD	-	-	-	-	3	2
DG	-	-	-	-	-	-
GG	-	-	4	2	-	-

#### Table 3: Genotype data of amino acid changing polymorphisms

#### Table 4: Haplotype frequencies of the eight SNPs around the MLPH exon 2

Haplotype		Wildtype color, 30	5 animals	Dilute color, 45 animals	
	Alleles <sup>1</sup>	Ν	%	Ν	%
Haplotype I	AAAAACCG	34	5.6	-	-
Haplotype 2	AGGGGTTG	80	13.1	52 <sup>2</sup>	57.8
Haplotype 3	CAAAACCA	493	80.8	38 <sup>3</sup>	42.2
Haplotype 4	AAAAGCCG	3	0.5	-	-

<sup>1</sup> The alleles correspond to the eight polymorphic positions between 163889 and 164397 in the genomic reference sequence. <sup>2</sup> Each of the 22 analyzed dilute Doberman Pinschers was homozygous for haplotype 2.

<sup>3</sup> Each of the 18 analyzed dilute German Pinschers was homozygous for haplotype 3.

position <sup>1</sup>	cDNA position <sup>2</sup>	Doberman Pinscher/ Large Munsterlander/ Beagle	Doberman Pinscher (American origin)	German Pinscher
157354 (exon I)	-139 (5'-UTR)	G	G	G
157471 (exon I)	-22 (5'-UTR)	А	A	А
157486 (intron 1)		А	A	А
163882 (intron 1)		С	С	т
163889 (intron 1)		А	A	С
163936 (intron 1)		G	G	А
163983 (intron 1)		G	G	А
164012 (intron 1)		G	G	А
164049 (intron 1)		G	G	А
164075 (intron 1)		т	Т	С
164258 (exon 2)	+106	т	Т	С
164397 (intron 2)		G	G	А
180908 (exon 3)	+223	G	G	А
180994 (exon 3)	+309	C	Т	С
180998 (exon 3)	+313	G	G	A
183047 (intron 4)		C	G	G
183254 (exon 5)	+461	c	T	c
183336 (intron 5)		c	T	c
184650 (intron 5)		c	T	c
184751 (exon 6)	+553	G	G	G
184818 (intron 6)		G	A	G
186072 (intron 6)		Т	C	т
186091 (intron 6)		T	c	т Т
186184 (exon 7)	+596	A	G	A
186329 (intron 7)	. 370	G	A	G
18744954 (ex. 8) <sup>4</sup>	+775 – +780	GAGGAG	del	GAGGAG
189560 (intron 8)	.,,5 .,66	G	G	G
189575 (intron 8)		C	A	C
189719 (exon 9)	+1032	C	C	C
189728 (exon 9)	+1041	n.d.	G	A
195548 (intron 9)	. 1011	n.d.	G	G
195720 (exon 10)	+1148	n.d.	G	A
195808 (exon 10)	+1236	n.d.	A	G
195888 (intron 10)	1230	n.d.	G	G
195891 (intron 10)		n.d.	G	G
· · · ·			G	G
195900 (intron 10)		n.d. n.d.	G	del
195901 (intron 10)			G	G
195932 (intron 10)	±1262	n.d.		
196097 (exon 11)	+1263	n.d.	G T	G
196354 (intron 11)		n.d.		C T
197343 (intron 11)		n.d.	Т	Т
197510 (intron 11)		n.d.	С	С
197673 (intron 12)		n.d.	С	С
197730 (intron 12)		n.d.	G	G
198080 (intron 12)		n.d.	A	n.d.
198126 (intron 12)		n.d.	A	n.d.
199847 (intron 13)		n.d.	A	A
202837 (exon 16)	+1801 (3'-UTR)	n.d.	Т	C

#### Table 5: dilute haplotypes within the canine MLPH gene

<sup>1</sup> Positions refer to the entire determined genomic sequence of 212.696 bp [EMBL:BN000728].

 $^{2}$  +1 corresponds to the adenosine of the translation start codon in the *MLPH* cDNA.

analyzed to confirm whether these polymorphisms are appropriate DNA markers for the coat color dilution in Beagles as well. The data clearly imply that *MLPH* is the causative gene for dilute coat color in several dog breeds. However, the causal mutation has not yet been conclusively identified.

The data are compatible with two alternative hypotheses: Dilute coat color could be caused by a single founder mutation in all investigated dog breeds. Under this scenario the observed haplotypes suggest a location of the causal mutation within the *MLPH* gene upstream of exon 2. The alternative hypothesis, which can not be ruled out at this time, would be that different independent *MLPH* mutations cause coat color dilution in dogs.

Although no single polymorphism affecting amino acids was associated with all dilute phenotypes, it is possible that multiple mutations in this gene or its promoter region are responsible as is true for the brown phenotype in dogs [13]. At this time a functional significance of a synonymous mutation, such as the C/T change in exon 2, can not be completely excluded as it has been reported that such synonymous polymorphisms may influence mRNA folding and stability thereby mediating functional effects. Such mutations can either act in isolation or in combination with other mutations in the same transcript [14]. All dogs with the TT genotype were of dilute coat color, even though the inverse was not true.

In order to fully explore the possibility of different functional *MLPH* mutations, mRNA from dogs of dilute phenotype of several breeds such as Great Dane, Newfoundland, Shar-Pei, Beagle, Doberman Pinscher and Large Munsterlander are being collected. If multiple mutations occurred or if the same mutation occurred at several points in time, the haplotypes would not be consistent in all individuals with dilute coat color. This trait is under selection in some breeds in which dilute coat color has minimal to no health associated problems and is under strong negative selection in other breeds such as Large Munsterlanders where the trait is typically associated with severe hair loss. This large variation in pleiotropic effects also suggests that multiple mutations may be involved.

In the mouse an independent gene termed suppressor of dilute (*Dsu*) is known that is able to suppress the effects of *Mlph* mutations. Mice carrying loss of function alleles at the *Mlph* and the *Dsu* loci have a coat color closely resembling the wildtype coat color [15]. So far, no equiv-

alent *DSU* mutations have been reported in the dog. However, it seems possible that unrecognized *DSU* mutations might confound our analysis, which is based on the assumption of a strictly monogenic autosomal recessive inheritance of coat color dilution in dogs.

# Conclusion

We characterized the canine *MLPH* gene and identified 48 polymorphisms of this gene that occur in Doberman Pinschers and/or German Pinschers. Eight of these 48 polymorphisms located around exon 2 are in strong linkage disequilibrium with coat color dilution in Doberman Pinschers. A R199H mutation is in strong linkage disequilibrium with the dilute phenotype in German Pinschers. These results indicate that mutations in or near the *MLPH* gene are responsible for the coat color dilution in Pinschers. The reported polymorphisms will allow genetic testing of Doberman and German Pinschers to facilitate the breeding of dogs with specific coat colors.

# Methods

# Cloning and sequencing the MLPH gene

For the isolation of a canine BAC clone with the MLPH gene the Doberman Pinscher RPCI-81 BAC library [16] was screened with a 32P-labeled PCR fragment derived from the conserved 5'-end of the human MLPH cDNA. The donor animal for the RPCI-81 BAC library was a black-and-tan Doberman Pinscher named Grumpy. Grumpy was heterozygous at the dilute locus (Dd) as he had blue offspring. The probe sequence was amplified from the IMAGE cDNA clone IRAKp961H0816 provided by the Resource Center/Primary Database of the German Human Genome Project [17]. Hybridization was performed according the RPCI protocols [18]. End sequences of the positive BAC clone RP81-203J24 were determined on a LI-COR 4200L-2 automated sequencer (MWG, Biotech, Ebersberg, Germany) using the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany) and comparatively mapped to the human genome. Additionally, the size of the BAC clone was determined by pulsed field gel electrophoresis (PFGE).

primer name	primer sequence	product size	Т <sub>М</sub>	comments
MLPH cDNA, first round amplification				
Mlph_cDNA_Ex1_F1	CCT TCC TTC CCC TGT AGG AC	513 bp	52°C	
Mlph_cDNA_Ex4_R	GGA TCA CCT TGG CAC TCC			
Mlph_cDNA_Ex4_F1	GTG AAG ATC GGC TCG GTA G	686 bp	52°C	
Mlph_cDNA_Ex9_R	GGA TGC TGA GAG GTG GTG			
Mlph_cDNA_Ex7_FI	CTT GGA CTT TGA GGC AGA C	985 bp	52°C	
Mlph_cDNA_Ex14_R	ACT GAA CTT CCT TCT GAG G			
Mlph_cDNA_Ex10_F	AGA GAA GAG GAG ACC CTC	651 bp	52°C	

Miph_cDNA_Exit6_R GCT GGG TCA TCA CAG GC   MIph_cDNA_second round amplification   Miph_cDNA_Exit_F2 CTG TAG GAC CGG AGA GAG C 502 bp 52°C   Miph_cDNA_Exit_R3 GGA TCG CCT TGG CAC TCC 507 bp 52°C   Miph_cDNA_Exit_R4 GGA TCG CTG TGG GAC TCC 679 bp 52°C   Miph_cDNA_Exit_R4 GGA TCG CTC TGA GAC GTC CAC TTG 967 bp 52°C   Miph_cDNA_Exit_R ACT GAC ACT CCT CT GA GAG GAC CCC CTC 651 bp 52°C same primers as in first round amplification   Miph_cDNA_Exit_R ACT GAG GC TCC AAG GCC CTC 651 bp 52°C same primers as in first round amplification   Miph_cDNA_Exit_F4 ACT GAG CTC CTG GAG ACC 591 bp 56°C 56°C   P_Miph_Exit_for1 AGT GAC CCG AGA CTC CTG 538 bp 56°C 56°C   P_Miph_Exit_rev ATG AGC TCC CTG AGA ACC 598 bp 58°C 58°C   Miph_Exit_rev CAG GCT GGA GAG TCC ATG 598 bp 58°C   Miph_Exit_rev CAG GCT GGA GGA CTC CTG 52 bp 53°C   Miph_Exit_for1 GAG AGT GA GGA CTC CTG GAG ACC 58°C 58°C   Miph_Exit_for GAG AGT GAG GAG CTC	Table 0. PCK Primers us	ed for the MEPH CONA amplification and	lor genomic	<i>MEPTI III</i>	ation analysis (continued)
round amplification Miph. cDNA_Ex1_F2 GGA TCA CCT TGG CAC TCG Miph. cDNA_Ex4_R GGA TCA CCT TGG CAC TCG GA TCA CGC TGG CAG AGT G GA TCA TGG GAC GGG TCAG AGT G Miph. cDNA_Ex4_F2 AGA TCG GCT CGG TAG AGT G Miph. cDNA_Ex7_F2 CTC TGA CGA CTC CAC TTG Miph. cDNA_Ex7_F2 CTC TGA CGA CTC CAC TTG Miph. cDNA_Ex7_F2 CTC TGA CGA CTC CAC TTG Miph. cDNA_Ex14_R ACT GAG CTT CCT TC TGA GG Miph. cDNA_Ex16_R CTG GG TCA TCA CAG GC Miph. cDNA_Ex16_R CTG TGC CTC AGG CCC TG Miph. cDNA_Ex16_R CTG TCC CTG CGA GGA CCC CTG Miph. Ex1_rev ATG AGC TCC CTG AGA ACC P_Miph. Ex1_for1 ATG AGC TCC CTG AGA ACC Miph. Ex1_for2 CTC CTC CCG CGA GG CCC TG Miph. Ex1_rev ATG AGC TCC CTG AGA ACC Miph. Ex1_rev ATG AGC TCC CTG AGA ACC Miph. Ex2_rev CG CG CCC GA GG CTC CTG Miph. Ex2_rev AGG GT GA AGG TCA CGA GTC Miph. Ex3_rev AGG CTC CTG CGG CAC TG CG TG Miph. Ex3_rev CG CGA CCT CTG CGC GAG CTC Miph. Ex3_rev CTG CAC CGA CAT TCC AAA GG TCA Miph. Ex3_rev CTG CAC CGA CAT TGC AAG GTCA Miph. Ex3_rev CTG CAC CGA CAT TGC AAG CGT Miph. Ex3_rev CTG CAC CGC TTT CGA AGG CTC Miph. Ex3_rev CTG CAC AGG CGT CTT AGC Miph. Ex3_rev CTG CAC AGG CGT CTT AGC Miph. Ex4_rev CTG CAC AGG CGT CTT AGC Miph. Ex4_rev CTG CAC AGG CGT CTT AGC Miph. Ex4_rev CTG CAC AGG CGT CTT AGC Miph. Ex1_rev TG CAC AGG CGT CCT AGG CTC Miph. Ex1_rev TG CAC AGG CGT CCT AGG CTC Miph. Ex1_rev TG CAC AGG CGT CCT AGG CTT Miph. Ex1_rev TG CAC CGG CGT CCT AGG CTT Miph. Ex1_rev TG CAC CGG CGT CCT GG CGC CGA CGC Miph. Ex1_rev TG CAC CGG CGT CCT GGG CCT Miph. Ex1_rev TG CAC CGG CGT CCT CGG GGC CCA AGG Miph. Ex1_rev TG CAC CGG CGC CGA CGC GGC CCA AGG Miph. Ex1_rev TG CAC GGG CTT GCC CGC GCC CGA CGC GGC CCAC GGC Miph. Ex1_rev TG CCC CGG GCC CGA GGC CGA CGA GGA CAC GG Miph. Ex1_rev T	Mlph_cDNA_Ex16_R	GCT GGG TCA TCA CAG GC			
round amplification Miph. cDNA_Ex1_F2 GGA TCA CCT TGG CAC TCG Miph. cDNA_Ex4_R GGA TCA CCT TGG CAC TCG GA TCA CGC TGG CAG AGT G GA TCA TGG GAC GGG TCAG AGT G Miph. cDNA_Ex4_F2 AGA TCG GCT CGG TAG AGT G Miph. cDNA_Ex7_F2 CTC TGA CGA CTC CAC TTG Miph. cDNA_Ex7_F2 CTC TGA CGA CTC CAC TTG Miph. cDNA_Ex7_F2 CTC TGA CGA CTC CAC TTG Miph. cDNA_Ex14_R ACT GAG CTT CCT TC TGA GG Miph. cDNA_Ex16_R CTG GG TCA TCA CAG GC Miph. cDNA_Ex16_R CTG TGC CTC AGG CCC TG Miph. cDNA_Ex16_R CTG TCC CTG CGA GGA CCC CTG Miph. Ex1_rev ATG AGC TCC CTG AGA ACC P_Miph. Ex1_for1 ATG AGC TCC CTG AGA ACC Miph. Ex1_for2 CTC CTC CCG CGA GG CCC TG Miph. Ex1_rev ATG AGC TCC CTG AGA ACC Miph. Ex1_rev ATG AGC TCC CTG AGA ACC Miph. Ex2_rev CG CG CCC GA GG CTC CTG Miph. Ex2_rev AGG GT GA AGG TCA CGA GTC Miph. Ex3_rev AGG CTC CTG CGG CAC TG CG TG Miph. Ex3_rev CG CGA CCT CTG CGC GAG CTC Miph. Ex3_rev CTG CAC CGA CAT TCC AAA GG TCA Miph. Ex3_rev CTG CAC CGA CAT TGC AAG GTCA Miph. Ex3_rev CTG CAC CGA CAT TGC AAG CGT Miph. Ex3_rev CTG CAC CGC TTT CGA AGG CTC Miph. Ex3_rev CTG CAC AGG CGT CTT AGC Miph. Ex3_rev CTG CAC AGG CGT CTT AGC Miph. Ex4_rev CTG CAC AGG CGT CTT AGC Miph. Ex4_rev CTG CAC AGG CGT CTT AGC Miph. Ex4_rev CTG CAC AGG CGT CTT AGC Miph. Ex1_rev TG CAC AGG CGT CCT AGG CTC Miph. Ex1_rev TG CAC AGG CGT CCT AGG CTC Miph. Ex1_rev TG CAC AGG CGT CCT AGG CTT Miph. Ex1_rev TG CAC CGG CGT CCT AGG CTT Miph. Ex1_rev TG CAC CGG CGT CCT GG CGC CGA CGC Miph. Ex1_rev TG CAC CGG CGT CCT GGG CCT Miph. Ex1_rev TG CAC CGG CGT CCT CGG GGC CCA AGG Miph. Ex1_rev TG CAC CGG CGC CGA CGC GGC CCA AGG Miph. Ex1_rev TG CAC GGG CTT GCC CGC GCC CGA CGC GGC CCAC GGC Miph. Ex1_rev TG CCC CGG GCC CGA GGC CGA CGA GGA CAC GG Miph. Ex1_rev T					
Miph_cDNA_Ext_P2CTG TAG GAC GGG AGA GAG C502 bp52°CMiph_cDNA_Ext_RGGA TGA CCT TGG CAC TCC679 bp52°CMiph_cDNA_Ext_RGGA TGA CTG TGG AGA GTG G679 bp52°CMiph_cDNA_Ext_RACT GAA CTC CAC TTG967 bp52°CMiph_cDNA_Ext_RACT GAA CT CCAC TTG967 bp52°CMiph_cDNA_Ext_RACT GAA GAG CTC CAC TG651 bp52°CMiph_cDNA_Ext6.RGCT GG TCA TCA GAG CC651 bp56°CP_Miph_Ext_foriAGT GGC CTC AAG CCC TG591 bp56°CP_Miph_Ext_foriAGT GGC CTG AGA GAC CCTG563 bp56°CP_Miph_Ext_foriAGT GGC TC CTG CGA GAG ACC725 bp56°CP_Miph_Ext_foriAGT GGC TC GGA GAG CC TG598 bp58°CMiph_Ext_revATG AGC TCC CTG AGA GTC725 bp56°CP_Miph_Ext_revCAG CAC CAG GAT CAG GAT C725 bp56°CMiph_Ext_revCAG CAC CAG GAT CGA GTC725 bp56°CMiph_Ext_revCAG CAC CAG GAT CGA GTC725 bp56°CMiph_Ext_revCAG CAC CAG GAG CAG CAC TG716 bp56°CMiph_Ext_revCAG CAC CAG GAG CAG GAG588 bp53°CMiph_Ext_revAGA AGC GAT CGA GAG GG588 bp53°CMiph_Ext_revGT GT GAG AGC TTC TG AAG588 bp53°CMiph_Ext_revGT GT GAG AGC TTC TG AAG588 bp53°CMiph_Ext_revGT GT GAG AGC TTC GA AGC588 bp53°CMiph_Ext_revGT GT GAG AGC TTC GA AGC588 bp53°CMiph_Ext	,				
Miph_cDNA_ExitGGA TCA CCT TGG CAC TCCMiph_cDNA_ExitAGA TCG GCT CGG TAG AGT G679 bp52°CMiph_cDNA_ExitAGA TCG GCT CA GAG GTG GTG967 bp52°CMiph_cDNA_ExitACT GAA GAG CT CCAC TTG967 bp52°CMiph_cDNA_ExitACT GAA CTT CCT TC TGA GGA967 bp52°CMiph_cDNA_ExitACT GAA CTT CCT TC TGA GGA957 bp52°CMiph_cDNA_ExitACT GAA CTC CTC TG AGG CTC651 bp52°CMiph_cDNA_ExitACT GGG CTC AAG CCC TG591 bp56°CP.Miph_Exit_foriAGT GGG CTC CTG AGG ACC56 bp56°CP.Miph_Exit_foriAGT GGG CTC CTG AGG CTC CTG563 bp56°CP.Miph_Exit_foriATG AGC TCC CTG AGG CTC CTG GAG ACC1000000000000000000000000000000000000					
Miph_cDNA_Ex4_F2AGA TCG GCT CG TAG AGT G679 bp52°CMiph_cDNA_Ex4_F2CTC TGA CGA CTC CAC TTG967 bp52°CMiph_cDNA_Ex14_RACT GAA CTT CCT TCT GAG G967 bp52°CMiph_cDNA_Ex16_RGCT GGG TCA TCA CAG GC651 bp52°CMIPh_cDNA_Ex16_RGCT GGG TCA TCA CAG GC591 bp56°CP_Miph_Ex1_orlAGT GGC CTC AAG CCC TG591 bp56°CP_Miph_Ex1_revATG AGC TCC CTG AGA ACC56°CP_Miph_Ex1_revATG AGC TCC CTG AGA ACC588 bpP_Miph_Ex1_revATG AGC TCC CTG AGA ACCMiph_Ex2_forCTC CCG GAG GTC AGA CCMiph_Ex2_forCTC ACG GAC ATT ATG TCA CAGMiph_Ex3_forAGA AGC GAC AGT AGA CCMiph_Ex4_forAGA GCT CC GA AGT CA GAT CMiph_Ex4_forGAG ACC AGG AT CA ATG CAC TCMiph_Ex4_forGAG ACC AGG AT CAG ATCMiph_Ex5_forAGA AGT GAT GGA GGT CA CTGMiph_Ex5_forAGA GGA CAG GAA CAG GAGMiph_Ex5_forAGA GGA CAC GAA CAG GAGMiph_Ex6_forAG CTG CTG CAGC GAT CTG GAC GTMiph_Ex6_forGTG CTC AGC ACT TCT GA CAGMiph_Ex6_forCGG CT CTG CAACMiph_Ex6_forCGG CT CTG CAAC GAG GACMiph_Ex7_forGTC CTG CAG CAT CTG GAC GTMiph_Ex8_forCAG CAG GAC CTG GA GAG CTMiph_Ex8_forCAG CAG GAC CTG GA GAG CTMiph_Ex8_forCAG CAG GAC CTG GA GAG CTCMiph_Ex8_forCAG CAG CTG CTG GA GAG CTCMiph_Ex8_forCAG CAG GAC CTG GAC CTGMiph_Ex8_forCAG CGG GT CT GA GAG C	-		502 bp	52°C	
Miph_cDNA_ExP_RGGA TGC TGA GAG GTG GTGMiph_cDNA_Ex7_P2CTC TGA CGA CTC CAC TTG967 bp52°CMiph_cDNA_Ex14_RACT GAA CTT CCT TCT GAG G651 bp52°CMiph_cDNA_Ex16_RGGG TCA TCA CAG GC651 bp52°CMIPh_cDNA_Ex16_RGGC CTC AGG CCC TG651 bp52°CMIPh_Ex1_for1AGT GGC CTC AGG CCC TG591 bp56°CP.Miph_Ex1_for2CTC CTC CG AGA ACC598 bp56°CP.Miph_Ex1_revATG AGC TCC CTG AGA ACC598 bp58°CMiph_Ex2_revCAG GCT GGA GGT CAG GGT C725 bp56°CP.Miph_Ex1_revATG AGC TCC CTG AGA GGT C725 bp56°CMiph_Ex2_revCAG GCT GGA AGG TCA GAG TC710 bp56°CMiph_Ex3&forGAG ACC CAG GAT CGA GGT C725 bp56°CMiph_Ex3&forGAG ACC CAG GAT CAC GG GT725 bp56°CMiph_Ex3&forGAG ACC CAG GAT CAC GG TC710 bp56°CMiph_Ex5FerwCAC TGA CAT TCC AAA GGAT C582 bp53°CMiph_Ex5_revACC GAA CAG GAA CAG GAG582 bp53°CMiph_Ex6_forAG CTT GC TG AGA ACG GAT GAT GG582 bp53°CMiph_Ex7_forGTC CTC AGC AGT TCT GAG CC588 bp53°CMiph_Ex8_revGTG TG GAC GAG GAG GAG GG55°CMiph_Ex8_revGTG TG GAC AGG GAG GAG GG55°CMiph_Ex8_forCAG CAG GAG GAG GC TC590 bp55°CMiph_Ex8_revGTG TG GAC GAG GGA GGG55°CMiph_Ex8_revGG TTG CCA AGG GAG GGG GG550 bp55°	Mlph_cDNA_Ex4_R	GGA TCA CCT TGG CAC TCC			
Miph_cDNA_Ex7_F2CTC TGA CGA CTC CAC TTG ACT GAA CTT CCT TC TG AG G ACT GAA CAG GAA GAG GAA CAC CTC GGT GGT CAT CA CAG GA967 bp52°Csame primers as in first round amplificationMiph_cDNA_Ex16_RGCT GGG TCA TCA CAG GC651 bp52°Csame primers as in first round amplificationMIPh_cDNA_Ex16_RGCT GGG TCA TCA CAG GC591 bp56°CP_Miph_Ex1_for1AGT GGC CTC AGA CCC TG591 bp56°CP_Miph_Ex1_revATG AGC TCC CTG AGA ACC563 bp56°CP_Miph_Ex1_revATG AGC TCC CTG AGA ACC588 bp58°CMiph_Ex2_forGTC ACC GGA TCA ATG TCA CAG598 bp58°CMiph_Ex2_forGAG ACC CAG GAT CA ATG CAC CAG725 bp56°CMiph_Ex3a4_forGAG ACC CAG GAT CA ATG CAC TG71 bp56°CMiph_Ex4_revCAC GAA CAG GAA CAG GAA CAG GA71 bp56°CMiph_Ex5_forAGC ATG CAT GG ATG GAT G582 bp53°CMiph_Ex6_forAG CTT GC TG GAA GC588 bp53°CMiph_Ex6_forAG CTT GC AG CAT CT GAA CG483 bp56°CMiph_Ex6_forCAG CGG GAT TTC TGA AAGC483 bp56°CMiph_Ex7_revGTG GAC AGT CAG AGT CAG CT716 bp56°CMiph_Ex8_forCAG GAG AGC CTT GGA AGC416 bp56°CMiph_Ex8_forCAG CAG CAG GAG CTT500 bp55°CMiph_Ex8_forCAG GAG AGC CTT GGA GGA AGC716 bp55°CMiph_Ex1_forCGC AGG AGA CGC GGGA AGC716 bp55°CMiph_Ex1_forCGC GG CTT CT GA AG CC519 bp55°C	Mlph_cDNA_Ex4_F2	AGA TCG GCT CGG TAG AGT G	679 bp	52°C	
Miph_cDNA_ExI4_RACT GAA CTT CCT TCT GAG G52°Csame primers as in first round amplificationMiph_cDNA_ExI6_RGCT GGT CA TCA CAG GC651 bp52°Csame primers as in first round amplificationMLPH genomic DNAP_Miph_ExI_for1AGT GGC CTC AGG CCC TG591 bp56°CP_Miph_ExI_for2ATG AGC TCC CTG AGA ACC591 bp56°CP_Miph_ExI_for2CTC CTC CCG AGG CTC CTG AGA ACC598 bp58°CMiph_Ex2_forGTC ACC GAC ATT ATG TCA CAG GS598 bp58°CMiph_Ex2_forGTC ACC GA AGG TCA GAT C725 bp56°CMiph_Ex3_forGAG AGC CAG GAT CGA GTC725 bp56°CMiph_Ex3_forGAG AGC CAG GAT CGA GTC725 bp56°CMiph_Ex4_forGAG ACC CAG GAT CGA GTC725 bp56°CMiph_Ex5_forAGA GAG GAG CAC TG471 bp56°CMiph_Ex5_forAGA GTT GCT GGA GG TCA CTG471 bp56°CMiph_Ex5_forAGC GAG CCT TCT GGA GC582 bp53°CMiph_Ex7_forGTC CTC AGC AGC TTC TGG AGC588 bp53°CMiph_Ex7_forGTC CTC AGC AGC TTC TGG AAGC483 bp56°CMiph_Ex9_forCAG CAG CAG GAG CT550 bp56°CMiph_Ex9_forCCC GC GTG GT CTC GAG600 bp56°CMiph_Ex9_forCCC GG GT CT CTG GAG AGC550 bp55°CMiph_Ex1_forAGG GGG CAG AGG CGT GG539 bp55°CMiph_Ex1_forAGG CGT CC AGG GT GGG GGG591 bp55°CMiph_Ex1_forCCC GG CT TC TGG AGA CT GG591 bp55°C<	Mlph_cDNA_Ex9_R	GGA TGC TGA GAG GTG GTG			
Miph_cDNA_ExI0_FAGA GAA GAG GAG ACC CTC GCT GGG TCA TCA CAG GC651 bp52°Csame primers as in first round amplificationMUPH_cDNA_ExI6_RGCT GGG TCA TCA CAG GCF91 bp56°CP_Miph_ExI_revAGT GGC CTC CTG AGA ACCF91 bp56°CP_Miph_ExI_revATG AGC TCC CTG AGA ACCF91 bp58°CP_Miph_ExI_revATG AGC TCC CTG GA GA ACCF81 bp58°CP_Miph_ExI_revATG AGC TCC CTG AGA ACCF81 bp58°CP_Miph_ExI_revATG AGC TCC CTG AGA ACCF81 bp58°CMiph_Ex2_forGC ACC GAC ATT ATG TCA CAG598 bp58°CMiph_Ex3_forGAG ACC CAG GAT GAG GT725 bp56°CMiph_Ex4_revCAC CAC ACT TCC AAA GGA TCMiph_Ex34_revCAC GAA ACG GAA GGA GAGMiph_Ex5_forAGA AGT GAT GGA GGT CAC TG471 bp56°CMiph_Ex6_forAG CTT GCC TG GA AG588 bp53°CMiph_Ex6_forAG CTT GCC TG GA AG588 bp53°CMiph_Ex7_revGTG TC GAC AGT CT GAG588 bp56°CMiph_Ex8_forCAG GGA ATTTC TG AAG483 bp56°CMiph_Ex9_revGTG TTG GAC AGT CAG AGT G500 bp56°CMiph_Ex9_revGTG GTG GT CTC GAG AGC TTT600 bp56°CMiph_Ex1_revGCC GCA CTG GTG TTG GAC AGC539 bp55°CMiph_Ex1_forCAG GGA AGC TTT TGC GAG AGC539 bp55°CMiph_Ex13_forCCT GCT GCA GAG GTG GAG519 bp55°CMiph_Ex13_forCCT GT TC CG GAG AGC519 bp55°CMiph_Ex1	Mlph_cDNA_Ex7_F2	CTC TGA CGA CTC CAC TTG	967 bp	52°C	
Miph_cDNA_Ex16_RGCT GGG TCA TCA CAG GCMLPH genomic DNAP_Miph_Ex1_for1AGT GGC CTC AAG CCC TG591 bp56°CP_Miph_Ex1_for2CTC CTC CCG AAG CCC CTG533 bp56°CP_Miph_Ex1_for2CTC CTC CCG AGG CTC CTG563 bp58°CMiph_Ex2_forGTC ACC GAC ATT ATG TCA CAG598 bp58°CMiph_Ex3&revCAG CTG GA AG TCA GAT CMiph_Ex3&revCAG CTGC AGG AGG TCA GAT CMiph_Ex3&revCAC TCA CAT TCC AAA GGA TCMiph_Ex3&revCAC TCA CAT TCC AAA GGA TCMiph_Ex3&revCAC TCA CAT TCC AAA GGA TCMiph_Ex5_revCAC TCA CAT TCC AAA GGA TCMiph_Ex5,forAGA ACT GAT GGA AGG GAGMiph_Ex5,revCTC CG GAA CAG GAG CAG GAGMiph_Ex5,revCTC CG GAA CAG GAG CAG GAGMiph_Ex6, forAG CTT GC CTG CA ACMiph_Ex7,revGTG AGA CTT CTG CAACMiph_Ex7, forGTC CTC AGC ACT TCT GA AGG CMiph_Ex8, forCAG GG AT TTC TGA AAG CMiph_Ex8, forCAG GG GAT TTC TGA AAG CMiph_Ex8, forCCG CG CTTT GC CTT AGCMiph_Ex8, forCCG CG CTTT GC CTT AGCMiph_Ex8, forCCG AGG AGG CTT CTGMiph_Ex8, forCCG CG CG CTT GG CT CG GA AGCMiph_Ex8, forCCG CG CG CTT GG CAG GG CG TC CTG AGGMiph_Ex8, forCCG AGG AGC CCT GG GA AGCMiph_Ex8, forCCG CG CG CT TG GG AGG CTT CGMiph_Ex8, forCCG CG CG CT TG GG AGG CT CG GG GA AGCMiph_Ex8, forCCG CG CG CT CT GG GG CC CG GG GG CG CG GG GG CG CG CG	Mlph_cDNA_Ex14_R	ACT GAA CTT CCT TCT GAG G			
MLPH genomic DNAP_HIph_Ex1_for1AGT GGC CTC AAG CCC TG591 bp56°CP_MIph_Ex1_revATG AGC TCC CTG AGA ACCP_MIph_Ex1_revATG AGC TCC CTG AGA ACCMiph_Ex2_revCAG GCT GGA AGG TCA CAG AGA CCMiph_Ex2_revCAG GCT GGA AGG TCA GAT CMiph_Ex3&frorGTG AGA CCC AG GAT CGA GTCMiph_Ex3&frorAGA AGT GAA GT CCACG AGA TCCMiph_Ex3&frorAGA AGT GAA GG TGA GAT CMiph_Ex3&frorAGA AGA GAA GAG GAA CCAG GAAMiph_Ex3&frorAGA AGA GAT GGA GGT CCACG ATT ATG CAAA GGA CCMiph_Ex5_forAGA AGA GAA GAG GAA CAG GAAMiph_Ex5_revACC GAA CAG GAA CAG GAAGMiph_Ex6_revCTG CAG CCT CTG CCA ACMiph_Ex7_revGTG AGA AGC TTC TGG AGG CCMiph_Ex7_revGTG CTC AGC ACT TCT GAA GCMiph_Ex8_forCAA GGA GAT TC TGG AAGCMiph_Ex8_forCAA GAG GAT TCT TGG AAGCMiph_Ex8_forCAG GGG GAT TCT TGG AAGCMiph_Ex8_revGTG TTG GAC AGT CAG AGT GMiph_Ex8_revGTG TTG GAC AGT CAG AGCMiph_Ex8_revGTG TTG GAC AGT CCT GAG 600 bpS6°CMiph_Ex8_revGTG CCC GCG CT CT GG AGA GCMiph_Ex1_revGC CTG CC CAG GGT TCT GG530 bpS5°CMiph_Ex1_revGG CTT CC CCA GGT GTG GTMiph_Ex1_revGG CTT CC CCA GGT GCT GGMiph_Ex1_revGG CTT CC CCA GGT GGCMiph_Ex13_revGG TTT GC CTG GGA CCAGMiph_Ex13_revGG TTT GC CTG GGA CCAGMiph_Ex14_forCTC CTT TAT GCT CTG GGAC CSMiph_Ex15_revTG	Mlph_cDNA_Ex10_F	AGA GAA GAG GAG ACC CTC	651 bp	52°C	same primers as in first round amplification
P_Mlph_Ex1_revAGT GGC CTC AAG CCC TG\$91 bp\$6°CP_Mlph_Ex1_revATG AGC TCC CTG AGA AACC*********************************	Mlph_cDNA_Ex16_R	GCT GGG TCA TCA CAG GC			
P_Mlph_Ex1_revAGT GGC CTC AAG CCC TG591 bp56°CP_Mlph_Ex1_revATG AGC TCC CTG AGA AACCP_Mlph_Ex1_revATG AGC TCC CTG AGA AACCP_Mlph_Ex1_revATG AGC TCC CTG AGA AACCMlph_Ex2_revCAG GCT GGA AGG TCA CAG589 bp58°CMlph_Ex3&4_rovCAC CAC GAC ATT ATG TCA CAG598 bp58°CMlph_Ex3&4_rovCAG CTC AGG ATG CA GAT C725 bp56°CMlph_Ex3&4_rovCAC TCA CAT TCC AAA GGAT C58° cb53°CMlph_Ex5_forAGA AGT GAT GGA GGT CAC TG71 bp56°CMlph_Ex5_forAGA AGT GAT GGA GGT CAC TG58° bp53°CMlph_Ex6_revCTG CAC CAC CAT CT CACA CAC588 bp53°CMlph_Ex6_revGTG CTC AGC ATT CT GAG ACG568 bp53°CMlph_Ex7_revGTG AGA AGC TTC TGG ACC568 bp53°CMlph_Ex7_revGTG AGA AGC TTC TGA GAC483 bp56°CMlph_Ex8_revGTG TG GAC AGT CAG AGG GT56°C56°CMlph_Ex8_revGTG TG GAC AGT CAG AGG GT50° bp55°CMlph_Ex8_revGTG TTG GAC AGT CTG GAG50° bp55°CMlph_Ex10_rovCAC CTG GGA CAG GGA AGCMlph_Ex12_revGGG CTT CTG AGA AGC539 bp55°CMlph_Ex12_revTGG ACT TGA GGC CTG GGG539 bp55°CMlph_Ex12_revTGG ACT TGA GGC CTG GGA CAC AGMlph_Ex13_rovGG CTT TGC GGA CAC AGGMlph_Ex14_forCTC CTT TAT GCT CTG GGA CC591 bp55°CMlph_Ex15_rov <td>MIPH genomic DNA</td> <td></td> <td></td> <td></td> <td></td>	MIPH genomic DNA				
P_Miph_Ex1_revATG AGC TCC CTG AGA ACCP_Miph_Ex1_revATG AGC TCC CTG AGA ACCMiph_Ex2_forGTC ACC GAC ATT ATG TCA CAG598 bp58°CMiph_Ex2_forGTC ACC GAC ATT ATG TCA CAG598 bp58°CMiph_Ex3&forGAG ACC CAG GAT CGA GTC725 bp56°CMiph_Ex3&forAGA AGT GAT GGA GGT CAC TG471 bp56°CMiph_Ex3&forAGA AGT GAT GGA GGT CAC TG588 bp53°CMiph_Ex5_forAGA AGT GAT GGA GGT CAC TG580 bp53°CMiph_Ex5_revACC GAA CAG GAA GG588 bp53°CMiph_Ex5_revGTG CTG AGC ACT TCT GAG568 bp53°CMiph_Ex6_revGTG CTG AGC ACT TCT GGA CC116 bp56°CMiph_Ex7_forGTC CTC AGC ACT TCT GGA CG568 bp53°CMiph_Ex6_revGTG GACA AGT CTT GGA CG418 bp56°CMiph_Ex7_revGTG TTG GAC AGT CAG AGT C56°CMiph_Ex8_revGTG TTG GAC AGT CGA GGG AGC550 bp55°CMiph_Ex9_revGCT GCA AGG AGG GGA AGC600 bp55°CMiph_Ex10_revCCC CCC TTT GC TAA GC539 bp55°CMiph_Ex11_forAGG CGT CCA GAT CTT GG GAC AGG539 bp55°CMiph_Ex13_forCCT GTC TCT GG GAC AG GCT GG539 bp55°CMiph_Ex13_forCCT GTC TTA GC CTG GCA CG591 bp55°CMiph_Ex13_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex13_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA CA GG591 bp	0		591 bo	56°C	
P_Miph_Ex1_for2CTC CTC CCG AGG CTC CTG563 bp56°CP_Miph_Ex1_revATG AGC TCC CTG AGA ACCMiph_Ex2_forGTC ACC GAC ATT ATG TCA CAG598 bp58°CMiph_Ex2_revCAG GCT GGA AGG TCA GAT CMiph_Ex3&4_forGAG ACC CAG GAT CGA GAT CMiph_Ex3&4_revCAC TCA CAT TCC AAA GGA TCMiph_Ex5_forAGA ACT GAT GGA GGT CAC TGMiph_Ex5_forAGA ACT GAT GGA GGT CAC TGMiph_Ex5_forAGA CT TCC CAG GAT GAT GMiph_Ex5_revACC GAA CAG GAA CAG GAGMiph_Ex6_revCTG CTC AGC ACT TCT GAGMiph_Ex7_forGTC CTC AGC ACT TCT GAGMiph_Ex8_revGTG TTG GAC AGT CAG AGG GATMiph_Ex8_revGTG TTG GAC AGT CAG AGG GATMiph_Ex9_forCCC GCC TTT GCC TTA AGCMiph_Ex9_forCCC GCC CTT GGA GGA AGCMiph_Ex9_forCCC GAC CTT GGA CCTMiph_Ex9_forCCC GGC TTT GCC TTA AGCMiph_Ex9_forCCC GGC CTT GGA AGG AGCMiph_Ex10_revCAC CTG GGA CAG GGA AGCMiph_Ex11_revGCC TTC TGG AGA GGC GT GGMiph_Ex12_forCGC CGC CCA AGG CTTC GGA AGCMiph_Ex12_forCGC CGC CCA AGG CCT GGA AGCMiph_Ex12_forCGC CGC CCA AGG CCT GGA AGCMiph_Ex12_revTGG ACT TGC CCA GGC TGGMiph_Ex12_forCCT CTT TAT GCT CTG GCA CMiph_Ex12_forCCT CT CT TAT GCT CTG GCA CMiph_Ex13_forCT CT TT AGC CT GGA CCMiph_Ex14_forCT CT TT AGC CT GGA CCMiph_Ex14_forCT CT TT AGC CT GGA CCMiph_Ex14_forCT CT CT TAG CT GGA CC			571.00	50 0	
P_Miph_Ex1_revATG AGC TCC CTG AGA ACCMiph_Ex2_revCAG GCT GGA AGG TCA CAG598 bp58°CMiph_Ex384_forGAG ACC CAG GAT CGA GTC725 bp56°CMiph_Ex384_revCAC TCA CAT TCC AAA GGA TC471 bp56°CMiph_Ex5_revAGC AAC GAG GAT GGA GGT CAC TG471 bp56°CMiph_Ex5_revACC GAA CAG GAG CAT GC S82 bp53°CMiph_Ex6_forAG CTT GCC TGG ATG GAT G582 bp53°CMiph_Ex6_revCTG CAG CCT CTG CAAC568 bp53°CMiph_Ex7_revGTG AGA ACT TCT GA ACC483 bp56°CMiph_Ex7_revGTG GGA AGC TTC TGG AAC56° CMiph_Ex7_revGTG GGA AGC TTC TGG ACC568 bp53°CMiph_Ex8_revGTG TTG GAC AGT CAG AGC TTC56° CMiph_Ex9_revGTG TG GGA CAG TAG GA GC TTC56° CMiph_Ex9_revGCT GCA AGG AGG AGC TTC550 bp55° CMiph_Ex10_forCAC GGG CAG AGG CTC550 bp55° CMiph_Ex11_forAGG CT CCC CAG AGT GTG539 bp55° CMiph_Ex12_revTGG ACT TGA GAC CTT TGG AGA AGC539 bp55° CMiph_Ex13_revGGG TTT GC CAAG GCT GGC591 bp55° CMiph_Ex13_revGG GTT GC CAA GG GAT GCA591 bp55° CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55° CMiph_Ex14_revTTG TCA CAC GAA GCT GAG591 bp55° CMiph_Ex14_revTTG TCA CAC GAA GAC AGG591 bp55° CMiph_Ex14_revTTG TCA CAC GGA GAA CAC G591 bp55° C <td></td> <td></td> <td>563 bp</td> <td>56°C</td> <td></td>			563 bp	56°C	
Miph_Ex2_forGTC ACC GAC ATT ATG TCA CAG598 bp58°CMiph_Ex2_revCAG GCT GGA AGG TCA GAT C725 bp56°CMiph_Ex384_revCAC TCA CAT TCC AAA GGA TC711 bp56°CMiph_Ex5_forAGA AGT GAT GGA GGT CAC TG471 bp56°CMiph_Ex5_forAGA AGT GAT GGA GGT CAC TG582 bp53°CMiph_Ex6_forAGC CAG CAC TCT G CCA AC582 bp53°CMiph_Ex6_forGTC CTC AGC ACT TCT GAG568 bp53°CMiph_Ex7_revGTG CAG CAG TC TG GCA AC483 bp56°CMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex9_revGTG TTG GAC AGT CAG GAG TC116 bp56°CMiph_Ex9_revGTG TG GAC AGT CAG GAG GAG550 bp55°CMiph_Ex9_revGCT GCA GGG GAT GTG GTG GTG GTG550 bp55°CMiph_Ex10_forCAG GGG CAG GGA AGC539 bp55°CMiph_Ex11_forAGG CTT CG GAG AGG CTG GT539 bp55°CMiph_Ex13_revGG TTT GC CTG GCA GG539 bp55°CMiph_Ex13_revGG TTT GC CTG GCA CG539 bp55°CMiph_Ex13_revGG TTT GC CTG GCA C591 bp55°CMiph_Ex13_revGG TTT GC CTG GCA CG591 bp55°CMiph_Ex14_revTTG CA CAG GAG AGC AGA GC591 bp55°CMiph_Ex14_revTTG CA CAG GAG AGC AGA GC591 bp55°CMiph_Ex13_revGG TTT GC CTG GCA CAG GCA GAG591 bp55°CMiph_Ex14_revTTG CA CAG GGA GAC AGA GC591 bp55°CMiph_Ex1	•		303 DP	50 0	
Miph_Ex2_revCAG GCT GGA AGG TCA GAT CMiph_Ex384_forGAG ACC CAG GAT CGA GTC725 bp56°CMiph_Ex384_revCAC TCA CAT TCC AAA GGA TCMiph_Ex5_forAGA AGT GAT GGA GGT CAC TG471 bpMiph_Ex5_forAGC AGA CAG GAA CAG GAG582 bp53°CMiph_Ex6_forAG CTT GCC TGG ATG GAT G582 bp53°CMiph_Ex7_forGTC CTC AGC ACT TCT GAG568 bp53°CMiph_Ex7_forGTC CTC AGC ACT TCT GG ACC418 bp56°CMiph_Ex7_revGTG AGA AGC TTC TGG ACC483 bp56°CMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex9_forCCG CGC CTT GC CTA AGC416 bp56°CMiph_Ex9_forCCG GCA TTC TGG AGA GT G550 bp55°CMiph_Ex10_forCAG AGC CTG GCT CCT GG AGA GC550 bp55°CMiph_Ex11_forAGG CGT CCA GAG GTT GG539 bp55°CMiph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex13_forCT GT CTG CC CA AG GCT GG539 bp55°CMiph_Ex13_revGG TTT GC AAG GCT GAG539 bp55°CMiph_Ex13_revGG TTT GC CAA GG CT GG591 bp55°CMiph_Ex14_forCTC CTT TAT GCT GG CAC GC S91 bp55°CMiph_Ex14_revTTG CC ACG GAG GCA GAC AGG591 bp55°CMiph_Ex14_revTTG TCA CAC GA GG CA CAC AG591 bp55°CMiph_Ex14_revTTG CC ACG GA GCA CAC G591 bp55°CMiph_Ex14_revTTG CC ACG GA GCA CAC G591 bp55°CMiph	•		598 ho	58°C	
Miph_Ex384_forGAG ACC CAG GAT CGA GTC725 bp56°CMiph_Ex384_revCAC TCA CAT TCC AAA GGA TC471 bp56°CMiph_Ex5_forAGA AGT GAT GGA GGT CAC TG471 bp56°CMiph_Ex6_forAG CTT GCC TGG ATG GAT GA S82 bp53°CMiph_Ex6_revCTG CAG CCT CTG CCA AC710 bp56°CMiph_Ex7_revGTC CT CAGC ACT TCT GAG568 bp53°CMiph_Ex7_revGTG AGA AGT TTC TGA AAG C483 bp56°CMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex9_forCCC GCC TTT GCC TTA AGC416 bp56°CMiph_Ex9_forCCC GCC TTT GCC TA AGC AGT CAG500 bp56°CMiph_Ex9_forCCC GC CTT GCC TA AGC AGT CAG500 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTC700 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGC700 bp55°CMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex12_forCGC CGA CCA AGT CTG GCA539 bp55°CMiph_Ex13_forCTC GTT TC CC CA GAT TCG539 bp55°CMiph_Ex13_revGG TTT GCC AAG GCT GAG700 bp55°CMiph_Ex13_revGG TTT GCC AAG GCT GAG591 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex15_forCTC CAG GAG GAC AAG641 bp55°CMiph_Ex15_revTG CTC AGG GAC AAG641 bp55°C	·		370 DP	30 C	
Miph_Ex384_revCAC TCA CAT TCC AAA GGA TCMiph_Ex5_forAGA AGT GAT GAA GGT GAC GGA GGT CAC TG471 bp56°CMiph_Ex5_forAGC TAG CAG GAA CAG GAG582 bp53°CMiph_Ex6_revCTG CAG CCT TG CCA ACMiph_Ex7_forGTC CTC AGC ACT TCT GAG568 bp53°CMiph_Ex7_revGTG AGA AGC TTC TGA AAG C483 bp56°CMiph_Ex8_revGTG TTG GAC AGT CAG AGT GAT G56° CMiph_Ex8_revGTG TTG GAC AGT CAG AGT GMiph_Ex9_forCCC GCC TTT GCC TTA AGC416 bp56° CMiph_Ex9_revGCT GCA AGG AGG AGC TTCMiph_Ex10_revCAC CTG GGA CAG GGA AGCMiph_Ex11_forAGG CGT CCA GAG GAT GTG550 bp55° CMiph_Ex12_revTGG ACT TGA GAG GCT GTGMiph_Ex13_revGGG TTT GCC AGG GT GTG539 bp55° CMiph_Ex13_revGGG TTT GCC CAG GGC GAGMiph_Ex13_revGGG TTT GCC AGG GCT GAGMiph_Ex13_revGGG TTT GCC AGG GCT GAGMiph_Ex13_revGGG TTT GCC AGG GCT GAGMiph_Ex14_forCTC CTC TTA AGC CGG GAC ACA G55° CMiph_Ex15_revTGC ACA GGA GAC ACA G461 bp55° C	-		725 hp	56°C	
Miph_Ex5_forAGA AGT GAT GGA GGT CAC TG471 bp56°CMiph_Ex5_revACC GAA CAG GAA CAG GAGS82 bp53°CMiph_Ex6_forAG CTT GC TGG CTG GG ATG GAT GAT GAT GAT GAT GAT GAT GA			725 Up	50 C	
Mlph_Ex5_revACC GAA CAG GAA CAG GAGMlph_Ex6_forAG CTT GCC TGG ATG GAT G582 bp53°CMlph_Ex7_forGTC CTC AGC ACT TCT GAG568 bp53°CMlph_Ex7_revGTG AGA AGC TTC TGG ACCMlph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMlph_Ex9_revGTG TTG GAC AGT CAG AGT GAGMlph_Ex9_revGCT GCA AGG AGG AGG TTCMlph_Ex10_forCAG AGC CTG GGT CCT GAG600 bp56°CMlph_Ex11_forAGG CGT CCA GAG GGA AGCMlph_Ex12_revGCC TGG GAT CTT TGG AGA GGMlph_Ex12_revGCC TGG TT TG GAC AGG CGT GTG550 bp55°CMlph_Ex12_revGCC TGC TCT GA GAG AGG CGT GTGMlph_Ex12_revGGG TTA GAG CCT GTGT TGC623 bp55°CMlph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMlph_Ex13_revGGG TTT GCC AAG GCT GAGMlph_Ex14_revTTG TCA CAC GAG AGA CAC AG591 bp55°CMlph_Ex15_revTGG TCA CGG GAG CAC AGG461 bp55°C	-		471 ha	۲4°C	
Miph_Ex6_forAG CTT GCC TGG ATG GAT G582 bp53°CMiph_Ex6_revCTG CAG CCT CTG CCA ACMiph_Ex7_forGTC CTC AGC ACT TCT GAG568 bp53°CMiph_Ex7_forGTC CTC AGC ACT TC TG ACCMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex8_revGTG TTG GAC AGT CAG AGG T GTMiph_Ex9_revGCT GCA AGG AGG AGC TTCMiph_Ex9_revGCT GCA AGG AGG AGC TTCMiph_Ex9_revGCT GCA AGG AGG AGC CTG GAG CTG GAG GAG CTGMiph_Ex10_revCAC CTG GGA CAG GAG AGCMiph_Ex11_revMiph_Ex11_revGCC TGC TTC TGG AGA AGCMiph_Ex11_revGCC TGC TTC TGG AGA AGCS50 bp55°CMiph_Ex12_revTGG ACT GAG GCT GTG539 bp55°CMiph_Ex13_forCCT GTC TCC CCA GAG TTCGMiph_Ex13_revGGG TTT GC CAAG GCT GAGMiph_Ex13_revGGG TTT GC CAAG GCT GAGS91 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA GMiph_Ex15_revTGC TCT GGG GTC CTA AGGMiph_Ex15_rev	·		do i ve	30 C	
Miph_Ex6_revCTG CAG CCT CTG CCA ACMiph_Ex7_forGTC CTC AGC ACT TCT GAG568 bp53°CMiph_Ex7_revGTG AGA AGC TTC TGG ACC483 bp56°CMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex8_revGTG TTG GAC AGT CAG AGT G116 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTC116 bp56°CMiph_Ex10_revCAG AGC CTG GGT CCT GAG600 bp56°CMiph_Ex11_revCAG CGT CCA GAG GGA AGC116 bp55°CMiph_Ex11_revGCC TGC TTC TGG AGA AGC110 bp55°CMiph_Ex12_revGGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GAG539 bp55°CMiph_Ex13_forCCT GT CT CC CAAG GCT GAG110 bp55°CMiph_Ex13_revGGG TTT GC AAG GCT GAG591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G416 bp55°CMiph_Ex14_revTG TCA CAC GGA GAC ACA G591 bp55°CMiph_Ex15_forCTC CTT AG GCA GAC AAG461 bp55°C	. – –		502 ha	52°C	
Miph_Ex7_forGTC CTC AGC ACT TCT GAG568 bp53°CMiph_Ex7_revGTG AGA AGC TTC TGG ACC483 bp56°CMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex8_revGTG TTG GAC AGT CAG AGT G416 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTC416 bp56°CMiph_Ex10_revCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGC416 bp55°CMiph_Ex11_revGCC TGC TTC TGG AGA AGC550 bp55°CMiph_Ex12_forAGG CGT CCA AGG AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAG539 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G461 bp55°CMiph_Ex15_forCTC AGG CAG GCA GAC ACA G591 bp55°CMiph_Ex15_revTGC TCT GGG GT CTA ACG591 bp55°C	-		362 UP	33 C	
Miph_Ex7_revGTG AGA AGC TTC TGG ACCMiph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex8_revGTG TTG GAC AGT CAG AGT G416 bp56°CMiph_Ex9_forCCC GCC TTT GCC TTA AGC416 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTC416 bp56°CMiph_Ex10_forCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGC416 bp55°CMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTG416 bp55°CMiph_Ex13_forCCT GT TC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GC CAA GCT GAG416 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G461 bp55°CMiph_Ex15_forCTC AGG CAG GCA AGC AAG461 bp55°C	•		549 hr	52°C	
Miph_Ex8_forCAG CGG GAT TTC TGA AAG C483 bp56°CMiph_Ex8_revGTG TTG GAC AGT CAG AGT GMiph_Ex9_forCCC GCC TTT GCC TTA AGC416 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTCMiph_Ex10_forCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGCMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex11_revGCC TGC TTC TGG AGA AGCMiph_Ex12_revTGG ACT TGA GGC CGT GTGMiph_Ex13_forCCT GT TC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC CAA GGC T GAGMiph_Ex14_forCTC TTT AT GCT CTG GCA C591 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°C	. – –		200 ph	33 C	
Miph_Ex8_revGTG TTG GAC AGT CAG AGT GMiph_Ex9_forCCC GCC TTT GCC TTA AGC416 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTC16 bp56°CMiph_Ex10_forCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGC16 bp55°CMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex12_revGCC TGC TTC TGG AGA AGC16 bp55°CMiph_Ex12_revGCC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex13_revGGG TTT GCC CAG GAT TCG539 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G461 bp55°CMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°C	. – –		102 ha	۲4°C	
Miph_Ex9_forCCC GCC TTT GCC TTA AGC416 bp56°CMiph_Ex9_revGCT GCA AGG AGG AGC TTC116 bp56°CMiph_Ex10_forCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGC116 bp55°CMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex12_revGCC TGC TTC TGG AGA AGC116 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTG117 CCC CCA GAT TCG539 bp55°CMiph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAG110 bp55°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G461 bp55°CMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°C			403 DP	30 C	
Miph_Ex9_revGCT GCA AGG AGG AGC TTCMiph_Ex10_forCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGC600 bp55°CMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex12_revGCC TGC TTC TGG AGA AGC600 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTG75°CMiph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAG75°CMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA G461 bp55°CMiph_Ex15_revTGC TCT GGG GTC CTA ACG55°C	. – –		414 6-	F4°C	
Miph_Ex10_forCAG AGC CTG GCT CCT GAG600 bp56°CMiph_Ex10_revCAC CTG GGA CAG GGA AGCMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex11_revGCC TGC TTC TGG AGA AGCMiph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTGMiph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAGMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA GMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°C	. – –		410 DP	30 C	
Miph_Ex10_revCAC CTG GGA CAG GGA AGCMiph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex11_revGCC TGC TTC TGG AGA AGCMiph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTGMiph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAGMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA GMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMiph_Ex15_revTGC TCT GGG GTC CTA ACG			600 h-	F4°C	
Miph_Ex11_forAGG CGT CCA GAG GTT GTG550 bp55°CMiph_Ex11_revGCC TGC TTC TGG AGA AGCMiph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTGMiph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAGMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA GMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMiph_Ex15_revTGC TCT GGG GTC CTA ACG	·		600 bp	36 C	
MIph_Ex11_revGCC TGC TTC TGG AGA AGCMIph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMIph_Ex12_revTGG ACT TGA GGC CGT GTGMIph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMIph_Ex13_revGGG TTT GCC AAG GCT GAGMIph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMIph_Ex14_revTTG TCA CAC GGA GAC ACA GMIph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMIph_Ex15_revTGC TCT GGG GTC CTA ACG	·			FF°C	
Miph_Ex12_forCGC CGA CCA AGT CTT TGC623 bp55°CMiph_Ex12_revTGG ACT TGA GGC CGT GTGMiph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMiph_Ex13_revGGG TTT GCC AAG GCT GAGMiph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMiph_Ex14_revTTG TCA CAC GGA GAC ACA GMiph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMiph_Ex15_revTGC TCT GGG GTC CTA ACG	•		550 pp	55 C	
MIph_Ex12_revTGG ACT TGA GGC CGT GTGMIph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMIph_Ex13_revGGG TTT GCC AAG GCT GAG591 bp55°CMIph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMIph_Ex14_revTTG TCA CAC GGA GAC ACA G461 bp55°CMIph_Ex15_revTGC TCT GGG GTC CTA ACG55°C	·		(22 h-	FE°C	
MIph_Ex13_forCCT GTC TCC CCA GAT TCG539 bp55°CMIph_Ex13_revGGG TTT GCC AAG GCT GAGMIph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMIph_Ex14_revTTG TCA CAC GGA GAC ACA GMIph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMIph_Ex15_revTGC TCT GGG GTC CTA ACG	·		623 DP	55 C	
Mlph_Ex13_revGGG TTT GCC AAG GCT GAGMlph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMlph_Ex14_revTTG TCA CAC GGA GAC ACA GMlph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMlph_Ex15_revTGC TCT GGG GTC CTA ACG	. – –		5201	rr°c	
Mlph_Ex14_forCTC CTT TAT GCT CTG GCA C591 bp55°CMlph_Ex14_revTTG TCA CAC GGA GAC ACA GMlph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMlph_Ex15_revTGC TCT GGG GTC CTA ACG	·		234 pp	55 C	
Mlph_Ex14_revTTG TCA CAC GGA GAC ACA GMlph_Ex15_forCTC AGG CAG GCA GAC AAG461 bp55°CMlph_Ex15_revTGC TCT GGG GTC CTA ACG	•		501.1	rr°c	
Mlph_Ex15_for CTC AGG CAG GCA GAC AAG 461 bp 55°C Mlph_Ex15_rev TGC TCT GGG GTC CTA ACG	·		2AI pb	55°C	
Mlph_Ex15_rev TGC TCT GGG GTC CTA ACG	·			FF°0	
			461 bp	55°C	
			4401	5300	
	P_MLph_Ex16_for	GCT TCA GAG CCT GAA ATT CT	469 bp	53°C	
P_Mlph_Ex16_rev GAC AAA AGA TCA GGC TGG AG	r_mpn_Ex16_rev	GAC AAA AGA TCA GGC TGG AG			

Table 6: PCR Primers used for the MLPH cDNA amplification and/or genomic MLPH mutation analysis (Continued)

For sequencing the BAC insert plasmid subclones were produced using the TOPO Shotgun Cloning Kit (Invitrogen, Karlsruhe, Germany). Plasmid DNA was isolated with the Montage Plasmid Miniprep<sub>96</sub> Kit (Millipore, Eschborn, Germany). Sequencing was done on a Mega-BACE capillary sequencing machine (Amersham Biosciences, Freiburg, Germany) using the Dyenamic<sup>™</sup> Terminator Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany) or on a LI-COR 4200L-2 automated sequencer using the Thermo Sequenase Primer Cycle Sequencing Kit. Shotgun sequences were collected until eight-fold coverage of the BAC clone was achieved. The sequences were assembled with Sequencher 4.2 (GeneCodes, Ann Arbor, MI, USA). Whole genome shotgun sequences from a Boxer were retrieved from the trace archive to fill gaps in the BAC clone sequence as well as for assembling the 3'-end of the canine *MLPH* gene that was not contained on the BAC clone [19]. Our experimental genomic sequences were deposited under accession [EMBL:AJ920047] in the EMBL database. The entire 212.696 bp contig consisting of an assembly from our experimental sequence reads as well as public WGS reads was also deposited in the EMBL database [EMBL:BN000728]. The exon/intron boundaries were determined by comparative alignment of the canine genomic sequence versus a canine cDNA sequence using LALIGN [20] and BLAST [21]. GC content and CpG islands were calculated with CpG plot [22]. The protein translation and calculation of protein molecular weight and pI was done with DNASTAR software (GATC, Konstanz, Germany).

# Sequencing the MLPH cDNA

Fresh skin biopsies (4 or 6 mm diameter  $\sim$  30 to 60 mg) were either frozen in liquid nitrogen and stored at -80 °C or stored in RNAlater (Qiagen, Hilden, Germany) at -20°C. RNA could be isolated and yielded cDNA from both storage methods. RNA of skin was isolated using the Trizol™ reagent (Invitrogen, Karlsruhe, Germany) or the the Qiagen RNAeasy 96 Universal Tissue Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using oligo-dT and the SuperScript<sup>™</sup>III reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For the subsequent PCR 2-3 µl of the cDNA were used in 50 µl reactions containing 20 pmol of each PCR primer, 200 µM dNTPs and 2.5 units of Taq DNA polymerase (Qiagen, Hilden, Germany). The entire coding sequence with the exception of the last two codons of the MLPH cDNA was amplified as four overlapping fragments. Two rounds of semi-nested PCR had to be performed in order to generate enough cDNA for DNA sequencing. The primers and conditions for the RT-PCRs are given in Table 6. The RT-PCR products were purified from agarose gels using QiaExII (Qiagen, Hilden, Germany) and directly sequenced with the Dyenamic<sup>™</sup> Terminator Cycle Sequencing Kit and a MegaBACE capillary sequencer. The cDNA sequence of the canine MLPH gene was submitted to the EMBL nucleotide database [EMBL:AJ920333].

# Mutation analysis

DNA from approximately 350 dogs was available for various aspects of this study (140 Doberman Pinschers, 143 German Pinschers, 12 Large Munsterlanders, 6 Beagles, and ~50 dogs from other breeds or crossings). Doberman Pinscher and German Pinscher samples were collected from European and North American dogs. As the Doberman Pinschers showed some genetic differences with regard to their origin, these samples were divided into Doberman Pinschers of American origin (38 animals) and European origin (102 animals). The coat color of the dogs was recorded based on their pedigree certificates. In Pinschers there were four colors, black-and-tan, brown or red, blue, and Isabella fawn, respectively. Black-and-tan and brown or red were classified as wildtype colors, whereas blue and Isabella fawn were classified as dilute colors. Genomic DNA was isolated from blood using the QiaAmp 96 DNA Kit (Qiagen, Hilden, Germany) or the Nucleon BACC2 kit (Amersham Biosciences, Freiburg, Germany). Genomic DNAs of tissue samples from Doberman Pinschers were isolated using the Puregene Kit (Gentra, Minneapolis, MN, USA). All kits were used according to the manufacturers' instructions.

The exons of the canine MLPH were sequenced in 11 dog samples from Pinscher families with segregating coat color phenotypes. Six samples belonged to a Doberman Pinscher family (Fig. 4A). The other five samples belonged to a German Pinscher family (Fig. 4B, animals 8-12). Exons 2-15 were individually amplified with specific PCR primer pairs (Table 6). For the first exon two rounds of semi-nested PCR amplification were necessary to generate enough product for DNA sequencing. PCR was carried out in 20 µl reactions containing 10 ng genomic DNA according to the standard protocol advised by the manufacturer of the Taq DNA polymerase (GLtaq, Bremen, Germany). The subsequent sequencing of the PCR products was performed using the Dyenamic<sup>™</sup> Terminator Cycle Sequencing Kit. The products were analyzed on a MegaBACE capillary sequencing machine. A set of eight SNPs around exon 2 was genotyped by DNA sequencing in most available DNA samples and statistically evaluated. The R199H mutation was either genotyped by DNA sequencing of the exon 7 PCR product or by RFLP analysis of this PCR product with HhaI on 1.5% agarose gels. The commercial use of MLPH based genotyping for diagnostic purposes in dogs is protected by the international patent EP04106291.

# Statistical methods

A set of eight SNPs around exon 2 was analyzed in 350 dogs of several breeds using Haplotyper 1.0 This software for haplotype inference uses the Bayesian algorithm [23]. Allele frequencies were tested for significant associations with the dilute phenotype using Fisher's Exact Test.

# **Authors' contributions**

UP performed the molecular genetic analyses and drafted the manuscript. HH performed the statistical analyses. LM provided initial ideas for candidate genes as well as dog samples. SN and EM established an experimental Doberman cross with segregating coat colors and provided samples from this colony. ARGA established an experimental Beagle cross with segregating coat colors and provided samples from these animals. SMS provided the BHFD samples from an experimental cross of Large Munsterlanders, performed some confirmative genotyping, and contributed to the writing of the manuscript. TL conceived the study, participated in the MLPH gene characterization, and finalized the manuscript.

#### Acknowledgements

The authors would like to thank Heike Klippert-Hasberg, Diana Seinige, and Stefan Neander for expert technical assistance. The authors would also like to thank Beat Indermaur and Sabine Schindler for the pictures of Doberman Pinschers and blood samples. Finally the tremendous support of numerous veterinarians as well as dog breeders and owners who donated samples is gratefully acknowledged.

#### References

- Laukner A: Coat color in dogs. 2: Clinical significance. Tierärztl Prax Ausg K Kleintiere Heimtiere 1998, 26:124-128.
- Schmutz SM, Moker JS, Clark EG, Shewfelt R: Black hair follicular dysplasia, an autosomal recessive condition in dogs. Can Vet J 1998, 39:644-646.
- 3. Carlotti DN: Canine hereditary black hair follicular dysplasia and color mutant alopecia: Clinical and histopathological aspects. Adv Vet Dermatol 1990, 1:43-46.
- 4. Laffort-Dassot C, Beco L, Carlotti DN: Follicular dysplasia in five Weimaraners. Vet Dermatol 2002, 13:253-260.
- Mercer JA, Seperack PK, Strobel MC, Copeland NG, Jenkins NA: Novel myosins heavy chain encoded by murine dilute coat colour locus. *Nature* 1991, 349:709-713.
- Wilson SM, Yip R, Swing DA, O'Sullivan TN, Zhang Y, Novak EK, Swank RT, Russel LB, Copeland NG, Jenkins NA: A mutation in Rab27A causes the vesicle transport defects observed in ashen mice. Proc Natl Acad Sci, USA 2000, 57:7933-7938.
- Matesic LE, Yip R, Reuss AE, Swing DA, O'Sullivan TN, Fletcher CF, Copeland NG, Jenkins NA: Mutations in Mlph, encoding a member of the Rab effector family, cause the melanosome transport defects observed in leaden mice. Proc Natl Acad Sci USA 2001, 98:10238-10243.
- Pastural E, Barrat FJ, Dufourq-Lagelouse R, Gertain S, Sanal O, Seger R, Griscelli C, Fischer A, de Saint Basile G: Griscelli disease maps to chromosome 15q21 and is associated with mutations in the myosin-VA gene. Nat Genet 1997, 16:289-292.
- Menasche G, Pastural E, Feldmann J, Gertain S, Ersoy F, Dupuis S, Wulfrat N, Bianchi D, Le Deist F, de Saint Basile G: Mutations in Rab 27A cause Griscelli Syndrome associated with hemaphagocytic syndrome. Nature Genet 2000, 25:173-176.
- Menasche G, Ho CH, Ozden S, Feldmann J, Tezcan I, Ersoy F, Houdusse A, Fischer A, de Saint Basile G: Griscelli syndrome restricted to hypopigmentation results from melanophilin defect (GS3) or a Myo5A F-exon deletion (GS1). J Clin Invest 2003, 112:450-456.
- Philipp U, Quignon P, Scott A, André C, Breen M, Leeb T: Chromosomal assignment of the canine melanophilin gene (MLPH): A candidate gene for coat color dilution in Pinschers. J Hered 2005: in press.
- Breen M, Jouquand S, Renier C, Mellersh CS, Hitte C, Holmes NG, Cheron A, Suter N, Vignaux F, Bristow AE, Priat C, McCann E, André C, Boundy S, Gitsham P, Thomas R, Bridge WL, Spriggs HF, Ryder EJ, Curson A, Sampson J, Ostrander EA, Binns MM, Galibert F: Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid /linkage map of the domestic dog genome to all chromosmes. *Genome Res* 2001, 11:1784-1795.
- Schmutz SM, Berryere TG, Goldfinch AD: TYRP1 and MC1r genotypes and their effects on coat color in dogs. *Mamm Genome* 2002, 13:380-387.
- Duan J, Wainwright MS, Comeron JM, Saitou N, Sanders AR, Gelernter J, Gejman PV: Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. Hum Mol Genet 2003, 12:205-216.
- O'Sullivan TN, Wu XS, Rachel RA, Huang JD, Swing DA, Matesic LE, Hammer JA 3rd, Copeland NG, Jenkins NA: dsu functions in a MYO5A-independent pathway to suppress the coat color of dilute mice. Proc Natl Acad Sci USA 2004, 101:16831-16836.
- Li R, Mignot E, Faraco J, Kadotani H, Cantanese J, Zhao B, Lin X, Hinton L, Ostrander EA, Patterson EF, de Jong PJ: Construction and characterization of an eightfold redundant dog genome bacterial artificial chromosome library. *Genomics* 1999, 58:9-19.
- 17. German Resource Center/Primary Database [http:// www.rzpd.de]
- 18. BACBAC Resources [http://www.chori.org/bacpac/]

- NCBI trace archive [<u>http://www.ncbi.nlm.nih.gov/Traces/</u> trace.cgi?]
- 20. LAlign [http://www.ch.embnet.org/software/LALIGN\_form.html]
- 21. NCBI BLAST home page [http://www.ncbi.nlm.nih.gov/blast/]
- 22. CpG plot [http://www.ebi.ac.uk/emboss/cpgplot/]
- Niu T, Oin, ZS Xu X, Liu JS: Bayesian haplotype inference for multiple linked single-nucleotide polymorphisms. Am J Hum Genet 2002, 70:157-169.
- 24. Kuroda TS, Ariga H, Fukuda M: The actin-binding domain of Slac2-a/melanophilin is required for melanosome distribution in melanocytes. *Mol Cell Biol* 2003, 15:5245-5255.

