THE PREVALENCE OF LEUCOCYTOZOON TODDI IN BIRD BLOOD SAMPLES IN ARAS-IĞDIR AND EVALUATION OF ITS PHYLOGENETIC RELATIONSHIPS

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THE PREVALENCE OF LEUCOCYTOZOON TODDI IN BIRD BLOOD SAMPLES IN ARAS-IĞDIR AND EVALUATION OF ITS PHYLOGENETIC RELATIONSHIPS

Today biological diversity is faced with high risks of extinction due to the overuse of natural resources. Studies of bird species constitutes a central theme in ecological investigations and for conservation of biological diversity. The identification of parasitic infections encountered in birds provide contributions to ecological studies with regards to the persistence of species. This aim of this study is the detection of the prevalance of *Leucocytozoon toddi* infection in birds of Aras-Iğdır region, using genetic methods. 401 blood samples belonging to 58 bird species of 25 different families were investigated. *L. toddi* infection was detected in 41 samples and five distinct haplotypes were obtained from six sequences. Phylogenetic trees were constructed using these five haplotypes along with 265 sequences of 76 species taken from GenBank and MalAvi databases. Four out of five haplotypes of Aras-Iğdır positive samples were distinct from those in literature. Again four of the five Aras-Iğdır haplotypes clustered very closely together, potentially suggesting some genetic isolation in this migratory pathway. The phylogenetic comparisons made using all sequences also support the idea of the presence of two cryptic species of *L. toddi*.

ARAS-IĞDIR' DAKİ KUŞ KANI ÖRNEKLERİNDE LEUCOCYTOZOON TODDI' NİN VARLIĞI VE FİLOGENETİK İLİŞKİLERİN DEĞERLENDİRİLMESİ

Doğal kaynakların aşırı kullanılması nedeni ile biyolojik çeşitlilik yok olma tehlikesi ile karşı karşıya kalmıştır. Kuş türlerinin korunması ekolojik çalışmalarda önemli bir yer tutmaktadır. Kuşlarda rastlanan parazit enfeksiyonlarının tespit edilmesi türlerin devamlılığı açısından ekolojik çalışmalara katkı sağlamaktadır. Bu çalışmanın amacı Aras-Iğdır bölgesine ait kuşlardaki *Leucocytozoon toddi* enfeksiyonunun varlığının genetik metodlar kullanılarak tespit edilmesidir. 25 farklı familyadan 58 kuş türüne ait 401 kan örneği incelenmiştir. 41 örnekte *L. toddi* enfeksiyonu saptanmış ve altı diziden beş farklı haplotip elde edilmiştir. Beş haplotip ile birlikte GenBank ve MalAvi veri tabanlarından alınan 76 türe ait 265 dizi kullanılarak filogenetik ağaçlar oluşturulmuştur. Aras-Iğdır bölgesinden elde edilen beş haplotipten dördü literatürde bulunanlardan farklıdır. Beş Aras-Iğdır haplotipinden dördü birbirine yakın konumlanmıştır, ki bu durum göç yolu üzerindeki olası genetik izolasyonu işaret etmektedir. Tüm diziler kullanılarak yapılan filogenetik karşılaştırmalar *L. toddi* içerisinde iki gizli türün varlığını desteklemektedir.

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Symbol	**Explanation**
asl	Adenylosuccinate Lyase
bp	Base Pair
clpc	Caseinolytic Protease
coI	Cytochrome Oxidase I
cyt *b*	Cytochrome *b*
DNA	Deoxyribonucleic Acid
DNTP	Deoxyribonucleotide Triphosphate
EBA	Endemic Bird Area
EDTA	Ethylenediaminetetraacetic Acid
Hap	Haplotype
IBA	Important Bird Area
IUCN	International Union for Conservation of Nature
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
TBE	Tris Base Boric Acid, EDTA
WHO	World Health Organisation

1. INTRODUCTION

The area of investigation of organismal relationships as well as their interaction with the environment is a fundamental concern of ecology. Since the studies of ecology contribute to an understanding of the fundamentals of biological diversity, they gain more attention as the diversity of species encounters an accelerating risk of extinction (Convention on Biological Diversity, 2010). The need to understand the causes of species extinction gives rise to the research of environmental correlates. Geographical regions, climate, and temperature are pivotal factors in the persistence of species, and along with organismal relationships determine the survival of species (Svenning and Condit, 2008).

Biological diversity is recognized to be comprised of three parameters: genetic diversity, species diversity and ecosystem diversity (Gaston and Spicer, 1998). Highly diversified groups of species inhabit discrete habitats which in total constitute complicated and interrelated structure of the biosphere. Because of its interrelated nature, a change in some parts of this system affects other elements both directly and indirectly. In a direct way, extinction of a species, for instance, may increase the survival rate of another species when there is a competition between them or decrease the survival chance of a dependent species in the case of a symbiotic or commensalistic relationship (Dobson et al., 2008).

Biodiversity is a forefront issue in ecological research especially due to its accelerating rate of loss (Pimm et al., 1995). Climate change, habitat destruction, deforestation, and urbanization are the main factors that result in the loss of biodiversity (Pounds and Puschendorf, 2004; Wilson, 1986). Most of these issues have something in common: being the negative outcome of human impression. Humans are degrading habitats, consuming natural resources for industrialization practices, undertaking uncontrolled city growth and road construction, logging, and maintaining agricultural and mining activities. Highly irreversible damages as a result of these activities have led to dire consequences for ecosystems (Vitousek et al., 1997).

Alterations of ecosystems, associated with the human activity,do not only cause declines in biodiversity, but also have direct impact on the spread of infectious pathogens. Correlation between reduced biodiversity with the increase of pathogen transmission have been analysed in various investigations (Keesing et al. 2010; Carlson et al., 2009). Keesing et al. (2010) revealed the effects of the loss of biodiversity on the transmission of infectious diseases through changes in the abundance or the condition of host/vector or changes in the behaviour of host, vector/parasite and they claimed that when extinct species is not responsible for the transmission of pathogens, its extinction increases the density of host species through which the possibility of infection increases. Birds, in particular, are highly affected from environmental changes irrevocably. Migratory birds, for instance, may change migratory routes due to habitat destruction resulting in the transmission of infectious pathogens to new and novel host species (Sehgal, 2010).

1.1. General Characteristics of Birds

Birds (Class: Aves), provide a wide range of ecological functions and can be considered as building blocks of biodiversity. Their ecological value both in human-dominated and pristine regions have been assessed in various studies. Birds are highly diversified especially in the tropical regions and their variability is an indication of ecosystem healthiness (Maurer, 1993). Birds serve as a complementary part for many ecological processes, such as contributing to the growth of plants through the transportation of seeds away from their parent trees thereby providing pollination (Wenny and Levey, 1998). Pest control is another natural service provided by birds, which is critical for health of plants and agriculture. Predation on vertebrates by raptors control rodent pests (Brown et al., 1988). Scavengers are the group of organisms responsible for the decomposition of carcasses (Villegas-Patraca et al., 2012). Hence extinctions of bird species may result in the failure of some ecological processes, especially unless an equivalent species replaces it (May, 1974). Studies on birds contribute not only to understanding biological diversity in general, but also help determine priorities for the future conservations of birds.

Birds are found in all types of major habitats on Earth (Newton, 2003). While some bird species are adapted to several habitats, most are only capable of living in a single one.

Forests are the most valuable habitat types for birds, where 75% of all bird species are found (BirdLife International, 2012). Artificial landscapes, shrublands, and grasslands supporting high richness of species are other significant habitats for birds (BirdLife International, 2012).

According to BirdLife International, 10,064 of bird species are recognized so far. Species are constantly evaluated regarding their conservation status in the IUCN Red List Categories (2012.1) and bird species are no exception. Currently 130 species are classified as Extinct, four as Extinct in the Wild, and 1313 are Threatened. To describe in greater detail, Threatened species are subclassified as Critically Endangered (197 species), Endangered (389 species), and Vulnerable (727 species). Furthermore, 880 species are classified as Near Threatened and 60 species are classified as Data Deficient. Among Class Aves, Passeriformes, comprising the largest order in the class, is the most negatively affected order with 42 species gone extinct, and 612 species in the Threatened category.

1.2. Turkish Bird Fauna

Turkey, situated at the intersection of Asia and Europe, is a major bird migration region for being at the crossroads of flyways. Birds from Eastern Europe fly through Bosphorus in Istanbul, therefore Turkey serves as a bridge between two continents for migratory birds whereas birds from Caucasus pass through Eastern and Southern Anatolia. Turkey is almost completely covered with parts of three biodiversity hotspots, the Caucasus, Irano-Anatolian, and Mediterranean (Conservation International, 2005; Şekercioğlu et al. 2011). Annual variation of temperature and rainfall across the country constitutes highly diversified ecosystems composed of agricultural land, forests of coniferous trees and broad-leaved trees, mountains, steppes, wetlands and marine systems, which makes it possible for Turkey to host a broad range species in its different biogeographic regions (The National Biological Diversity Strategy and Action Plan, 2007).

The Turkish avifauna includes 468 species with 331 being migratory (Şekercioğlu et al., 2011). The total avifauna does not include any endemic species. In Turkey, three species are classified as Critically Endangered, three as Endangered, eight as Vulnerable, and 17 as Near Threatened (IUCN, 2011). Decline of bird populations mainly started

around 1960s (The National Biological Diversity Strategy and Action Plan, 2007), with the drainage of wetlands (BirdLife International, 2004), and since then agricultural practices threatened bird species. To illustrate, due to the reduction in the amount of water in Lake Burdur, the wintering population of white-headed duck (*Oxyura leucocephala*) is in decline as the lake is its most significant wintering area (Erciyas and Kartal, 2012).

There are also examples of globally threatened species, which are found in Turkey. For instance, the endangered Egyptian vulture (*Neophron percnopterus*) and vulnerable lesser kestrel (*Falco naumanni*) were observed in the cities of Ankara and Izmir, respectively (Balkız et al., 2008). As bird populations suffer from extinction risks, Turkey hosts multiple species which are declining in Europe and Middle East. Brown fish-owl (*Ketupa zeylonensis*), accepted as the most uncommon bird at the European level, was thought to be extinct in the continent, but was observed in Taurus Mountains in Turkey (Doğa Derneği, 2010). The Dalmatian pelican (*Pelecanus crispus*) as another globally threatened bird is observed in wetlands of Turkey and is listed as Endangered at the country level (The National Biological Diversity Strategy and Action Plan, 2007).

Aras-Iğdır, the collection region of this study's blood samples, is an area located in north-eastern Turkey and acts as a transition region on the migratory pathway between Anatolia and Caucasus. The region's habitat consists of wetlands, steppe, agricultural area and the vegetation can be defined as sparse (Kılıç and Eken, 2004). The region is characterized by a rich avian species diversity, with 313 bird species having been recorded in Kars-Iğdır so far. Lake Kuyucuk in Kars is one of the 13 Ramsar sites in Turkey (The Ramsar Convention, 2009). The enlargement of agricultural areas is the main threat to the region's biodiversity (Kılıç and Eken, 2004).

1.3. Conservation of Birds

The attempts to maintain effective conservation plans to protect birds have first of all resulted in the estimation of number of birds under the risk of extinction. Objectives of the identification and conservation of sites criticial for birds led BirdLife International to recognise approximately 11.000 Important Bird Areas (IBAs) worldwide. In Turkey, 177 IBAs have been designated.

Some bird species occur in different areas, however most species are restricted to specific regions, sometimes being endemic. Those regions are identified as Endemic Bird Areas (EBAs) by BirdLife International. 356 EBAs were identified in the world. Turkey is in one EBA (Caucasus), with Armenia, Azerbaijan, Georgia, Iran, and Russia. Three north-eastern IBAs in Turkey are included in the Caucasus EBA.

1.4. General Characteristics of Bird Haemosporidians

The need of understanding the factors that precipitate the emergence and spread of infectious diseases constitute one of the complicated issues in ecological research. Most infectious diseases are thought to have originated in Africa, but the knowledge about the spread of pathogens across host species and geographical regions has not been fully studied yet. Since today climatic changes and species extinction risks are occuring in an accelerating rate, the nature of pathogenic diseases need to be studied in detail. Interactions between biotic and abiotic factors are responsible in the transmission of infectious diseases (Loiseau et al., 2010). Abiotic factors such as climatic and habitat alterations affect diversity and abundance of vectors and hosts, hence modifying parasite prevalence and virulence (Loiseau et al., 2010). To what extent pathogenic diseases will be affected from external changes is difficult to determine, as each type of pathogen behaves in a novel way. Research based on infectious diseases might help to assess the risk of infection of birds at large geographic scales since birds live in all major types of habitats (BirdLife International, 2012).

Despite the fact that the exact origin of bird haemosporidians is not known, they have most probably originated from haemosporidians of reptiles. The similarity of development type of haemosporidians between reptiles and birds and the use of relatively ancient groups of blood-sucking dipteran insects as vectors highlight the origin. Some reptilian haemosporidians share common features with leucocytozoids supporting the view that they are ancestors of *Leucocytozoon* spp., as both do not produce malarial pigment during their development and can develop in leukocytes (Valkiunas, 2005).

Avian blood parasites are a special group as their abundance and distribution are highly affected by climatic changes and geographical conditions (Zamora-Vilchis et al., 2012). These parasites are widely investigated in genetic studies especially owing to their function of revealing examples of host-parasite relations. Most of the research on avian blood parasites are based on the species in the genus *Plasmodium*. The main reason of this scientific interest is that among haemosporidians,only the species of *Plasmodium*, in particular *P. falciparum*, gives rise to malaria in humans (WHO, 2012). Malaria is transmitted to humans *via Anopheles* mosquitoes and causes illnesses which may result in death (Martinsen et al., 2007). Malaria infections and related deaths most commonly take place in Africa (WHO, 2012).

Leucocytozoids are taxonomically classified under the order Haemosporida. Order Haemosporida is divided into four families including Haemoproteidae, Plasmodiidae, Garniidae, and Leucocytozoidae. Haemoproteidae includes one genus, *Haemoproteus*, which is composed of two subgenera. Plasmodiidae contains one genus, *Plasmodium*, with five subgenera. Garniidae is composed of one genus, *Fallisia*, with one subgenus. Leucocytozoidae includes one genus, *Leucocytozoon*, with two subgenera. Among all three other families listed above, Leucocytozoidae is recognized to be genetically closer to Garniidae (Valkiunas, 2005).

The fauna of bird haemosporidians consists of 206 species, with 35 of them belonging to the family Leucocytozoidae (Valkiunas, 2005). The vectors of avian haemosporidian parasites are blood-sucking dipteran insects (Valkiunas, 2005). *Plasmodium* spp. are transmitted by Culicidae mosquitoes,whereas *Haemoproteus* spp. are transmitted by biting midges of Ceratopogonidae and louse flies of Hippoboscidae (Valkiunas, 2005).

Avian haemosporidian parasites are obligately heteroxenous and are vector-borne parasites infecting both domestic and wild birds. Haemosporidian parasites generally exist in birds for a lifetime with relapses during the energy demanding processes such as migration and breeding periods of hosts (Valkiunas, 2005). Birds behave as both reservoirs and vectors of infectious pathogens. Infection with haemosporidians may result in death, however the pathogenicity of the infections mainly depend on the response of the hosts (Szymanski and Lovette, 2005). Two kinds of reproduction accomplished in the

development of haemosporidians are sexual reproduction in vectors and asexual reproduction in vertebrate hosts (birds) (Valkiunas, 2005).

1.5. Life Cycle of Leucocytozoidae Species

Leucocytozoon spp., discovered by Danilewsky (1884), are intracellular blood parasites and their vectors are blood-sucking simuliid blackflies (Diptera: Simuliidae). As an exception, *L.caulleryi* is transmitted by biting midges of the order Ceratopogonidae (Valkiunas, 2005). Leucocytozoids have been recorded in all continents except Antarctica (Valkiunas, 2010). *Leucocytozoon* spp. infect both red and white blood cells and other organs within the vertebrate hosts (Hellgren et al., 2004). *Leucocytozoon toddi* was discovered by Sambon (1908). Among leucocytozoids, *L. toddi* is the sole species that infects birds of the order Falconiformes (Valkiunas, 2005). The life cycle of *L. toddi* has not been reported in detail. The following life cycle belongs to the genera as a whole. Infection of birds occurs through the inoculation of sporozoites by simuliid flies. Sporozoites are inoculated to avian hosts through salivary gland secretions during feeding. Sporozoites cause the development of hepatic meronts in the parenchymal cells of the liver (hepatocytes). During the growth of meronts, uninuclear merozoits are created by divisions of the nucleus, due to formation of invaginations from the extended cytoplasm. After being released to the blood, meronts penetrate into erythrocytes and cause the development of gametocytes. Apart from hepatic meronts, syncytia and cytoplasmic fragments spread into many organs and cause the formation of megalomeronts or megaloschizonts. Megalomeronts contain merozoites which cause the formation of gametocytes in the lymphocytes. The forms of the gametocytes of *Leucocytozoon* are roundish or oval, while host cells are of roundish and fusiform. In *L. toddi*, gametocytes in fusiform host cells are more common, and gametocytes in roundish host cells are rarely detected (Valkiunas, 2005).

Microgametocytes release from erithrocytes when the blood is exposed to air. During exflagellation, chromatin divides and some part of it penetrates into microgamete. After fertilization, zygote transforms into a motile ookinete. Ookinetes transform into oocysts after moving toward the layer of epithelial cells of midgut. Sporozoites formed in oocysts are released and they get into the haemocoele and penetrate into the salivary glands.

Pigment granules (hemozoin) are not included in the development of Leucocytozoidae because they digest hemoglobin in red blood cells, and the opposite is observed at the developmental stages of Haemosporidae and Plasmodiidae (Valkiunas, 2005). Hemozoin pigment is the product of digestion of hemoglobin molecules within the infected blood cell, formed by crystallization of the porphyrin (Martinsen et al., 2008).

1.6. Host-Switching

The discovery of Leucocytozoidae dates back a century, however taxonomy and host-parasite specificity are still problems not only related to this family, but also to other avian haemosporidians. The knowledge of associations among avian hamosporidian parasites and their hosts reveal the issues of host-specificity and host-switching, thereby contributing to the clearance of evolutionary history and host-parasite relations and to the tracking of emerging diseases (Krizanauskiene et al., 2006; Ricklefs and Fallon, 2002). The traditional view regarding the host-parasite relations used to emphasize that there was a natural host range for avian haemosporidian parasites and taxonomic characters were generally managed according to the morphology of blood stages using microscopic examinations, life-history traits and host taxa (Sehgal et al., 2006; Krizanauskiene et al., 2006; Njabo et al., 2010; Martinsen et al., 2008). Recent PCR-based methods have made this view controversial as host-switching has been determined to occurin avian blood parasites (Ricklefs and Fallon, 2002; Bensch et al., 2000). When more than one host species harbour parasite lineages sharing identical cyt *b* sequences, the phenomenon is referred to as host-switching (Ricklefs and Fallon, 2002). In contrast, the occurence of a parasite species in only one host species indicates they share a long evolutionary history (Hoberg et al., 1997). Among avian haematozoa, *Leucocytozoon* spp. are thought to be most host-specific and *Plasmodium* is less host-specific than *Haemoproteus*. When a parasite invades a new host species, the initial virulence is generally much higher, after several generations the host species is adapted to the parasite. The reason of reduction in the virulence is the selection on the immune system of host (Bensch et al., 2000). Because host-switching and specificity patterns are closely investigated with detection and identification analyses, these issues are scrutinized in greater detail in Literature Review section, below.

1.7. Objective of the Thesis

The aim of this study is to determine the prevalence of the avian blood parasite, *Leucocytozoon toddi* in blood samples of bird individuals in Aras-Iğdır region and use a phylogenetic approach to assess patterns of host specialization and determine if host-switching occurs by PCR-based methods and sequencing. The research area of avian species, Aras-Iğdır, is located in a transition zone for migratory birds. By evaluating host-switching patterns of *L. toddi*, a better understanding on the parasitic infections of the birds of the region, can also be developed.

2. LITERATURE REVIEW

Historically, the detection of leucocytozoids were based upon microscopic examination of blood films. Since PCR-based methods were developed, both microscopy and PCR methods have been used in the detection and identification of *Leucocytozoon* spp. infection. Prevalence of *Leucocytozoon* spp. in PCR-based diagnostics is generally quite greater than microscopic detection, although the difference is acceptable. Reasons of the differences include microscopic examination not being able to detect infections with light parasitemia and PCR methods amplifying DNA of sporozoites (Waldenström et al., 2004; Valkiunas et al., 2009; Garamszegi, 2010). As long as sensitivity increases in molecular methods, the amplification of "nonspecific" genes appear as a problem (Szöllosi et al., 2008). To overcome this problem, sequencing helps to reveal whether the target gene is amplified or not.

The first survey of the detection and identification of avian haemosporidians with PCR, targeting the amplification of mitochondrial DNA was made by Bensch et al. (2000). Phylogenetic reconstructions made after the amplification of mt- cyt *b* gene of *Plasmodium* and *Haemoproteus* from 12 passerine species revealed poorly matched trees between parasites and their hosts indicating that host shifts had occurred during evolutionary history. In another study by Perkins and Schall (2002) phylogenetic reconstruction of haemosporidian parasites from mammals, birds, and reptiles was made in which *Leucocytozoon* was included as an out-group. *Plasmodium* and *Hepatocystis* parasites in mammals clustered together and *Plasmodium* and *Haemoproteus* parasites of birds and lizards were included in another clade in which lizard parasites formed separate clusters. Waldenström et al. (2002) investigated host shifts of *Plasmodium* and *Haemoproteus* between African resident and European migratory songbirds in North-eastern Nigeria. In this study, detection of certain haemosporidian lineages in multiple host species provided evidence for host-sharing. Furthermore, infection of species in different host families with the same *Plasmodium* lineages revealed that parasites infect a wide range of hosts.

The nested PCR method was first applied to avian haemosporidians by Waldenström et al. (2004). The comparison of old techniques with nested PCR revealed that nested PCR provided a more precise and sensitive detection of haemosporidians. The first general PCR-based protocol for the detection of *Leucocytozoon* spp. was established by Hellgren et al. (2004). *Leucocytozoon* was separated from *Haemoproteus* and *Plasmodium* simultaneously with the combination of nested PCR with a second PCR step. In the latter two studies, the repeatibility of the methods was tested and detected to be high, indicating the reliability of the nested PCR. Another study by Cosgrove et al. (2006) examined 89 blood samples of breeding blue tits. In this study, the significance of bidirectional sequencing was revealed. Sequencing of 57 out of 89 samples did not have clear chromatograms with the forward primer, whereas reverse primer produced good quality sequences.

The sensitivity of microscopy and PCR-based methods in the estimation of parasite infections of host individuals were compared in recent investigations. In a comparative analysis by Garamszegi (2010), data were collected from published papers for the investigation of detection discrepancies of avian blood parasites *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. For the prevalence of *Plasmodium* spp., PCR methods provided higher prevalence rates than microscopic examinations, whereas for *Haemoproteus* spp. and *Leucocytozoon* spp. there was a positive correlation between screening methods. The underestimation by the microscopic methods in most papers had been explained by Valkiunas et al. (2008) as the probable improper application of the method and suggested that when proper application is established, similar prevalence rates between visual and molecular screening methods could be achieved. In contrast to studies supporting PCR method to give more accurate prevalence estimation than microscopy, in the study by Valkiunas et al. (2008), the prevalence of haemosporidian infections among 472 birds of 11 species were tested and prevalence rate was 54.2% with PCR and 53.6% with microscopy, whereas the combined result was 60%, indicating that both methods underestimated the prevalence.

The molecular analyses of bird haemosporidians are mostly based on single-gene phylogenies. A study by Martinsen et al. (2008) involved sequence data of *Plasmodium*, *Haemoproteus* and *Hepatocystis* from four genes of cytochrome *b*, cytochrome oxidase I,

adenylosuccinate lyase, and caseinolytic protease (cyt*b*, coI, asl, clpc). This multi-gene phylogeny of parasites from mammals, lizards, and birds revealed the relationships of these three genera with *Leucocytozoon* used as an outgroup taxa. *Haemoproteus* spp. diversified into two clades, one being a sister group to *Plasmodium* and *Hepatocystis*, and other clade being sister to all ingroup taxa. *Plasmodium* diversified into two major clades, one of them representing mammalian parasites and the other clade including both parasites of lizards and birds which did not diversify into distinct clades. In addition, *Leucocytozoon* spp. was found to be distantly related to other genera.

In another study, *Leucocytozoon* spp. prevalance in blue and great tits were estimated in nine locations in Europe (Jenkins and Owens, 2011). The overall prevalence of *Leucocytozoon* spp. across Europe was 24% in blue tits and 27% in great tits. Among the fourteen lineages of *Leucocytozoon* spp. found, three of them were found in both host species. Parasite lineages grouped into two major clades in which *L. toddi* lineage were in the same clade with *L. mathisi* and *L. buteonis* (Jenkins and Owens, 2011).

In another study, the effects of deforestation on the prevalence of avian haemosporidians in yellow-whiskered greenbul and the olive sunbird was studied in Southern Cameroon. The prevalence of *Leucocytozoon* spp. and *Haemoproteus* spp. was higher in undisturbed habitats than in disturbed habitats. The olive sunbird was found to host higher prevalence of haemosporidians than the yellow-whiskered greenbul. In olive sunbirds, effects of season, year, and site were observed in *Leucocytozoon* spp., whereas in *Haemoproteus* spp. and *Plasmodium* spp. infections, no differences were observed in terms of these effects. In greenbuls, season effect was observed on both *Plasmodium* spp. and *Haemoproteus* spp. (Chasar et al., 2009).

In a study by Silva-Iturriza et al. (2012), the determination of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* in *Hypsipetes philippinus,* an endemic bird to the Philippine Archipelago, provided information about an insular host-parasite system. Of all individuals, 48% had *Haemoproteus* infections and 2% were infected with either *Leucocytozoon* or *Plasmodium*. The comparison of levels of genetic divergences revealed that two levels of the variation of the host occurred, between subspecies (*H. philippinus guimarasensis* and *mindorensis*) and within species (*H. philippinus guimarasensis*),

whereas for *Haemoproteus* most of the variation occurred within populations, which is an indication of gene flow.

The prevalence of haematozoa in wild birds of a mountain forest of Japan over a three-year period was reported by Imura et al. (2012). Among 415 wild birds investigated, 13.5% were infected with *Leucocytozoon*, and 1.4% were infected with either *Plasmodium* or *Haemoproteus*. The reason for the low prevalence of *Plasmodium* and *Haemoproteus* was suggested as a result of the low level or absence of vectors at the region. Seven lineages of *Leucocytozoon* were found to infect different bird species. Three individual hosts were reported to have identical lineages whereas two *Leucocytozoon* lineages were found in all the other three host individuals. Of the 26 recaptured birds, six of them were found to be infected in each capture.

Cryptic speciation have also been analyzed in various studies (Bensch et al., 2004; Sehgal et al., 2006). Bensch et al. (2004) analysed bird species and compared the nuclear dihydrofolate reductase-thymidylate synthase (DHFR-TS) and mitochondrial DNA sequences of *Haemoproteus payevskyi* and *H. belopolskyi* which were determined to include similar cyt *b* parasite lineages. Mt cyt *b* lineages of parasites were associated with different sequences at the DHFR-TS locus, and it was suggested that they could represent distinct biological species. Sehgal et al. (2006) investigated cryptic speciation of *Leucocytozoon* spp. in diurnal raptors. Of the total 610 bird individuals of *Buteo* spp., *Accipiter* spp. and *Circus* spp. from California, Kazakhstan and Baltics, 189 were infected and the species of parasite was determined to be *L. toddi* in microscopic examinations. Parasite lineages of *Accipiter* spp. were grouped in a distinct clade with 10.9% sequence divergence from the clade of parasite lineages of *Buteo* spp. Even lineages from same locations, for example parasite lineages of *Accipiter cooperii* and *Buteo jamaicensis* from California, were found in different clades, suggesting that *L. toddi* might comprise a group of cryptic species.

3. MATERIALS AND METHODS

3.1. Collection of Blood Samples

Blood samples were collected in Aras-Iğdır which is located in north-eastern Turkey. Coordinates of the study site are 39º24'15"N and 45º21'55" E (Figure 3.1).

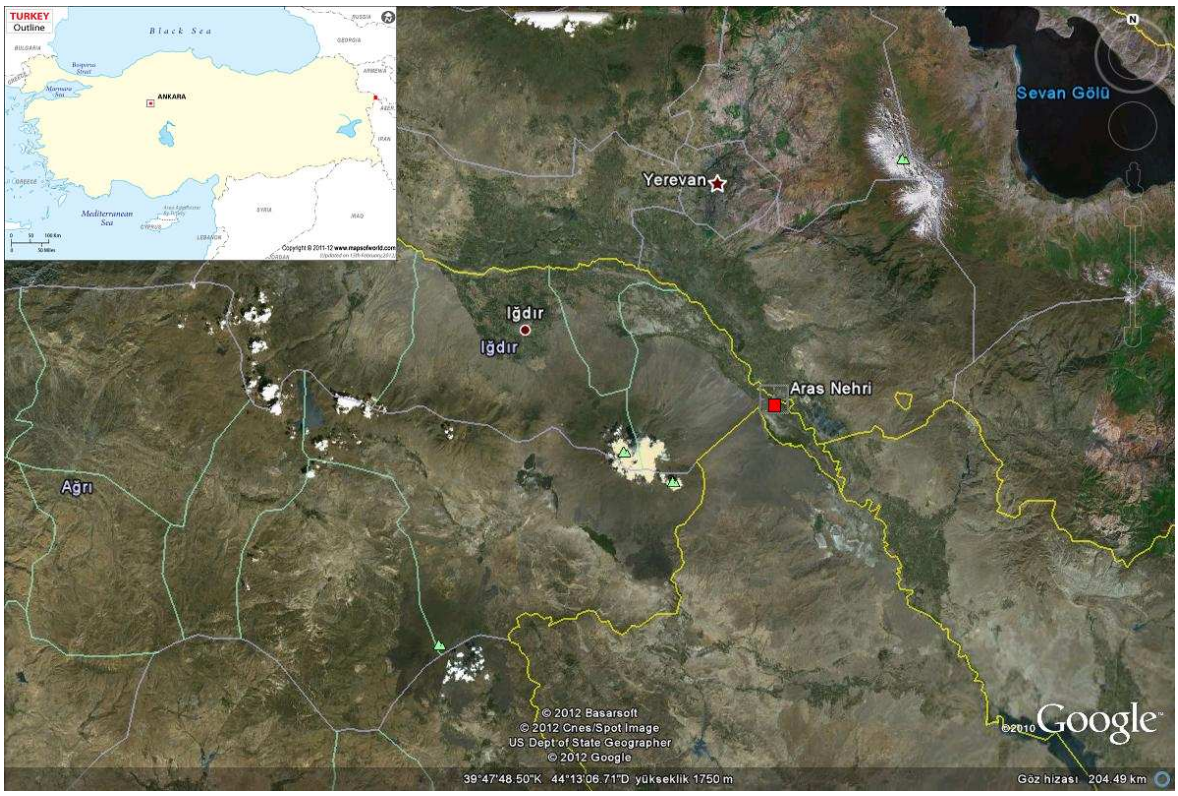


Figure 3.1. Map of Aras-Iğdır region in the north-eastern of Turkey where avian blood samples of this study were collected. The study area is indicated with the red square.

Samples were collected in May, June, August, September, and October of 2009. Collection of blood samples was performed by members of KuzeyDoğa Society. Birds were captured with very thin nets, and brachial vein of birds was punctured using a needle and with the aid of a suction device, blood was first taken into 50 microliter glass tubes and was then transferred to 1.5 ml. Eppendorf tubes containing Longmire buffer. Blood samples were

stored at -20°C until further processing. Avian families that were sampled belonged to the orders, Passeriformes, Coraciiformes, Caprimulgiformes, Galliformes, Cuculiformes, Piciformes, Ciconiiformes, and Columbiformes.

3.2. DNA Extraction

To obtain total DNA, blood samples were extracted using genomic DNA kits (Invitrogen or Roche) following the manufacturers'protocols. A mixture of 2 µl of extracted DNA mixed with 2 µl of Loading Dye were run on 1% agarose gels, stained with ethidium bromide and were visualized under ultraviolet light (Figure 3.2).

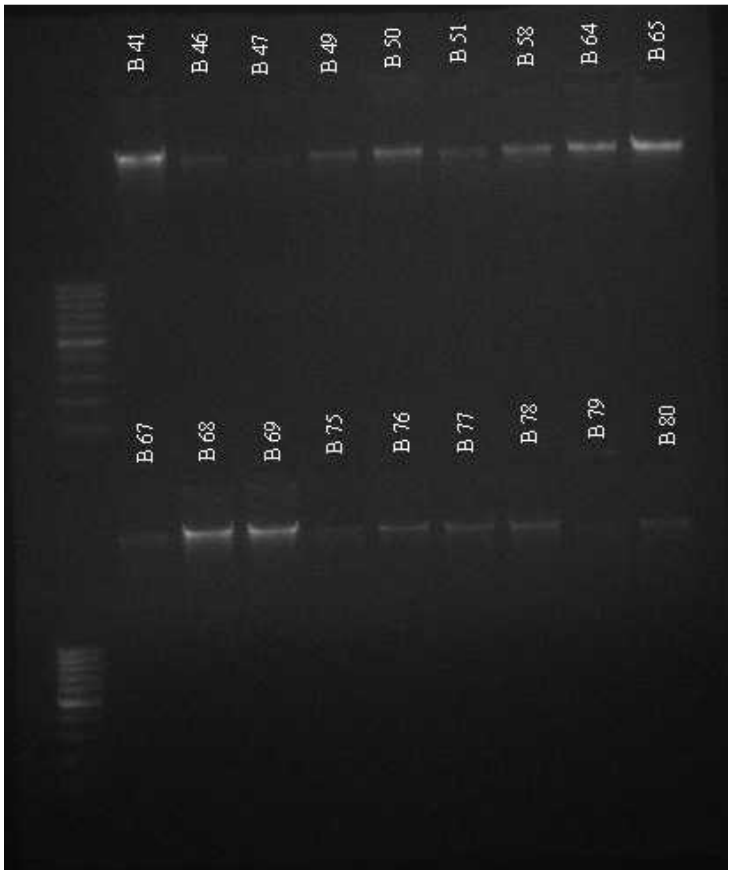


Figure 3.2. The agarose gel image showing the results of an extraction on DNA samples extracted from avian blood.

3.3. PCR Screening

Brain-derived neurotrophic factor (BDNF) primers were used as a positive control to test whether extractions were successful (Richard et al., 2002). The primers used were ChickBDNF5' (ATGACCATCCTTTTCCTTACTATG) and ChickBDNF3' (TCTTCCCCTTTTAATGGTTAATGTAC). 25 µl of reaction mixture contained 2 µl of genomic DNA, 0.1 µl of Taq DNA Polymerase (5U/µl), 2 µl of $MgCl_2$ (25mM), 1.25 µl of 10X Taq buffer (Thermo Scientific, Fermentas, Pure Extreme), 0.5 µl of each primer (10 µM), 0.5 µl of dNTPs (10 mM). The cycling profile consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles including denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec followed by final extension at 72°C for 7 min. All samples were tested with the same primers at least two times. The image showing the results of PCR reactions is given in Figure 3.3.

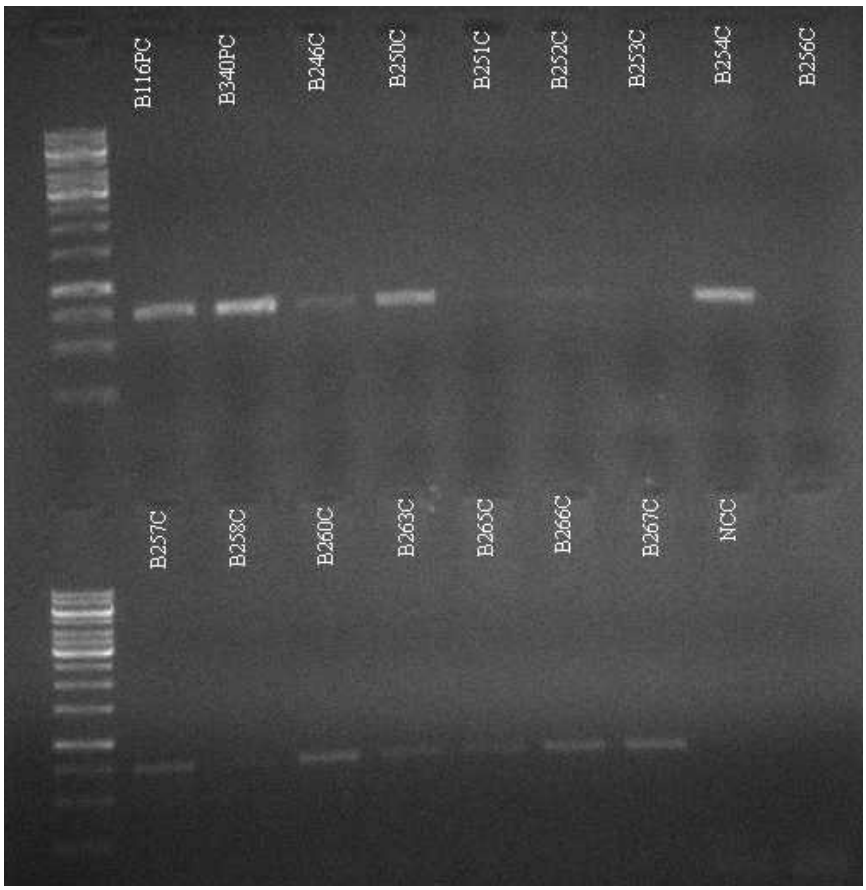


Figure 3.3. The agarose gel image showing the results of PCR products amplified with the primer pair ChickBDNF3-ChickBDNF5.

Extracted DNA was used in nested PCR reactions to amplify a portion of the mitochondrial cytochrome *b* gene (Sehgal et al., 2006). For the first amplification, the primer pair LeucoF: 5'-TCTTACTGGTGTATTATTAGCAAC-3', and LeucoR: 5'-AGCATAGAATGTGCAAATAAACC-3' were used. The initial PCR reaction was performed with the following conditions: 50 µl reaction mixture consisted of 2 µl of genomic DNA, 0.250 µl of Taq DNA Polymerase (5U/µl), 6 µl of $MgCl_2$ (25mM), 5 µl of 10X Taq buffer (Thermo Scientific, Fermentas, Pure Extreme), 1 µl of each primer (10 µM), 1 µl of dNTPs (10 mM). The cycling profile consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 47°C for 30 sec, and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. A picture showing the results of the PCR for the region amplified with this set of primers is given in Figure 3.4.

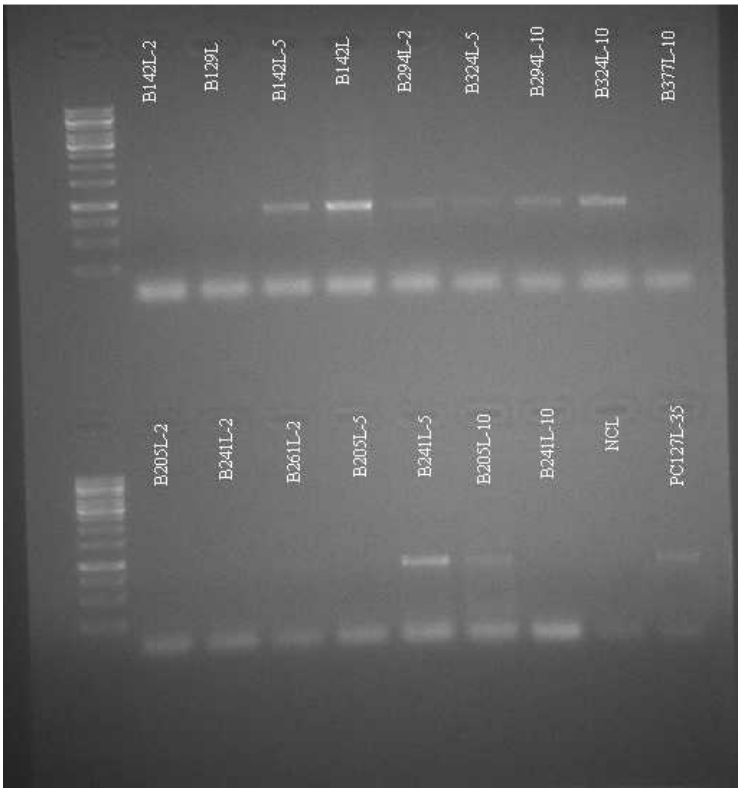


Figure 3.4. The agarose gel image showing the results of PCR products amplified with the primer pair Leuco F-Leuco R.

For the second PCR reaction, primers DW2: 5'-TAATGCCTAGACGTATTCCTGATTATCCAG-3', and DW4: 5'-TGTTTGCTTGGGAGCTGTAATCATAATGTG-3' were used (Sehgal et al., 2006). The PCR reaction included the following conditions: 50 µl reaction mixtures consisted of 2 µl of genomic DNA, 0.250 µl of Taq DNA Polymerase (5U/µl), 6 µl of $MgCl_2$ (25mM), 5 µl of 10X Taq buffer (Thermo Scientific, Fermentas, Pure Extreme), 1 µl of each primer (10 µM), 1 µl of dNTPs (10 mM). The cycling profile consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec, and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. A picture showing the results of the PCR for the region amplified with this set of primers is given in Figure 3.5. For nine samples, the third PCR reaction was performed using 2-10 µl of previous PCR products as template, with the same PCR profile as above.

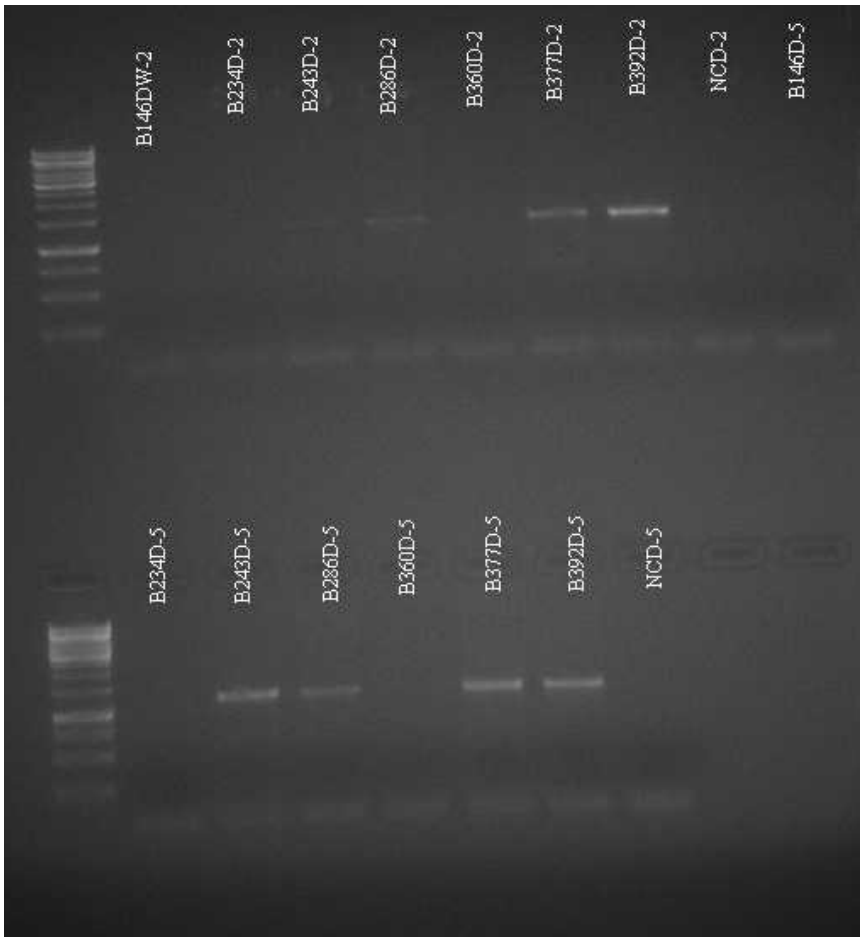


Figure 3.5. The agarose gel image showing the results of PCR products amplified with the primer pair DW2-DW4.

The PCRs were run approximately as sets of ten samples, with positive and negative controls used in each run. Samples from birds confirmed as infected were used as positive controls and reaction mixtures without any DNA template were used as negative controls to control for possible contamination. Three μl of the PCR products were run out on 1.0% agarose gels prepared with 1×TBE, and stained with ethidium bromide and were visualized under ultraviolet light for evaluation of the amplifications. The DNA of *L. toddi* positive PCR products were concentrated using an Epigentek kit and following the manufacturer's protocol prior to sequencing.

3.4. Sequencing

The amplified PCR products were commercially sequenced at Macrogen (Korea), using the same primers as used for PCR. The sequences were assembled and aligned using Sequencher. Chromatograms were screened for the presence of double nucleotide peaks which are indicators of infections with at least two different parasite lineages.

3.5. Phylogenetic Analysis

Phylogenetic analyses were performed using 397 bp cytochrome *b* sequences of six samples, for which we were able to acquire clean chromotograms. The estimation of phylogenetic relationships in a global context was made between the newly generated six mitochondrial cytochrome *b* sequences of *L. toddi* and 265 published sequences of *Leucocytozoon* spp. taken from the GenBank database via the National Center for Biotechnology Information (NCBI) nucleotide BLAST search and MalAvi database (Bensch et al., 2009). Phylogenetic analyses were conducted using MEGA 5 (Tamura et al., 2011). Neighbor-joining and maximum-likelihood trees were constructed, using the Kimura 2-parameter distance model, and bootstrapping 1000 times to determine node support.

4. RESULTS

4.1. Identification of Samples and Results of PCR Screening

DNA was initially extracted from 401 blood samples belonging to 58 avian species of 25 families (Table 4.1). Out of the 401 samples, 298 tested as positive in the control PCR with the ChickBDNF primers. 12 of these blood samples were collected in May and June, and 286 blood samples were collected in August, September and October in 2009. 41 individuals out of these 298 samples were determined as PCR positive for the *L. toddi* infection. Of all 41 positives, 25 were infants, 10 were adults, and four were naive juveniles and the age category of two individuals were not identified. 10 of the positive samples were male, and two were female, and the sex of the rest of the positives were unknown. The mean parasite prevalence was 10.2% and the prevalence of *L. toddi* in host species ranged from 0% to 100% (Table 4.1). The highest number of tested individuals was in *Sylvia borin* (n=36) in which the prevalence was 2.8%, whereas three species (*Locustella fluviatilis*, *Galerida cristata*, and *Coracias garrulus*) (n=1 each) had a prevalence of 100%. In *Sylvia curruca* (n=5) and *Motacilla flava* (n=17) moderate levels (40.0% and 47.1%, respectively) of infection rates were obtained. Infected individuals from Order Passeriformes included species *Acrocephalus arundinaceus*, *A. palustris*, *A. scirpaceus*, *Locustella fluviatilis*, *L. luscinioides*, *Phylloscopus trochilus*, *Sylvia borin*, *S. communis*, *S. curruca* of Family Sylviidae, species *Motacilla flava* of Family Motacillidae, species *Carpodacus erythrinus* of Family Fringillidae, species *Erithacus rubecula*, *Luscinia luscinia*, *L. svecica*, *Saxicola rubetra* of Family Muscicapidae, species *Galerida cristata* of Family Alaudidae, species *Lanius collurio* of Family Laniidae, and species *Passer domesticus* and *P. montanus* of Family Passeridae. Other infected individuals belonged to *Coracias garrulus* of Family Coraciidae of Order Coraciiformes, *Cuculus canorus* of Family Cuculidae of Order Cuculiformes, and *Ixobrychus minutus* of Family Ardeidae of Order Ciconiiformes. Detailed information on the age, sex, and collection date of bird samples are given in the Appendix A, and gel images of PCR results are given in Appendix B.

Table 4.1. Prevalence of *Leucocytozoon toddi* in avian host species as determined by PCR and sequencing. The individuals for which sequence data were obtained are highlighted.

Order	Family	Species	Number of individuals	Number of infections	Prevalence (%)
Passeriformes	Sylviidae	*Acrocephalus agricola*	2		
		Acrocephalus arundinaceus	21	1	4.8
		Acrocephalus palustris	29	1	3.4
		Acrocephalus schoenobaenus	7		
		Acrocephalus scirpaceus	30	2	6.7
		Cettia cetti	17		
		Hippolais pallida	1		
		Locustella fluviatilis	1	1	100
		Locustella luscinioides	7	1	14.2
		Phylloscopus collybita	16		
		Phylloscopus lorenzii	1		
		Phylloscopus trochilus	10	2	20
		Phylloscopus sibilatrix	1		
		Sylvia atricapilla	5		
		Sylvia borin	36	1	2.8
		Sylvia communis	23	5	21.7
		Sylvia curruca	5	2	40
		Sylvia nisoria	1		
	Motacillidae	*Anthus trivialis*	3		
		Motacilla flava	17	8	47.1
	Fringillidae	*Carpodacus erythrinus*	7	2	28.6
	Emberizidae	*Emberiza citrinella*	1		
		Emberiza hortulana	3		
		Emberiza schoeniclus	2		
		Miliaria calandra	1		
	Muscicapidae	*Erithacus rubecula*	7	1	14.3
		Ficedula parva	2		
		Luscinia luscinia	6	1	16.7
		Luscinia svecica	10	1	10
		Muscicapa striata	2		
		Oenanthe hispanica	1		
		Phoenicurus phoenicurus	12		
		Saxicola maura	1		
		Saxicola rubetra	6	3	50
	Alaudidae	*Galerida cristata*	1	1	100
	Hirundinidae	*Hirundo rustica*	16		
		Riparia riparia	6		
	Laniidae	*Lanius collurio*	17	3	17.6
		Lanius minor	1		

	Oriolidae	*Oriolus oriolus*	2		
	Passeridae	*Passer domesticus*	5	1	20
		Passer montanus	7	1	14.3
	Paridae	*Parus major*	3		
	Corvidae	*Pica pica*	1		
	Prunellidae	*Prunella modularis*	1		
	Remizidae	*Remiz pendulinus*	6		
	Troglodytidae	*Troglodytes troglodytes*	1		
	Turdidae	*Turdus merula*	1		
Coraciiformes	Alcedinidae	*Alcedo atthis*	4		
	Coraciidae	*Coracias garrulus*	1	1	100
	Meropidae	*Merops apiaster*	16		
Caprimulgiformes	Caprimulgidae	*Caprimulgus europaeus*	3		
Galliformes	Phasianidae	*Coturnix coturnix*	2		
Cuculiformes	Cuculidae	*Cuculus canorus*	2	1	50
Piciformes	Picidae	*Dendrocopos syriacus*	1		
		Jynx torquilla	7		
Ciconiiformes	Ardeidae	*Ixobrychus minutus*	2	1	50
Columbiformes	Columbidae	*Streptopelia turtur*	1		
	Total	58 species	401	41	10.2

4.2. Results of Phylogenetic Analysis

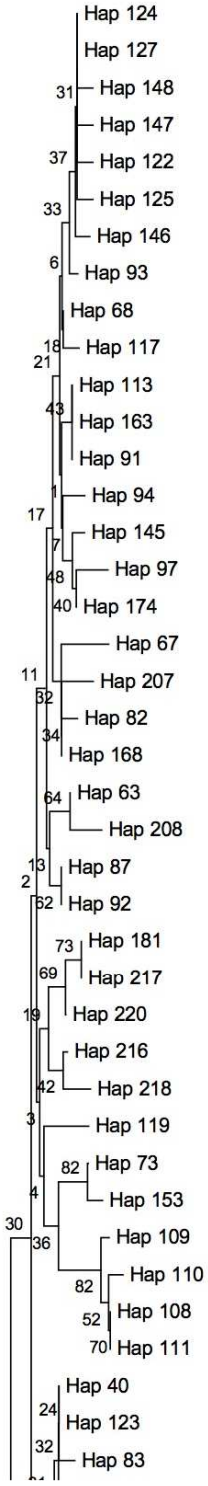
Of all PCR positive samples in this study, clean chromatograms were obtained only in six samples belonging to six species of six genera; the codes for the samples were B04 in the Great Reed-warbler (*Acrocephalus arundinaceus*), B129 in the common cuckoo (*Cuculus canorus*), B142_2 in the European robin (*Erithacus rubecula*), B294_5 in the willow warbler (*Phylloscopus trochilus*), B324_1 in the whinchat (*Saxicola rubetra*), and B396 in the lesser whitethroat (*Sylvia curruca*). Combined with sequence data from the literature, the phylogenetic relationships of a total of 271 mitochondrial sequences of *Leucocytozoon* recovered from 76 species were also analyzed. The trimmed data set was composed of 397 bp and a total of 220 distinct haplotypes were defined. Both neighbor-joining and maximum-likelihood analyses produced phylogenetic trees with similar topologies (Figures 4.1 and 4.2). The constructed phylogenetic tree grouped the haplotypes of *Leucocytozoon* into two major distinct clades of Clade A and Clade B (Table 4.2, and Figures 4.1 and 4.2). 198 haplotypes of 67 species were grouped into Clade A and the number of haplotypes *per* species in this clade was 2.95. In Clade B, 22 haplotypes of 14 species were grouped and the number haplotypes *per* species was 1.57. Both clades

included different species of hosts, and only *Milvus milvus* and *Sylvia atricapilla* were found in both Clade A and Clade B.

Table 4.2. The species of host birds included in phylogenetic analyses, grouped based on the clade they were clustered in.

Clade A	**Clade B**
Acrocephalus scirpaceus (3)	*Accipiter brevipes* (2)
Aegolius funereus (1)	*Accipiter cooperii* (1)
Ailuroedus buccoides (1)	*Accipiter nisus* (4)
Andropadus latirostris (7)	*Acrocephalus arundinaceus* (1)
Anthus berthelotii (1)	*Buteo buteo* (2)
Aphrastura spinicauda (2)	*Buteo jamaicensis* (10)
Aplonis cantoroides (1)	*Buteo regalis* (1)
Asio otus (3)	*Cuculus canorus* (1)
Bubo bubo (1)	*Erithacus rubecula* (1)
Bubo virginianus (5)	*Milvus migrans* (1)
Carduelis spinus (1)	*Milvus milvus* (1)
Catharus ustulatus (5)	*Saxicola rubetra* (1)
Circus aeruginosus (1)	*Sylvia atricapilla* (2)
Cracticus quoyi (1)	*Sylvia curruca* (1)
Cyanistes caeruleus (10)	
Emberiza spodocephala (2)	
Foudia madagascariensis (2)	
Fringilla montifringilla (1)	
Gallus gallus (2)	
Gavia immer (1)	
Glaucidium sjostedti (1)	
Hypsipetes borbonicus (3)	
Hypsipetes madagascariensis (4)	
Hypsipetes parvirostris (3)	
Ixos philippinus (2)	
Loxia curvirostra (1)	
Luscinia svecica (5)	
Milvus milvus (1)	
Nectarinia comorensis (1)	
Nectarinia coquerelli (1)	
Nectarinia humbloti (3)	
Nectarinia notata (4)	
Nectarinia souimanga (1)	
Otus scops (1)	

Parus caeureus (1)
Parus major (6)
Parus montanus (1)
Passer domesticus (1)
Phrygilus alaudinus (1)
Phylloscopus collybita (4)
Phylloscopus trochilus (11)
Pitohui kirhocephalus (1)
Ploceus cucullatus (2)
Pycnonotus jocosus (2)
Saxicola tectes (2)
Sephanoides sephanoides (1)
Sicalis luteola (1)
Streptopelia picturata (1)
Strix aluco (1)
Strix occidentalis (2)
Strix occidentalis caurina (4)
Strix occidentalis occidentalis (9)
Strix varia (2)
Sylvia atricapilla (11)
Sylvia borin (3)
Symposiachrus manadensis (1)
Turdus falcklandii(2)
Turdus merula azorensis (2)
Turdus obscurus (1)
Tyto alba (1)
Zosterops abyssinicus (2)
Zosterops borbonicus (17)
Zonotrichia capensis (2)
Zosterops chloronothos (3)
Zosterops maderaspatanus (6)
Zosterops mauritianus (3)
Zosterops olivaceus (5)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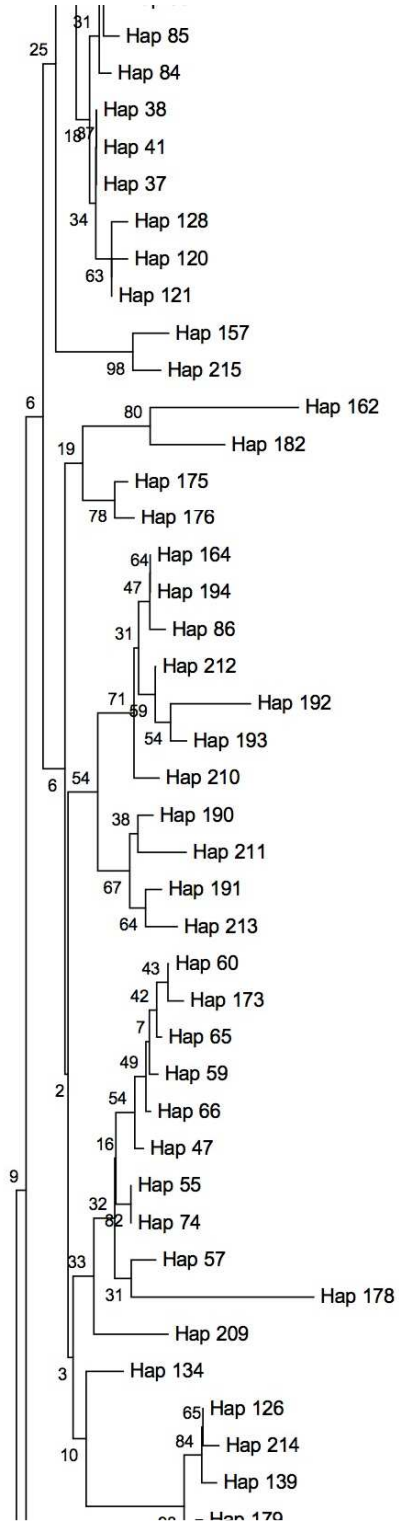
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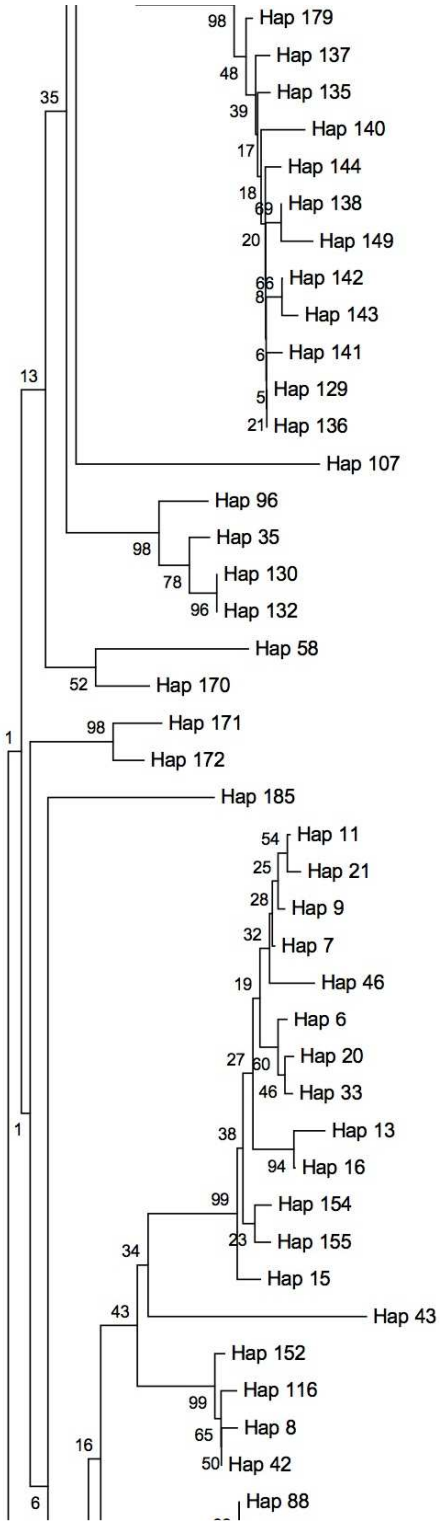
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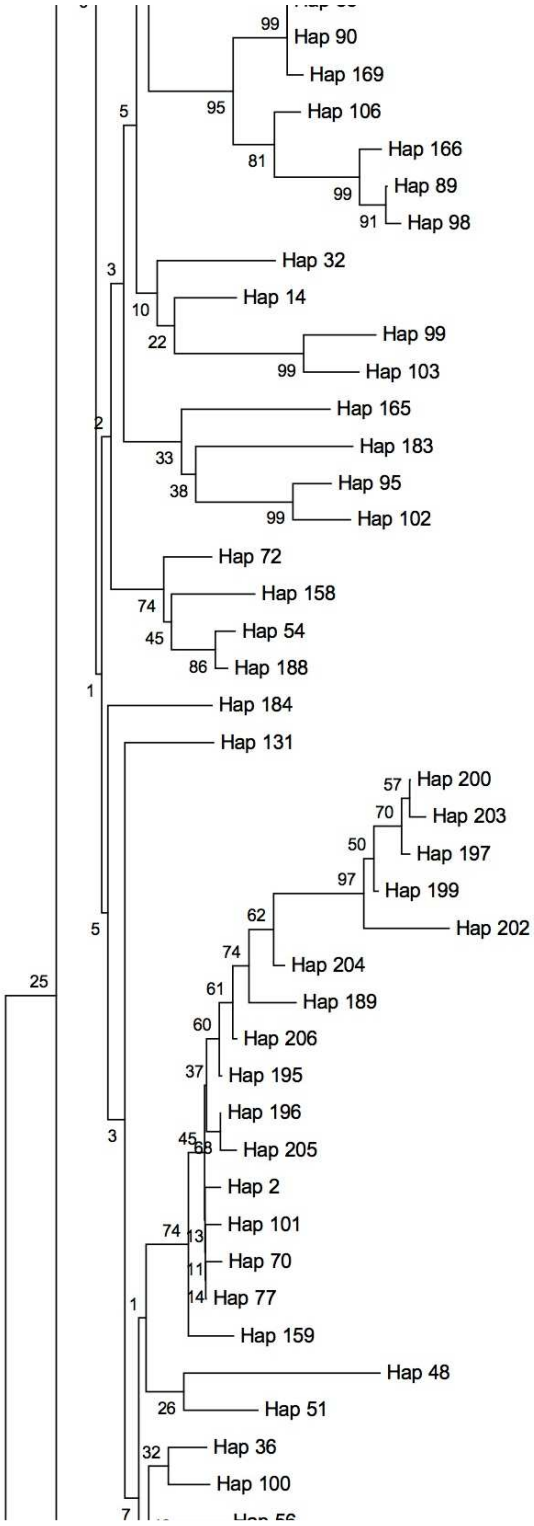
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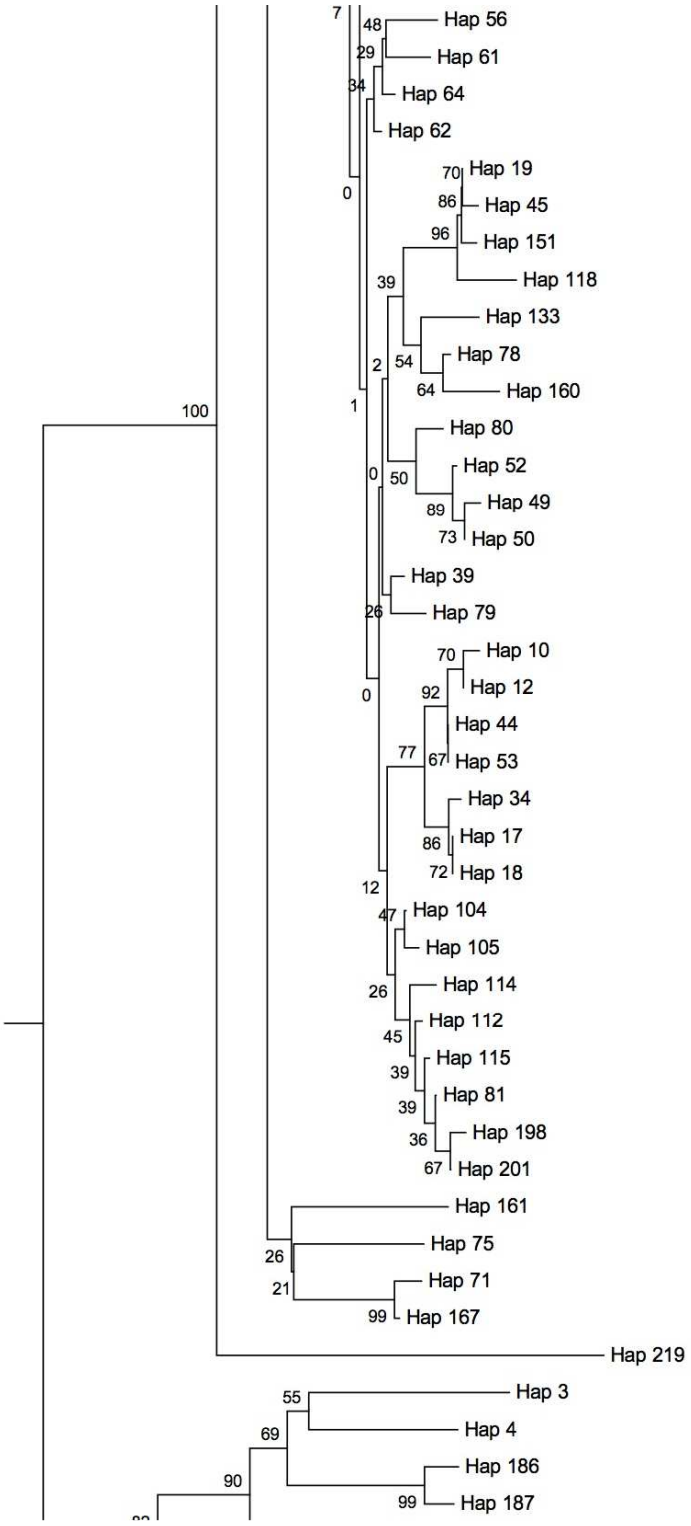
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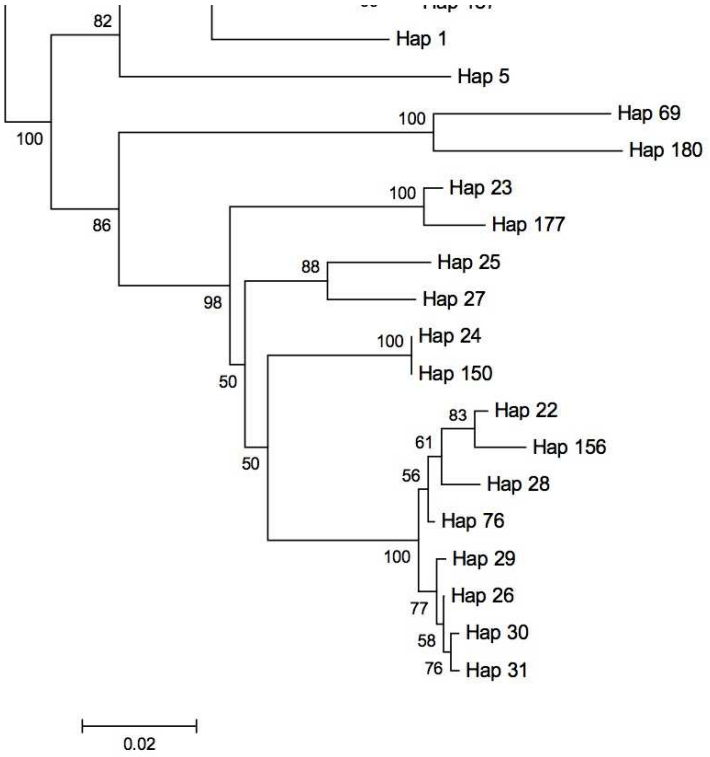
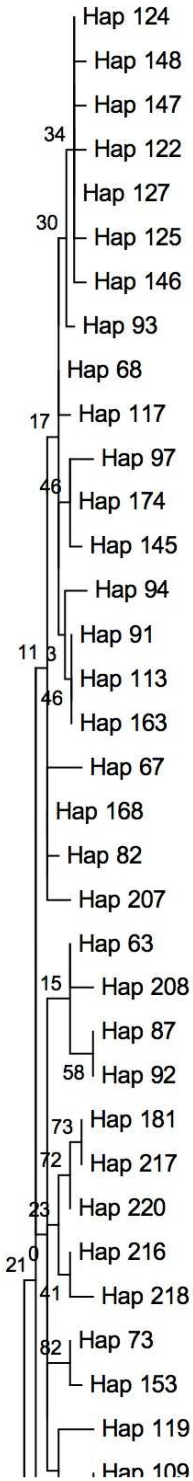


Figure 4.1. Phylogenetic relationships of 220 haplotypes of *Leucocytozoon* parasites based on mitochondrial cytochrome *b* gene. The phylogenetic tree was constructed using neighbor-joining method. Numbers in the branch indicate bootstrap values on 1000 replicates.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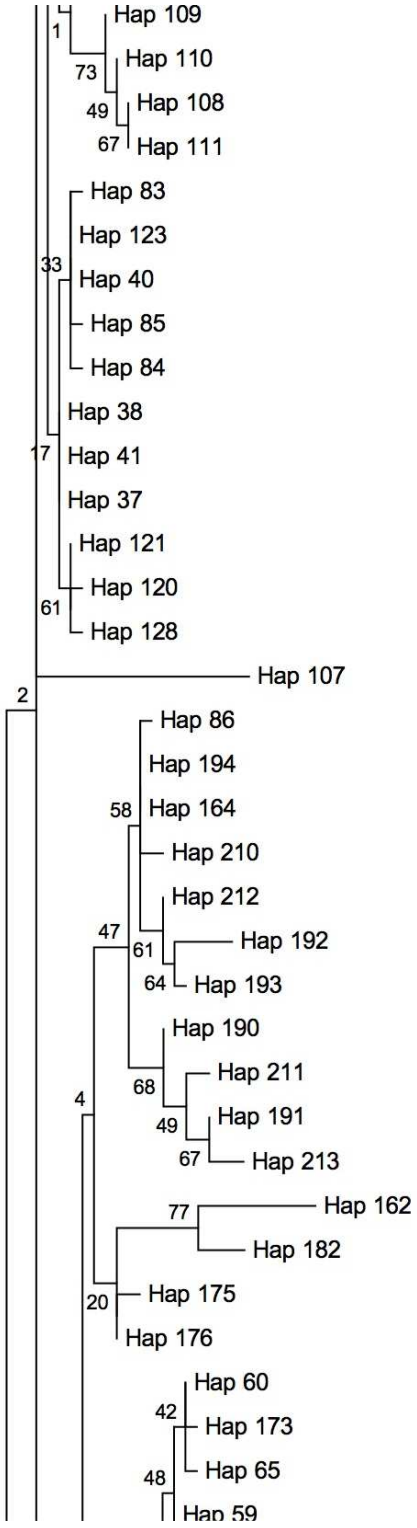
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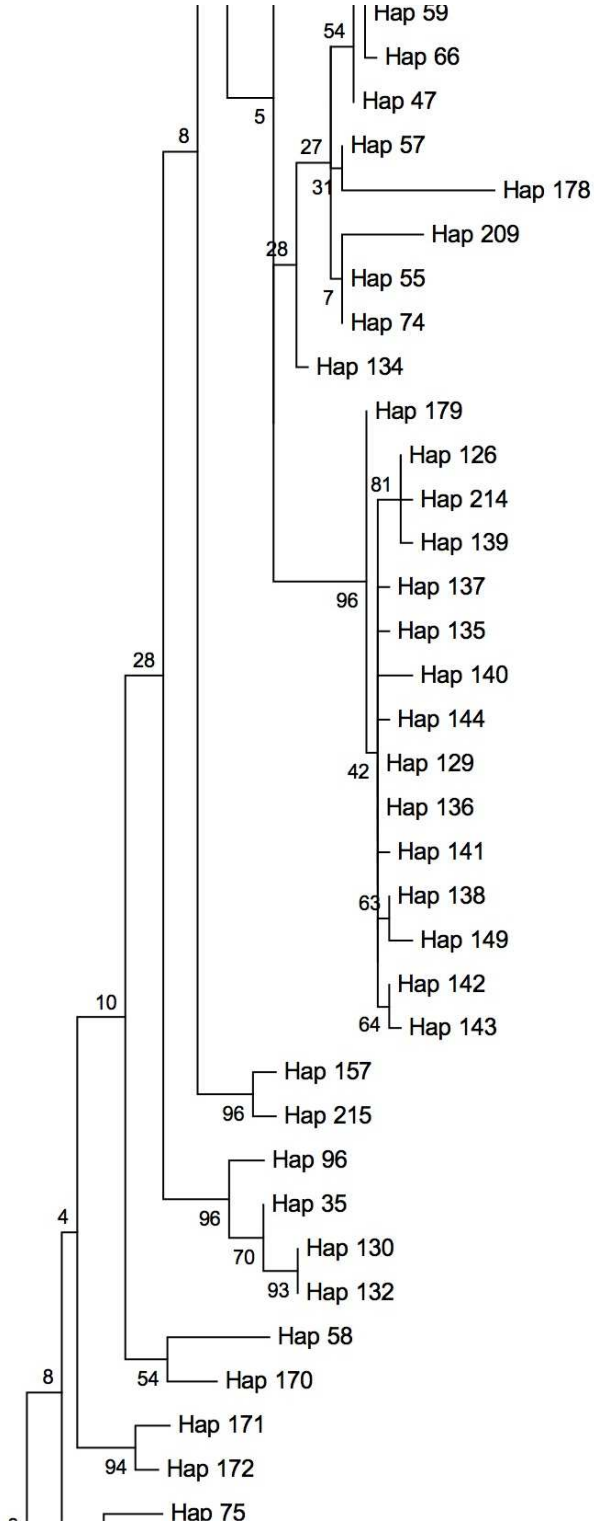
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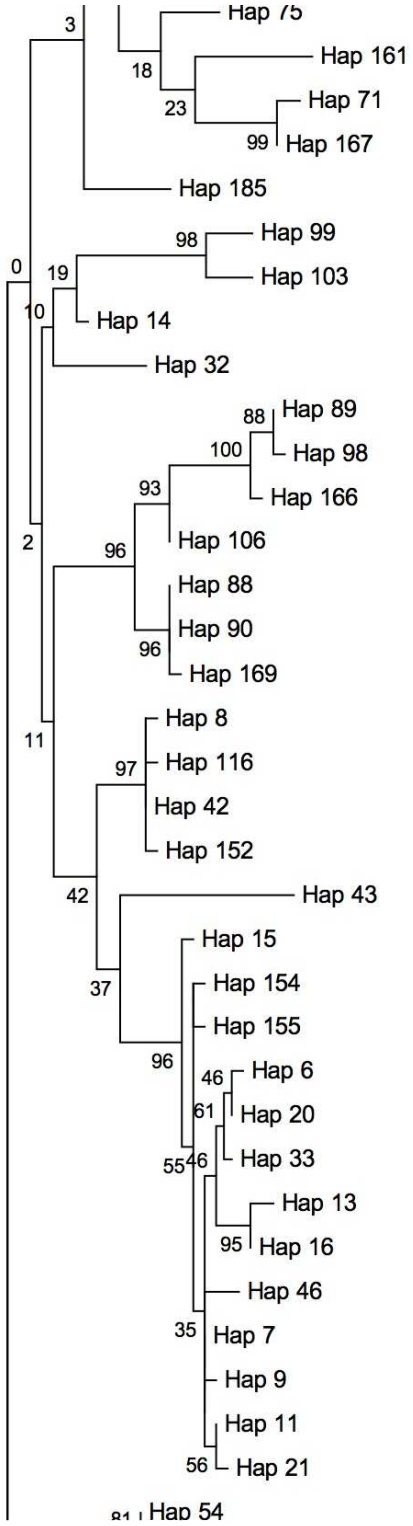
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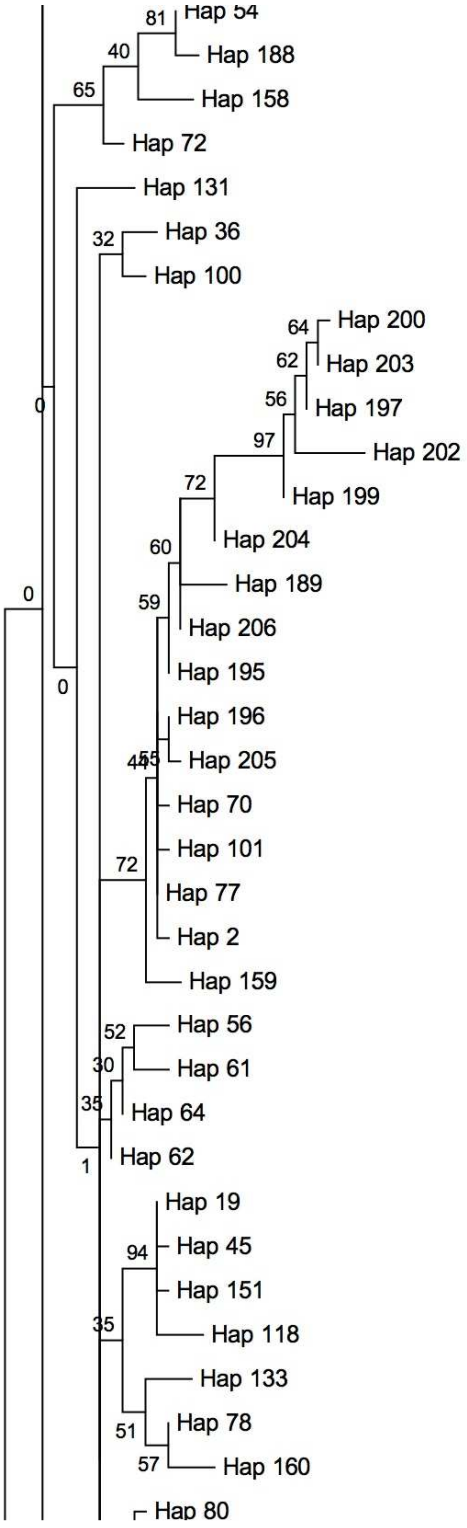
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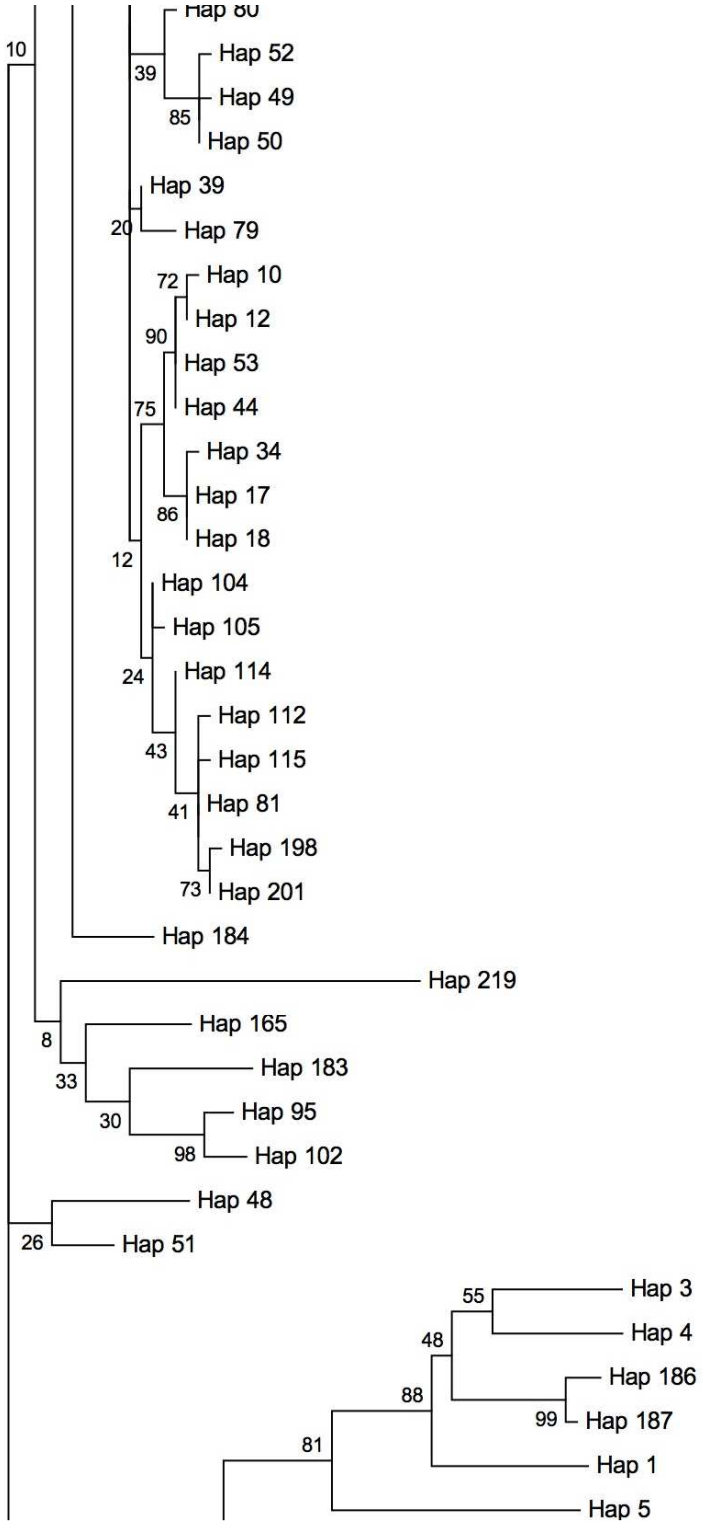
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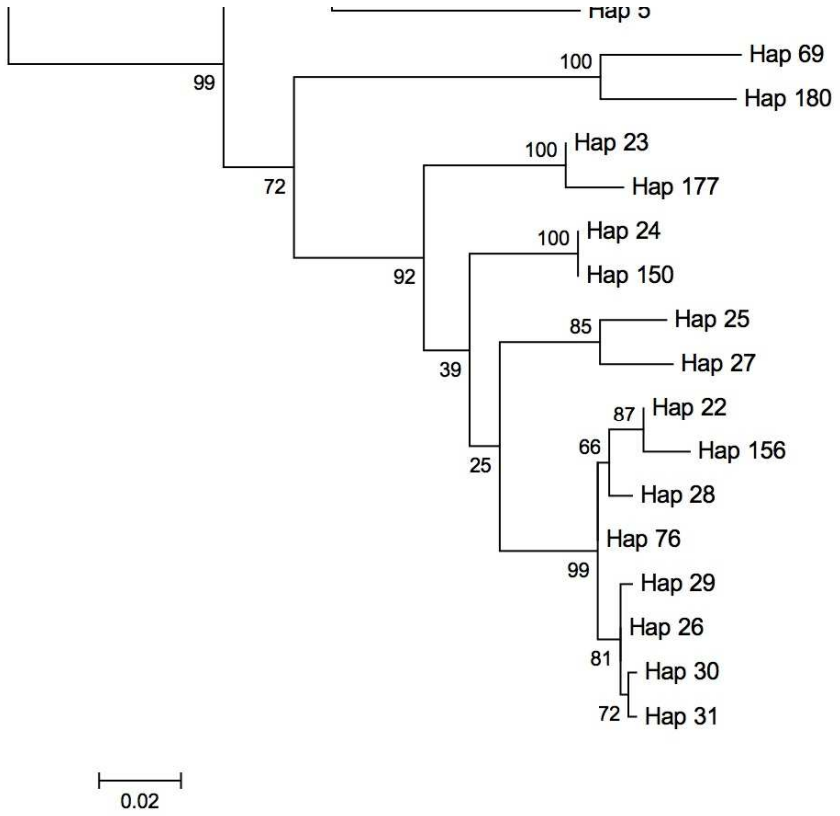

Figure 4.2. Phylogenetic relationships of 220 haplotypes of *Leucocytozoon* parasites based on mitochondrial cytochrome *b* gene. The phylogenetic tree was constructed using maximum-likelihood method. Numbers in the branch indicate bootstrap values on 1000 replicates.

The sequences we obtained in this study corresponded to six lineages representing five distinct cyt *b* haplotypes, with four of them also being distinct from those in the literature. Three lineages of *L. toddi* were restricted to a single host species each (*Acrocephalus arundinaceus*, *Cuculus canorus*, and *Sylvia curruca*). One of the parasite lineages was found in two bird species (*Erithacus rubecula* and *Saxicola rubetra*) of Aras-Iğdır samples and one of the lineages (in *Phylloscopus trochilus*) was previously detected in avian blood based on literature. Out of our five haplotypes, four (except B294_5 *Phylloscopus trochilus*) clustered into clade B. All four haplotypes in clade B clustered together with a strong bootstrap support (80%) including a species of *Leucocytozoon* identified previously from *Sylvia atricapilla*. On the other hand, the parasite lineage of B294_5 *Phylloscopus trochilus* clustered with lineages of *Leucocytozoon* from *Luscinia svecica* and two unidentified hosts from GenBank in clade A.

5. DISCUSSION

The molecular advances help to identify parasitic infections in animals and to reveal phylogenetic relationships among species. In this study, the prevalence of *Leucocytozoon toddi* in bird species inhabiting Aras-Iğdır, located in north-eastern Anatolia in Turkey, was investigated. The survey of 25 avian families of infections with *L. toddi* represent a wide range of possibly infected host species in a local community where no information on the prevalence of *L. toddi* infections have been reported previously. The region is on a migratory pathway, so the abundance of parasites might affect a broad range of bird species.

Previous studies based on the prevalence of *Leucocytozoon* in bird blood samples resulted in a wide range of rates. In the first nested PCR protocol for the detection of *Leucocytozoon* spp., the prevalence rate was 48% in adult blue throats (n=86) (Hellgren et al., 2004). Jones et al. (2005) recorded *Leucocytozoon* spp. in 3.6% of 828 passerines in West Africa. Sehgal et al. (2006) found 29.9% of 591 bird individuals from *Accipiter* spp., *Buteo* spp., and *Circus* sp. infected with *L. toddi*. Hellgren et al. (2007) identified 334 (8.6%) *Leucocytozoon* spp. infections in 3886 tested birds of 41 species. Valkiunas et al. (2008) compared *Leucocytozoon* spp. infection in 11 species of 472 birds and found 25.2% prevalence with microscopic examination and 29.9% with PCR screening. 109 samples of *Andropadus latirostris* were tested and infection rate with *Leucocytozoon* spp. was 3.7% with microscopic screening and 17.4% with the PCR method (Valkiunas et al., 2009). Ortego and Cordero (2009) investigated *Leucocytozoon* spp. infection in 203 nestlings of *Bubo bubo* and reported 107 (52.7%) positive individuals. As an example of detection of haemosporidian parasites in potential vectors, Sato et al. (2009) screened 490 black flies of six species in the alpine regions of Japan and found 1.6% positive samples infected with *L. lovati*. Ishak et al. (2010) screened 446 birds of three hawk species and found 30% *Leucocytozoon* spp. infection. Jenkins and Owens (2011) screened *Leucocytozoon* spp. in *Cyanistes caeruleus* and *Parus major* from several regions of Europe. Of the total 191 *C.caeruleus* individuals, 24% were infected and in 153 samples of *P. major*, the infection

rate was 27%. Imura et al. (2012) screened 415 birds from several species and reported 56 (13.5%) *Leucocytozoon* spp. positive samples in Japan.

41 individuals (10.2%) of all Aras-Iğdır samples were infected with *L. toddi* parasite and they belong to 22 species of 15 genera and 10 families. A large difference in *L. toddi* prevalence was also observed between different bird taxa. It can be concluded that our study is in concordance with studies that report low levels of infection. Several explanations might account for the low prevalence of *L. toddi* among examined birds. First, low level of prevalence may be due to low abundance of the blackfly vectors or the amount of vectors infected. In this study, parasites were only analysed in birds, but it should be noted that vectors also have crucial roles in the relationship of parasite-host systems. Second, blackfly vectors of *Leucocytozoon* have also been reported to have strong host preferences (Malmqvist et al., 2004). Third, Aras-Iğdır being a migratory stop-over area, rather than for instance a nesting region for resident birds where transmission of the parasite might be easier, is likely to have contributed to this result. To determine whether these issues are interrelated, it will be necessary to include the analysis of the abundance and dispersal ability of blackfly vectors and to determine the host preferences of vectors as well.

The prevalence of haemosporidians in bird hosts is affected from seasonal variation, especially in the breeding periods and therefore fluctuates yearly (Wood et al., 2007). In this study, samples were collected in spring and in autumn periods, however due to limited sampling for the spring season, comparison of prevalence rates between seasons could not be made. The parasite prevalence is also affected from the age of the birds, as older birds or naive juveniles are thought to be less resistent to parasitic infections (White et al., 1996). Our study is in concordance with this study as a result of the relatively higher infections of both infants and naive juveniles (n=29) than adults (n=10) (Table A.1).

Phylogenetic analyses revealed that birds were infected with unique lineages of *Leucocytozoon*, and therefore various relationships of hosts and parasites exist. Phylogenetic trees constructed showed two major clades, which we refer to as Clade A and Clade B. Considering the debate over *L. toddi* representing a group of cryptic species

(Sehgal et al., 2006; Valkiunas et al., 2010), the finding of sequences of Aras-Iğdır samples in two distinct clades in this study suggest the presence of more than one taxon in *L. toddi*.

Clade A includes a wide range group of haplotypes when compared to Clade B and the number of haplotypes *per* number of species is higher in Clade A (2.95) than Clade B (1.57). The low resistancy of bird hosts to *Leucocytozoon* spp. infection in Clade A might cause this relatively high ratio. Four out of our five haplotypes, found in hosts from several orders and families, were placed together in the same clade (Clade B) with strong boostrap values. These included two species (*Acrocephalus arundinaceus* and *Sylvia curruca*) from Family Sylviidae and two species (*Erithacus rubecula* and *Saxicola rubetra*) from Family Muscicapidae of Order Passeriformes, and one species (*Cuculus canorus*) from Family Cuculidae of Order Cuculiformes. These four haplotypes were closely grouped with two haplotypes found in *Sylvia atricapilla* from GenBank. *A.arundinaceus*, *S.curruca*, *E.rubecula*, *S.rubetra*, *C. canorus* and *S. atricapilla* are native species in Turkey and in the meantime they migrate in a route from Europe to several regions of Africa to spend the winter. The reason for clustering of *Leucocytozoon* haplotypes from such different species indicates a lack of host-specificity, but also the possibility of evolution of the parasite in species sharing migratory pathways. *Phylloscopus trochilus* of our study in Clade A is also native in Turkey and migrates to Africa, however the parasite lineage of this sample clustered apart from the rest of the Aras-Iğdır samples.

Parasite lineages from different hosts containing identical cytochrome *b* sequences are indicators of host-switching (Ricklefs and Fallon, 2002). Two out of five haplotypes in *L. toddi* were found in more than one bird species each and these are indicators of relatively low host specificity of *L. toddi*. Specifically Haplotype 4 is reported both in *Erithacus rubecula* and *Saxicola rubetra* of Aras-Iğdır samples, and both species belong to different genera, which is an indication of switching between genera in the same family. Haplotype 2 is found in two host species belonging to *Phylloscopus trochilus* and an unidentified host species from literature. In other words, Haplotype 2 is found both in Aras-Iğdır and an unidentified region. The sharing of one haplotype in this study with another from literature enabled to assess the host-switching of parasite lineages to possible different host species and distinct geographical regions.

We also made a comparison on whether the hosts of our six sequences have been recorded to harbor *L. toddi* infection previously by searching GenBank database for the prevalence of the parasite. *L.toddi* has never been recorded in any species that it tested positive for in this study. *P.trochilus* as a migratory Sylviidae was reported to harbor not *L. toddi*, but an unidentified *Leucocytozoon* spp. Since Sylviidae hosts in our study are migratory species, they might have originated from closely related regions.

6. CONCLUSION

In conclusion, although a wide species range of avian hosts was investigated in a local community in north-eastern of Anatolia, the analyses showed a low level of prevalence in terms of *L. toddi* infections. The relatively high infection rate of infants and juveniles are in concordance with that recorded in previous studies. Phylogenetic reconstructions resulted in two distinct clades and Aras-Iğdır samples were clustered all in one clade with the exception of one lineage. The detection of *L. toddi* lineages in distinct clades supports the idea that this parasite species represents a cryptic species group.

In our study, sex determination had been made for a few number of birds, but the sex of bird individuals might be determined with molecular methods. A comparison between seasons regarding infection rates, which was not possible due to low number of samples collected in spring, can be made with a greater sampling effort in the future. Future studies might also be extended toward the tracking of haemosporidian parasites in the migration route of bird hosts. The investigation of parasitic infections among birds would also contribute to the development of an effective conservation program.

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APPENDIX A : INFORMATION OF THE SAMPLES OF THE STUDY

Table A.1. Information of the code, species name, age, sex, and collection date of the study's samples. The codes of *Leucocytozoon toddi* positive samples are highlighted.

Ring Code/Lab Code	Species	Age	Sex	Date of Collection
JB 24031/B01	*Acrocephalus agricola*	i		06.09.2009
JB 24015/B02	*Acrocephalus agricola*	a		05.09.2009
FA 06746/B04	*Acrocephalus arundinaceus*	n		01.06.2009
FA 06728/B05	*Acrocephalus arundinaceus*	n		31.05.2009
FA 06743/B09	*Acrocephalus arundinaceus*	n		31.05.2009
FA 06800/B10	*Acrocephalus arundinaceus*	i		18.08.2009
FA 06793/B12	*Acrocephalus arundinaceus*	a		17.08.2009
FA 07439/B13	*Acrocephalus arundinaceus*			23.08.2009
FA 06829/B14	*Acrocephalus arundinaceus*	i		22.08.2009
FA 06828/B15	*Acrocephalus arundinaceus*	i		22.08.2009
FA 06787/B16	*Acrocephalus arundinaceus*	i		15.08.2009
FA 06840/B18	*Acrocephalus arundinaceus*	i		25.08.2009
FA 07906/B19	*Acrocephalus arundinaceus*	a		13.09.2009
FA 06891/B20	*Acrocephalus arundinaceus*	i		07.09.2009
FA 07904/B21	*Acrocephalus arundinaceus*	i		11.09.2009
FA 06898/B22	*Acrocephalus arundinaceus*	a		10.09.2009
JB 23215/B24	*Acrocephalus palustris*	n		31.05.2009
JB 23375/B25	*Acrocephalus palustris*	i		18.08.2009
JB 23441/B26	*Acrocephalus palustris*	i		21.08.2009
JB 23452/B28	*Acrocephalus palustris*	i		21.08.2009
JB 23453/B29	*Acrocephalus palustris*	i		21.08.2009
JB 23363/B30	*Acrocephalus palustris*	i		17.08.2009
JB 23447/B31	*Acrocephalus palustris*	i		21.08.2009
JB 23300/B32	*Acrocephalus palustris*	i		14.08.2009
JB 23472/B33	*Acrocephalus palustris*	i		23.08.2009
JB 23470/B34	*Acrocephalus palustris*	i		23.08.2009
JB 23335/B35	*Acrocephalus palustris*	i		16.08.2009
JB 23471/B36	*Acrocephalus palustris*	i		23.08.2009
JB 23473/B37	*Acrocephalus palustris*	i		23.08.2009
JB 23423/B38	*Acrocephalus palustris*	i		22.08.2009
JB 23440/B39	*Acrocephalus palustris*			21.08.2009
JB 23540/B40	*Acrocephalus palustris*	i		27.08.2009
JB 23589/B41	*Acrocephalus palustris*	i		29.08.2009
JB 23552/B42	*Acrocephalus palustris*	i		28.08.2009
JB 23517/B43	*Acrocephalus palustris*	i		26.08.2009
JB 23521/B44	*Acrocephalus palustris*	i		26.08.2009
JB 23527/B45	*Acrocephalus palustris*	i		26.08.2009
JB 24097/B46	*Acrocephalus palustris*	i		08.09.2009
JB 23528/B47	*Acrocephalus palustris*	i		26.08.2009
JB 23976/B49	*Acrocephalus palustris*	i		03.09.2009

JB 23739/B50	*Acrocephalus palustris*	i		01.09.2009
JB 23554/B54	*Acrocephalus schoenobaenus*	i		28.08.2009
JB 23516/B55	*Acrocephalus schoenobaenus*	i		26.08.2009
JB 23216/B59	*Acrocephalus scirpaceus*	i		01.06.2009
JB 23309/B62	*Acrocephalus scirpaceus*	a		14.08.2009
JB 23311/B63	*Acrocephalus scirpaceus*	i		15.08.2009
JB 23304/B65	*Acrocephalus scirpaceus*	a		14.08.2009
JB 23479/B66	*Acrocephalus scirpaceus*	i		23.08.2009
JB 23205/B67	*Acrocephalus scirpaceus*	n		31.05.2009
JB 23467/B68	*Acrocephalus scirpaceus*	i		22.08.2009
JB 23333/B69	*Acrocephalus scirpaceus*	n		16.08.2009
JB 23588/B70	*Acrocephalus scirpaceus*	i		29.08.2009
JB 23543/B71	*Acrocephalus palustris*	n		27.08.2009
JB 23529/B72	*Acrocephalus palustris*	n		26.08.2009
JB 23525/B74	*Acrocephalus scirpaceus*	a		26.08.2009
JB 24029/B76	*Acrocephalus scirpaceus*	i		06.09.2009
JB 24043/B77	*Acrocephalus scirpaceus*	i		07.09.2009
JB 24088/B80	*Acrocephalus scirpaceus*	i		08.09.2009
JB 23998/B81	*Acrocephalus scirpaceus*	i		04.09.2009
JB 23590/B82	*Acrocephalus scirpaceus*	i		29.08.2009
JB 24001/B83	*Acrocephalus scirpaceus*	i		04.09.2009
JB 24105/B87	*Acrocephalus scirpaceus*	i		09.09.2009
JB 24131/B89	*Acrocephalus scirpaceus*	i		10.09.2009
JB 23731/B90	*Acrocephalus scirpaceus*	i		31.08.2009
YH 03027/B91	*Alcedo atthis*	a	f	17.08.2009
YH 03042/B92	*Alcedo atthis*	i	f	09.09.2009
YH 03043/B93	*Alcedo atthis*	i	m	09.09.2009
YH 03053/B94	*Alcedo atthis*	i		14.09.2009
JB 24042/B97	*Anthus trivialis*	n		07.09.2009
JB 23218 /B98	*Carpodacus erythrinus*	n		01.06.2009
JB 23307/B100	*Carpodacus erythrinus*	a	m	15.08.2009
JB 23738/B101	*Carpodacus erythrinus*	i		01.09.2009
JB 24153/B102	*Carpodacus erythrinus*			11.09.2009
JB 24143/B103	*Carpodacus erythrinus*	n		11.09.2009
JB 24142/B104	*Carpodacus erythrinus*	n		10.09.2009
DA 03759/B105	*Caprimulgus europaeus*	a	f	27.08.2009
DA 03762/B106	*Caprimulgus europaeus*	i	m	03.09.2009
DA 03764/B107	*Caprimulgus europaeus*	i	f	04.09.2009
JB 22445/B108	*Cettia cetti*			31.05.2009
JB 23457/B109	*Cettia cetti*	n		22.08.2009
JB 23321/B110	*Cettia cetti*	n		21.08.2009
JB 23236/B111	*Cettia cetti*	n		18.08.2009
JB 22339/B112	*Cettia cetti*	n		31.05.2009
JB 23368/B113	*Cettia cetti*	i	m	15.08.2009
JB 23458/B114	*Cettia cetti*	n		22.08.2009

JB 23249/B115	*Cettia cetti*	n		16.08.2009
JB 02346/B116	*Cettia cetti*	n		08.09.2009
JB 24049/B117	*Cettia cetti*	n		07.09.2009
JB 23256/B118	*Cettia cetti*	n		07.09.2009
JB 12968/B119	*Cettia cetti*	a		12.09.2009
JB 23961/B120	*Cettia cetti*	n		15.09.2009
JB 24108/B121	*Cettia cetti*	n		09.09.2009
JB 24163/B122	*Cettia cetti*	n		12.09.2009
JB 23243/B123	*Cettia cetti*	n		09.09.2009
JB 23321/B124	*Cettia cetti*	n		10.09.2009
CS 00258/B125	*Coturnix coturnix*		f	25.08.2009
CS 00257/B126	*Coturnix coturnix*		f	24.08.2009
CA 00379/B127	*Coracias garrulus*	i		18.08.2009
CA 00372/B128	*Cuculus canorus*	i		16.08.2009
CA 00378/B129	*Cuculus canorus*	i		17.08.2009
HA 15790/B131	*Emberiza citrinella*	i	m	24.10.2009
JB 23482/B132	*Emberiza hortulana*	i		24.08.2009
JB 23483/B133	*Emberiza hortulana*	a		24.08.2009
JB 23997/B134	*Emberiza hortulana*	i		04.09.2009
JB 25231/B135	*Emberiza schoeniclus*	a	f	30.10.2009
JB 25226/B136	*Emberiza schoeniclus*	i	f	28.10.2009
JB 25186/B138	*Erithacus rubecula*	i		24.10.2009
JB 25205/B141	*Erithacus rubecula*	i		26.10.2009
JB 25148/B142	*Erithacus rubecula*	i		24.10.2009
FA 06900/B146	*Galerida cristata*	i		11.09.2009
JB 23550/B147	*Hippolais pallida*	i		28.08.2009
JB 23299/B148	*Hirundo rustica*	i		14.08.2009
JB 23362/B149	*Hirundo rustica*	i		17.08.2009
JB 23301/B150	*Hirundo rustica*	i		14.08.2009
JB 23439/B151	*Hirundo rustica*	i		11.08.2009
JB 23338/B152	*Hirundo rustica*	i		16.08.2009
JB 23302/B153	*Hirundo rustica*	i		14.08.2009
JB 23511/B154	*Hirundo rustica*	i		25.08.2009
JB 24074/B155	*Hirundo rustica*	i		07.09.2009
JB 24188/B156	*Hirundo rustica*	i		13.09.2009
JB 24044/B157	*Hirundo rustica*	i		07.09.2009
JB 24047/B158	*Hirundo rustica*	i		07.09.2009
JB 24215/B159	*Hirundo rustica*	i		14.09.2009
JB 23726/B160	*Hirundo rustica*	i		31.08.2009
JB 24208/B161	*Hirundo rustica*	a	f	14.09.2009
BS 00283/B164	*Ixobrychus minutus*	i	m	31.05.2009
BS 00282/B165	*Ixobrychus minutus*	i	m	31.05.2009
FA 03764/B166	*Jynx torquilla*	i		30.08.2009
FA 06847/B167	*Jynx torquilla*	a		28.08.2009
FA 06841/B168	*Jynx torquilla*	a		25.08.2009

FA 06850/B169	*Jynx torquilla*	i		28.08.2009
FA 07910/B170	*Jynx torquilla*	i		13.09.2009
FA 06873/B171	*Jynx torquilla*	i		01.09.2009
FA 06826/B173	*Lanius collurio*	i		21.08.2009
FA 06824/B174	*Lanius collurio*	i		21.08.2009
FA 06751/B175	*Lanius collurio*	i		22.08.2009
FA 06799/B176	*Lanius collurio*	i		18.08.2009
FA 06781/B177	*Lanius collurio*	a	m	14.08.2009
FA 06848/B178	*Lanius collurio*	i		27.08.2009
FA 06852/B179	*Lanius collurio*	i		28.08.2009
FA 07909/B180	*Lanius collurio*	i		13.09.2009
FA 07905/B187	*Lanius collurio*	i		12.09.2009
FA 06892/B189	*Lanius collurio*	i		08.09.2009
DA 03757/B190	*Lanius minor*			22.08.2009
JB 23548/B191	*Locustella fluviatilis*	a		28.08.2009
JB 23374/B192	*Locustella luscinioides*	i		18.08.2009
JB 23371/B193	*Locustella luscinioides*	i		18.08.2009
JB 24183/B194	*Locustella luscinioides*	i		13.09.2009
JB 24045/B195	*Locustella luscinioides*	n		07.09.2009
JB 24024/B196	*Locustella luscinioides*	i		06.09.2009
JB 24189/B197	*Locustella luscinioides*	i		13.09.2009
HA 15716/B199	*Luscinia luscinia*	a		21.08.2009
HA 15719/B200	*Luscinia luscinia*	i		23.08.2009
HA 15717/B201	*Luscinia luscinia*	i		22.08.2009
HA 15730/B202	*Luscinia luscinia*	i		31.08.2009
HA 15730/B203	*Luscinia luscinia*	i		09.09.2009
JB 23486/B205	*Luscinia svecica*	i	f	24.08.2009
YH 03032/B215	*Merops apiaster*	i		29.08.2009
YH 03030/B217	*Merops apiaster*	i		27.08.2009
YH 03048/B218	*Merops apiaster*	i		09.09.2009
YH 03038/B219	*Merops apiaster*	i		06.09.2009
YH 03051/B220	*Merops apiaster*	i		10.09.2009
YH 03039/B221	*Merops apiaster*	i		06.09.2009
YH 03035/B222	*Merops apiaster*	a		29.08.2009
YH 03036/B223	*Merops apiaster*	a	f	01.09.2009
YH 15747/B224	*Merops apiaster*	a		09.09.2009
YH 03040/B225	*Merops apiaster*	i		08.09.2009
YH 03044/B226	*Merops apiaster*	i		09.09.2009
YH 15746/B227	*Merops apiaster*	i		09.09.2009
YH 03052/B228	*Merops apiaster*	i		13.09.2009
YH 03049/B229	*Merops apiaster*	i		10.09.2009
FA 07907/B231	*Miliaria calandra*	n		13.09.2009
JB 24232/B232	*Motacilla flava*	i		16.09.2009
JB 24064/B233	*Motacilla flava*	a	m	07.09.2009
JB 24063/B234	*Motacilla flava*	a	m	07.09.2009

JB 24050/B235	*Motacilla flava*	i	m	07.09.2009
JB 24110/B236	*Motacilla flava*	a	f	09.09.2009
JB 24069/B237	*Motacilla flava*	a	m	07.09.2009
JB 24067/B238	*Motacilla flava*	a	m	01.09.2009
JB 24203/B239	*Motacilla flava*	i		13.09.2009
JB 24207/B240	*Motacilla flava*	i		14.09.2009
JB 24219/B241	*Motacilla flava*	i		14.09.2009
JB 24066/B242	*Motacilla flava*	i	m	08.09.2009
JB 24062/B243	*Motacilla flava*	i	m	07.09.2009
JB 24141/B244	*Motacilla flava*	i	m	10.09.2009
JB 24070/B245	*Motacilla flava*	i	m	07.09.2009
JB 24155/B246	*Motacilla flava*	a	m	11.09.2009
JB 24068/B247	*Motacilla flava*	a	f	07.09.2009
JB 24071/B248	*Motacilla flava*	i	m	07.09.2009
JB 23973/B249	*Muscicapa striata*	i		03.09.2009
JB 23181/B250	*Muscicapa striata*	n		13.09.2009
DA 03761/B252	*Oriolus oriolus*	i		01.09.2009
HA 15705/B254	*Passer domesticus*	i	m	16.08.2009
HA 15706/B255	*Passer domesticus*	i	m	16.08.2009
HA 15743/B257	*Passer domesticus*	i	m	17.09.2009
JB 24139/B258	*Parus major*	i	f	10.09.2009
JB 24034/B259	*Parus major*	a	m	06.09.2009
JB 24138/B260	*Parus major*	a	m	10.09.2009
JB 23303/B261	*Passer montanus*	n		14.08.2009
JB 23456/B263	*Passer montanus*	n		21.08.2009
JB 23477/B264	*Passer montanus*	n		23.08.2009
JB 23466/B265	*Passer montanus*			22.08.2009
JB 24019/B266	*Passer montanus*	n		05.09.2009
JB 24230/B267	*Passer montanus*	n		15.09.2009
RA 26332/B268	*Phylloscopus collybita*	n		16.08.2009
RA 26774/B270	*Phylloscopus collybita*	i		27.10.2009
RA 26786/B271	*Phylloscopus collybita*	a		28.10.2009
RA 26779/B272	*Phylloscopus collybita*	n		28.10.2009
RA 26732/B273	*Phylloscopus collybita*	i		24.10.2009
RA 26785/B274	*Phylloscopus collybita*	i		28.10.2009
RA 26772/B275	*Phylloscopus collybita*	a		27.10.2009
RA 26776/B276	*Phylloscopus collybita*	i		27.10.2009
RA 26777/B277	*Phylloscopus collybita*	a		27.10.2009
RA 26775/B278	*Phylloscopus collybita*	a		27.10.2009
RA 26787/B279	*Phylloscopus collybita*	a		27.10.2009
RA 26760/B280	*Phylloscopus collybita*	i		26.10.2009
RA 26773/B283	*Phylloscopus collybita*	i		27.10.2009
RA 26357/B284	*Phylloscopus lorenzii*	n		06.09.2009
RA 26334/B285	*Phylloscopus trochilus*	i		23.08.2009
RA 26335/B286	*Phylloscopus trochilus*	n		23.08.2009

RA 26342/B287	*Phylloscopus trochilus*	a		27.08.2009
RA 26359/B290	*Phylloscopus trochilus*	a		11.09.2009
RA 26361/B291	*Phylloscopus trochilus*	i		14.09.2009
RA 26363/B292	*Phylloscopus trochilus*	a		15.09.2009
RA 26360/B293	*Phylloscopus trochilus*	i		13.09.2009
RA 26358/B294	*Phylloscopus trochilus*	a		11.09.2009
JB 23549/B295	*Phoenicurus phoenicurus*	n	f	28.08.2009
JB 23538/B296	*Phoenicurus phoenicurus*	i	m	27.08.2009
JB 24310/B297	*Phoenicurus phoenicurus*	i	m	17.09.2009
JB 24311/B298	*Phoenicurus phoenicurus*	i	f	17.09.2009
JB 23735/B299	*Phoenicurus phoenicurus*	i	m	01.09.2009
JB 24318/B300	*Phoenicurus phoenicurus*	i	f	17.09.2009
JB 24222/B301	*Phoenicurus phoenicurus*	a	m	15.09.2009
JB 24239/B302	*Phoenicurus phoenicurus*	i		15.09.2009
JB 24151/B303	*Phoenicurus phoenicurus*		f	11.09.2009
JB 24083/B304	*Phoenicurus phoenicurus*	i	m	08.09.2009
JB 24176/B306	*Phoenicurus phoenicurus*		f	13.09.2009
RA 26348/B307	*Phylloscopus sibilatrix*	i		30.08.2009
BS 00289/B308	*Pica pica*	i		30.10.2009
JB 25203/B309	*Prunella modularis*	i		25.10.2009
JB 23695/B310	*Oenanthe hispanica*	i	f	30.08.2009
JB 23560/B311	*Remiz pendulinus*	i		28.08.2009
JB 23580/B312	*Remiz pendulinus*	i		29.08.2009
JB 23561/B313	*Remiz pendulinus*	i		28.08.2009
JB 23559/B314	*Remiz pendulinus*	i		28.08.2009
JB 23492/B315	*Remiz pendulinus*	i		24.08.2009
JB 23730/B316	*Remiz pendulinus*	i		31.08.2009
JB 24221/B317	*Riparia riparia*	i		15.09.2009
JB 23995/B318	*Riparia riparia*	n		04.09.2009
JB 24204/B320	*Riparia riparia*	i		13.09.2009
JB 24205/B321	*Riparia riparia*	a		13.09.2009
JB 24218/B322	*Riparia riparia*	i		14.09.2009
JB 23537/B324	*Saxicola rubetra*	i	m	27.08.2009
JB 23487/B325	*Saxicola rubetra*	i	m	24.08.2009
JB 24236/B326	*Saxicola rubetra*	i		16.09.2009
JB 24119/B327	*Saxicola rubetra*	i	m	09.09.2009
JB 23722/B328	*Saxicola rubetra*	i	f	31.08.2009
JB 23725/B331	*Sylvia atricapilla*	a	f	31.08.2009
JB 24144/B333	*Sylvia atricapilla*	i	f	11.09.2009
JB 24152/B334	*Sylvia atricapilla*	i	f	11.09.2009
JB 23210/B336	*Sylvia borin*			31.05.2009
JB 23469/B337	*Sylvia borin*	a		23.08.2009
JB 23331/B338	*Sylvia borin*	a		16.08.2009
JB 23449/B339	*Sylvia borin*	a		21.08.2009
JB 23460/B340	*Sylvia borin*	i		22.08.2009

JB 23438/B341	*Sylvia borin*	i		21.08.2009
JB 23314/B342	*Sylvia borin*	i		15.08.2009
JB 23344/B345	*Sylvia borin*	i		17.08.2009
JB 23330/B347	*Sylvia borin*	a		16.08.2009
JB 23526/B349	*Sylvia borin*	a		26.08.2009
JB 23523/B353	*Sylvia borin*	i		26.08.2009
JB 24120/B355	*Sylvia borin*	i		09.09.2009
JB 24098/B366	*Sylvia borin*	i		08.09.2009
JB 24112/B367	*Sylvia borin*	i		09.09.2009
JB 24113/B370	*Sylvia borin*	i		09.09.2009
JB 23450/B372	*Sylvia communis*	i		21.08.2009
JB 23312/B374	*Sylvia communis*	i	m	15.08.2009
JB 23313/B375	*Sylvia communis*	i		15.08.2009
JB 23476/B377	*Sylvia communis*	i		23.08.2009
JB 23315/B379	*Sylvia communis*	i		15.08.2009
JB 23553/B380	*Sylvia communis*	i		28.08.2009
JB 23493/B381	*Sylvia communis*	i		24.08.2009
JB 23484/B382	*Sylvia communis*	a	m	24.08.2009
JB 23522/B383	*Sylvia communis*	i		26.08.2009
JB 23485/B385	*Sylvia communis*	i		24.08.2009
JB 23541/B386	*Sylvia communis*	i		27.08.2009
JB 24091/B388	*Sylvia communis*	i		08.09.2009
JB 24053/B389	*Sylvia communis*	i		07.09.2009
JB 23728/B391	*Sylvia communis*	i		31.08.2009
JB 23583/B392	*Sylvia communis*	a		28.08.2009
JB 23733/B393	*Sylvia communis*	i		31.08.2009
JB 24145/B394	*Sylvia communis*	i	m	11.09.2009
JB 23547/B395	*Sylvia curruca*	i		28.08.2009
JB 23515/B396	*Sylvia curruca*	a		25.08.2009
JB 23581/B397	*Sylvia curruca*	a		29.08.2009
JB 23509/B398	*Sylvia curruca*	n		25.08.2009
HA 15729/B400	*Sylvia nisoria*	i		30.08.2009
JB 25206/B402	*Turdus merula*	i		26.10.2009

APPENDIX B: GEL IMAGES OF PCR REACTIONS AMPLIFIED WITH THE PRIMER PAIR LEUCOF-LEUCOR

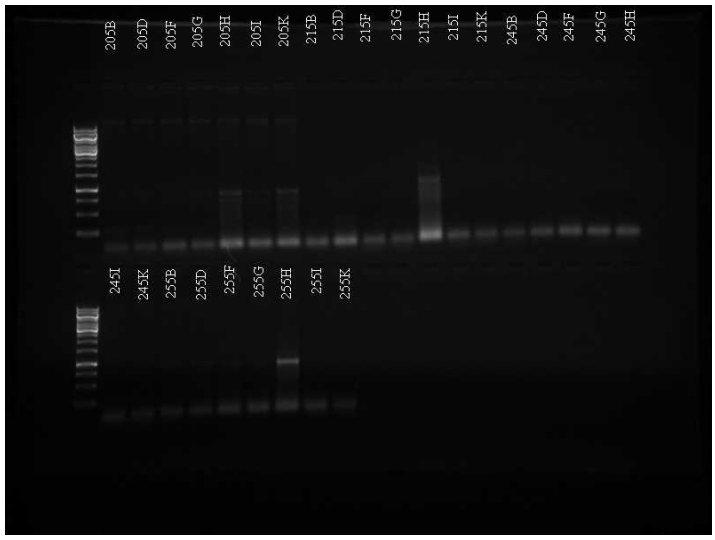

Figure B.1. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #1

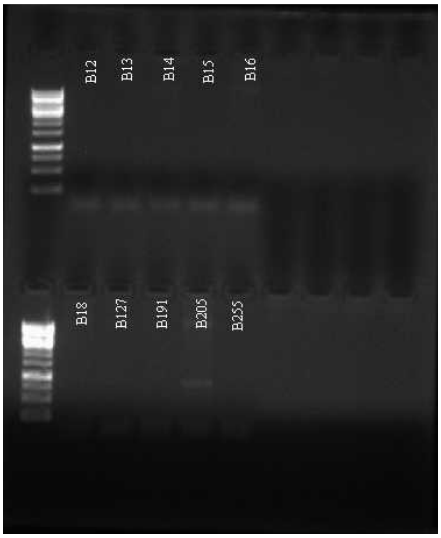

Figure B.2. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #2

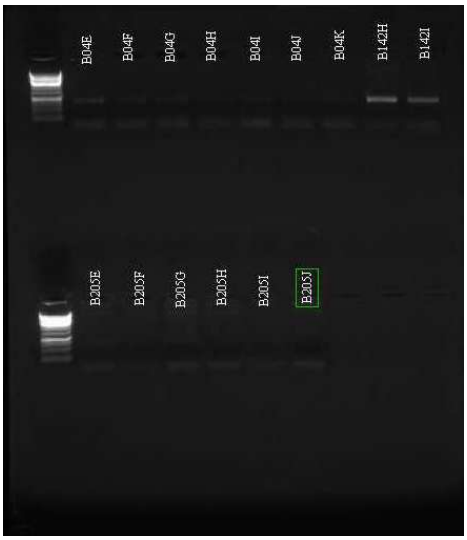

Figure B.3. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #3

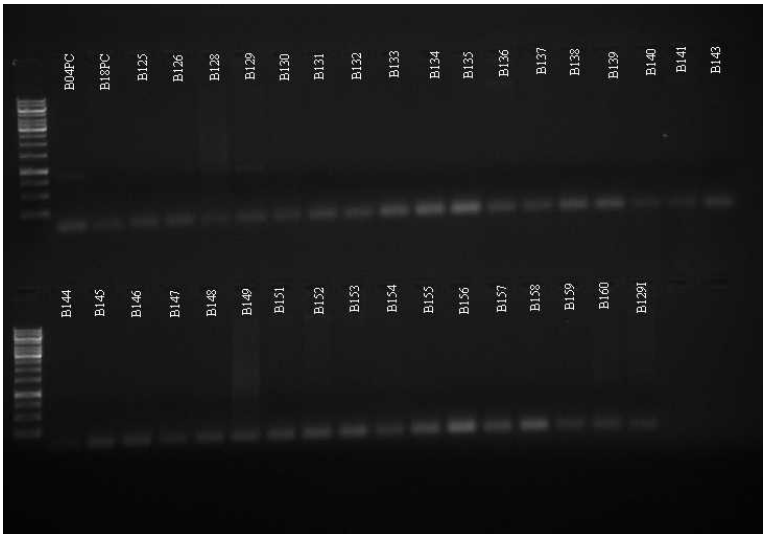

Figure B.4. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #4

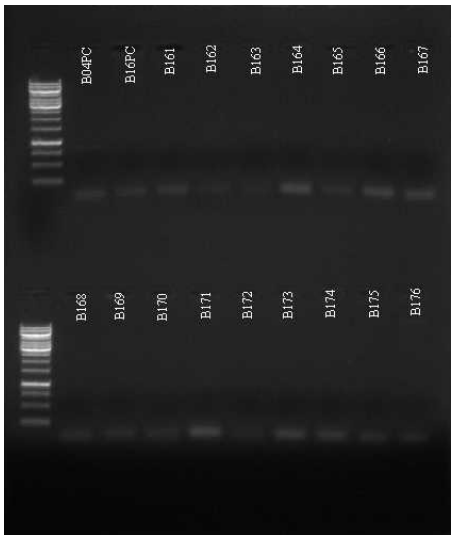

Figure B.5. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #5

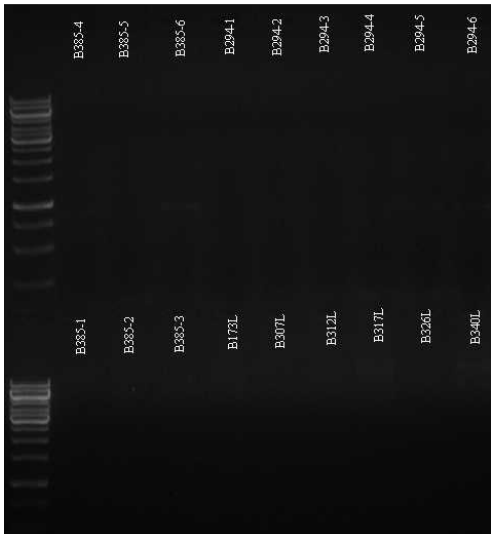

Figure B.6. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #6

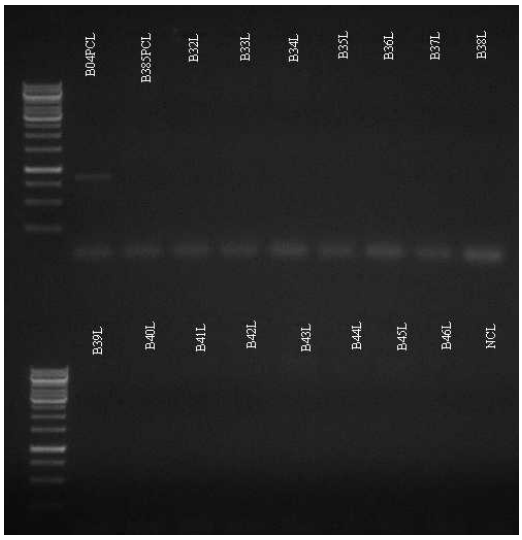

Figure B.7. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #7

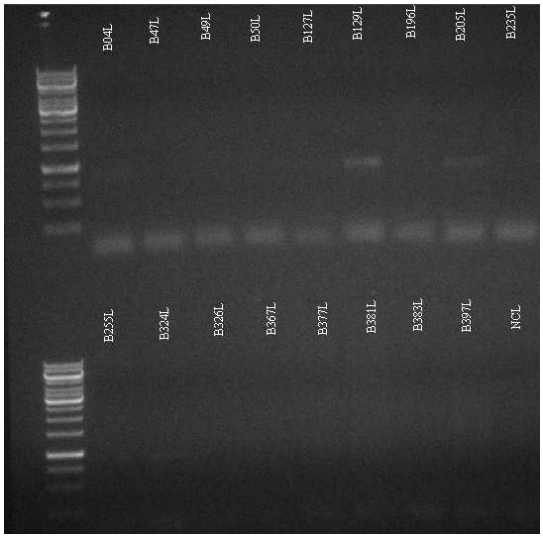

Figure B.8. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #8

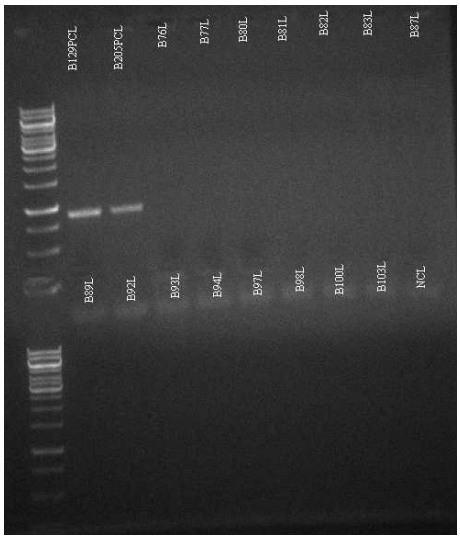

Figure B.9. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #9

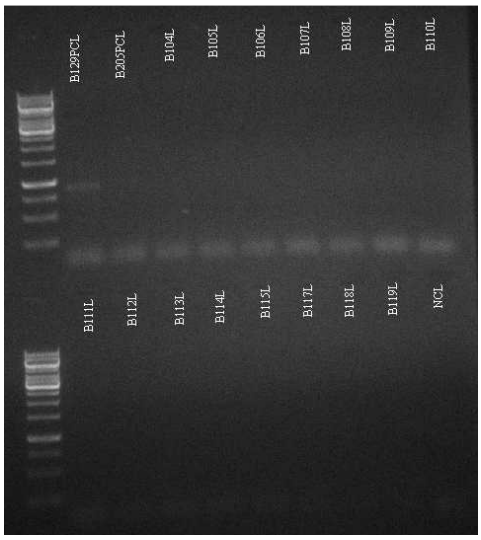

Figure B.10. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #10

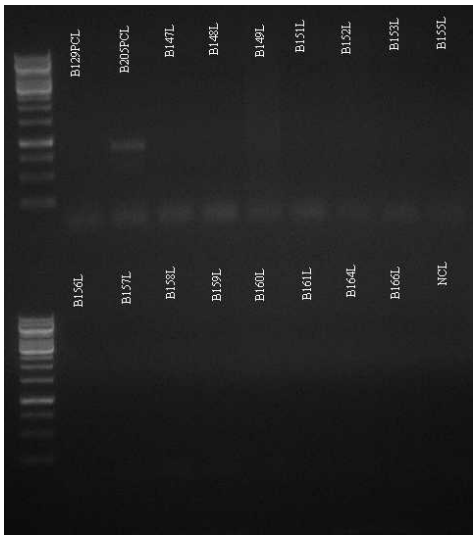

Figure B.11. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #11

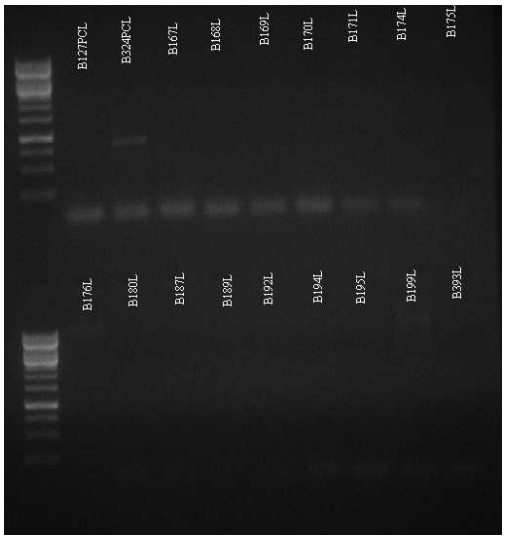

Figure B.12. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #12

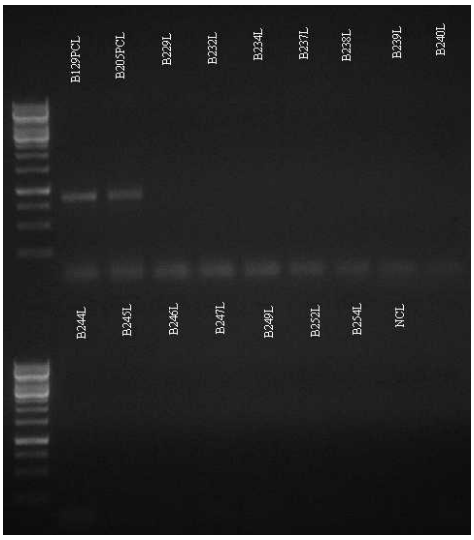

Figure B.13. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #13

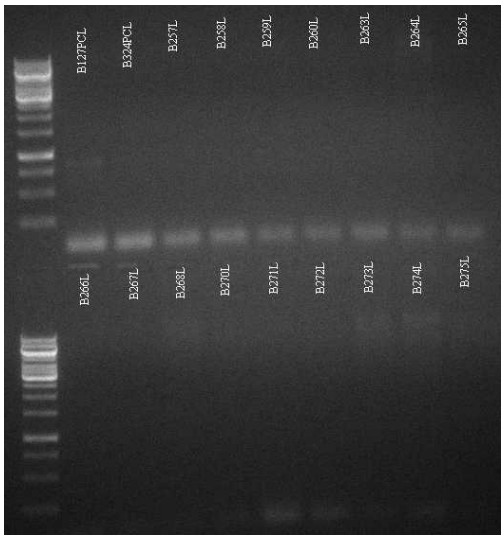

Figure B.14. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #14

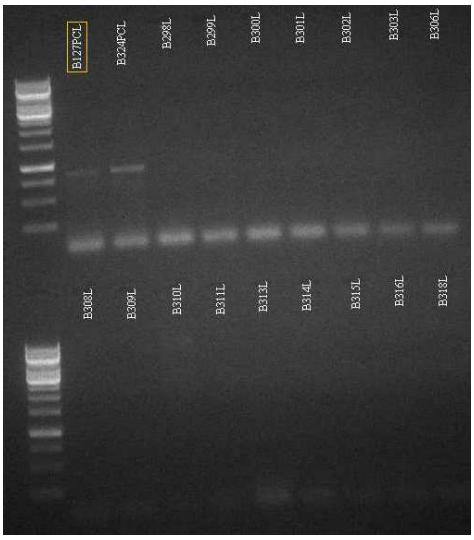

Figure B.15. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #15

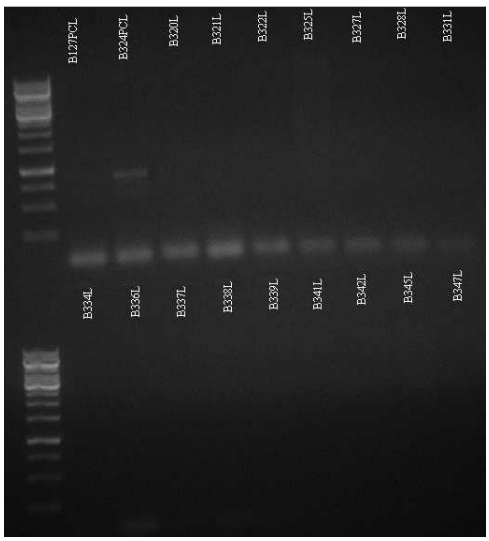

Figure B.16. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #16

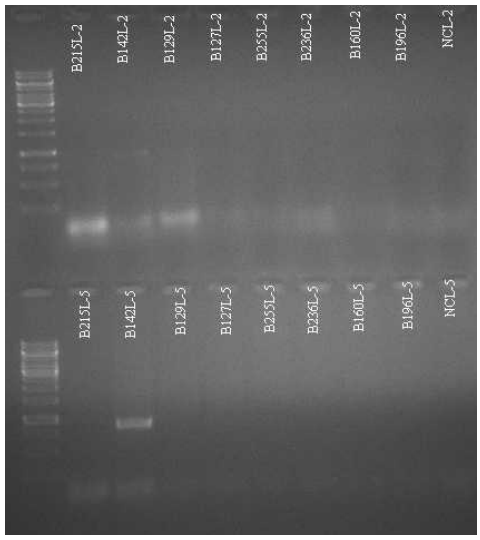

Figure B.17. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #17

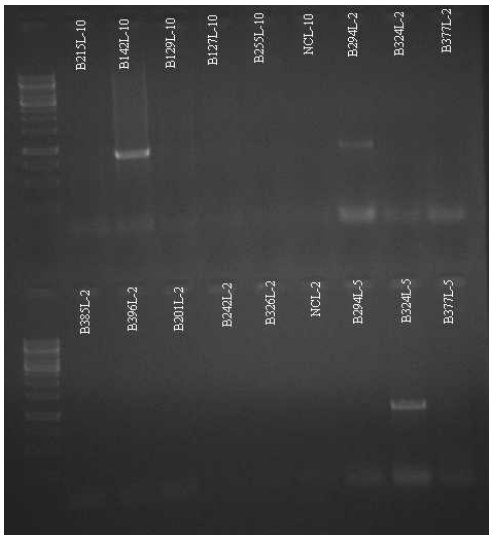

Figure B.18. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #18

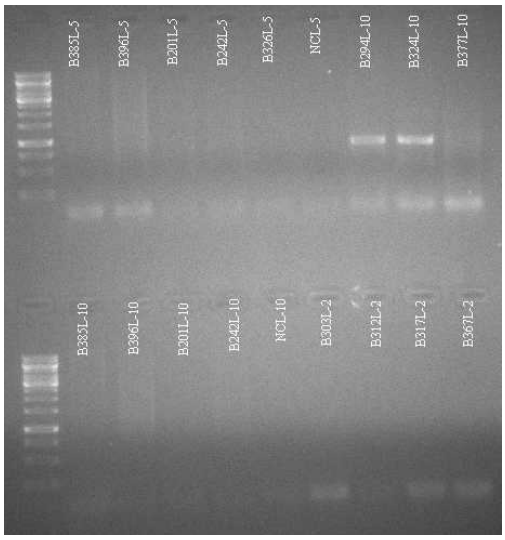

Figure B.19. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #19

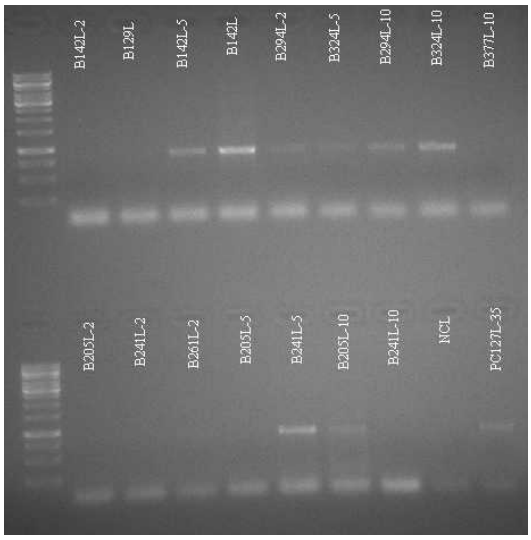

Figure B.20. Gel image of PCR reactions amplified with the primer pair LeucoF-LeucoR #20

APPENDIX C: GEL IMAGES OF PCR REACTIONS AMPLIFIED WITH THE PRIMER PAIR DW2-DW4

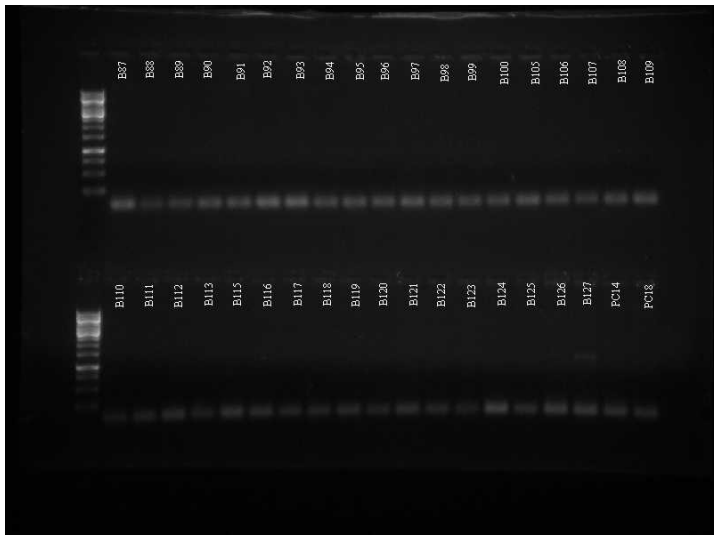

Figure C.1. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #1

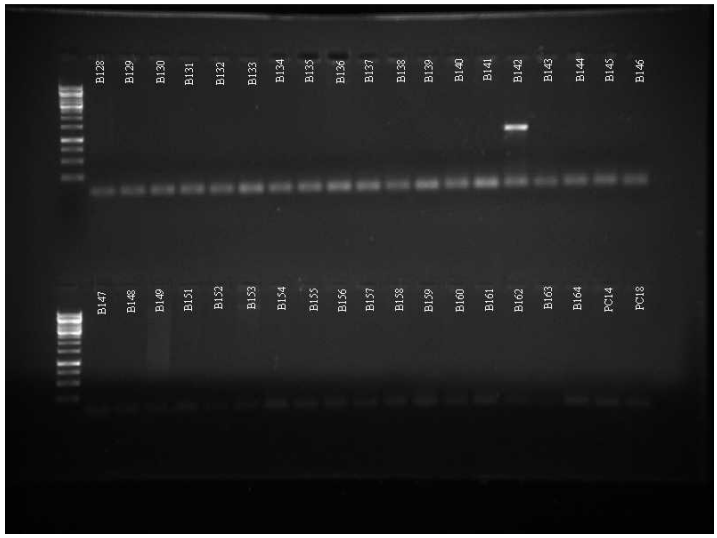

Figure C.2. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #2

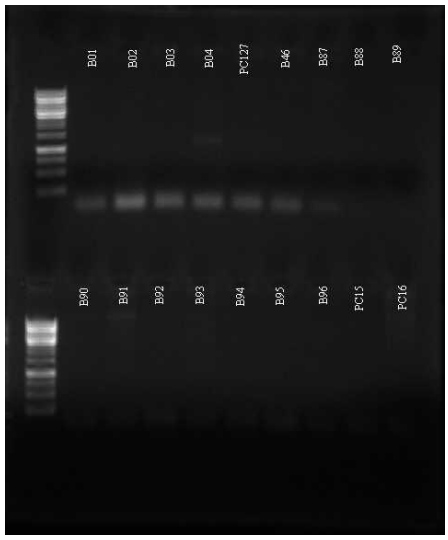

Figure C.3. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #3

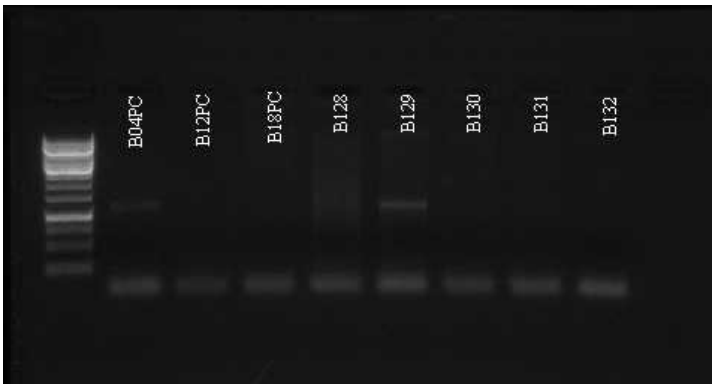

Figure C.4. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #4

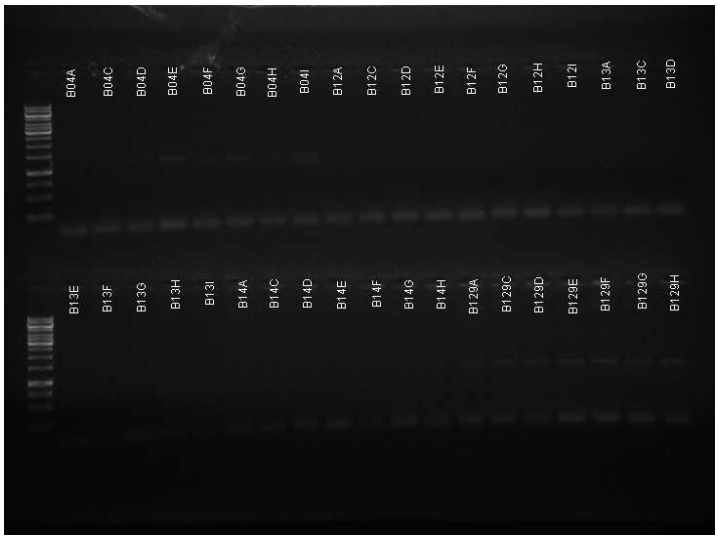

Figure C.5. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #5

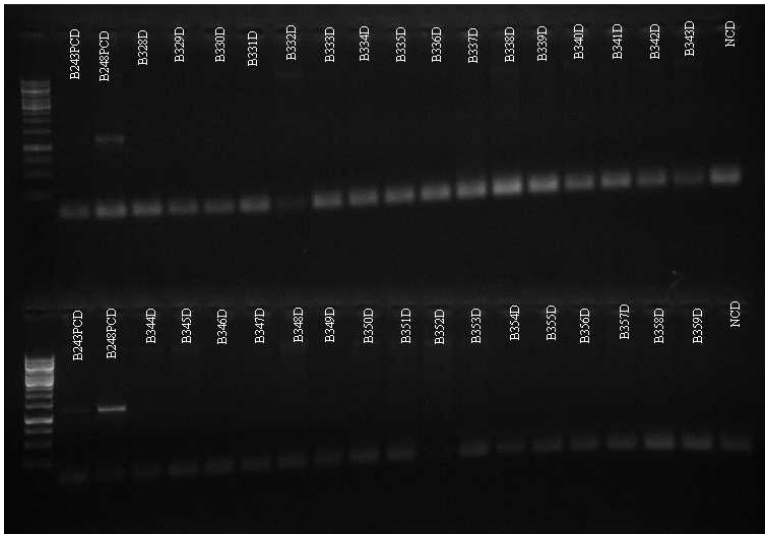

Figure C.6. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #6

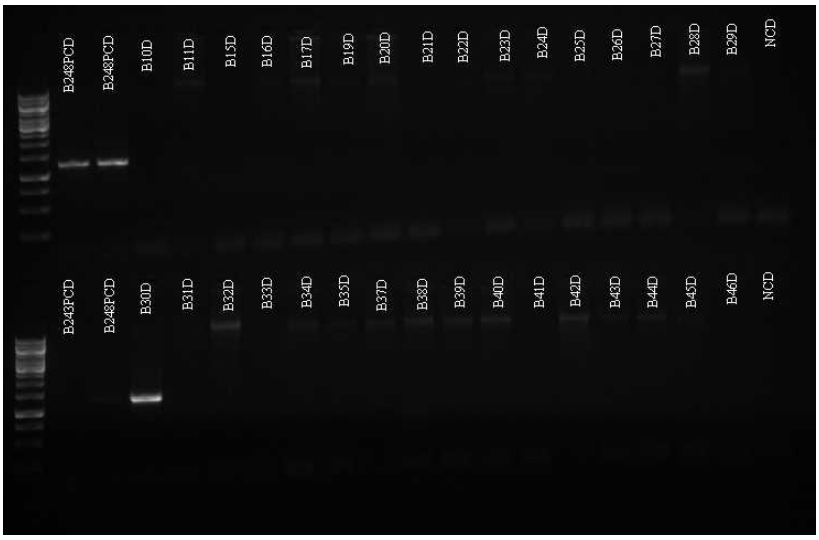

Figure C.7. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #7

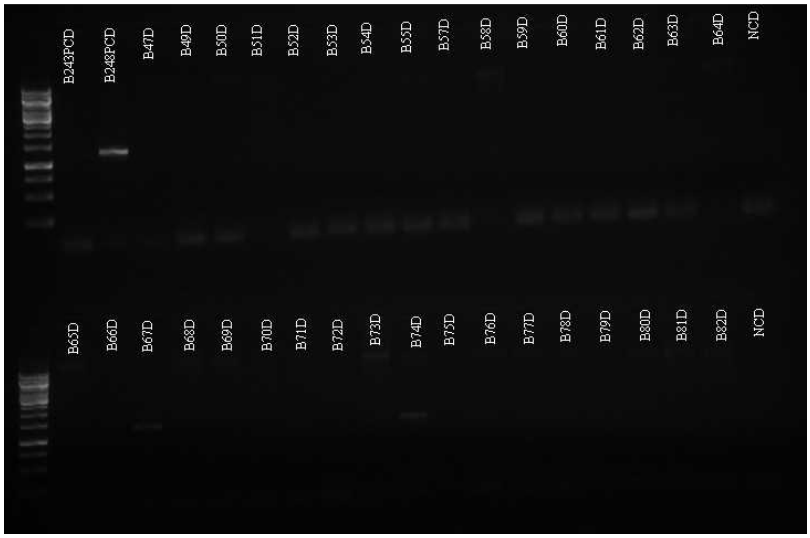

Figure C.8. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #8

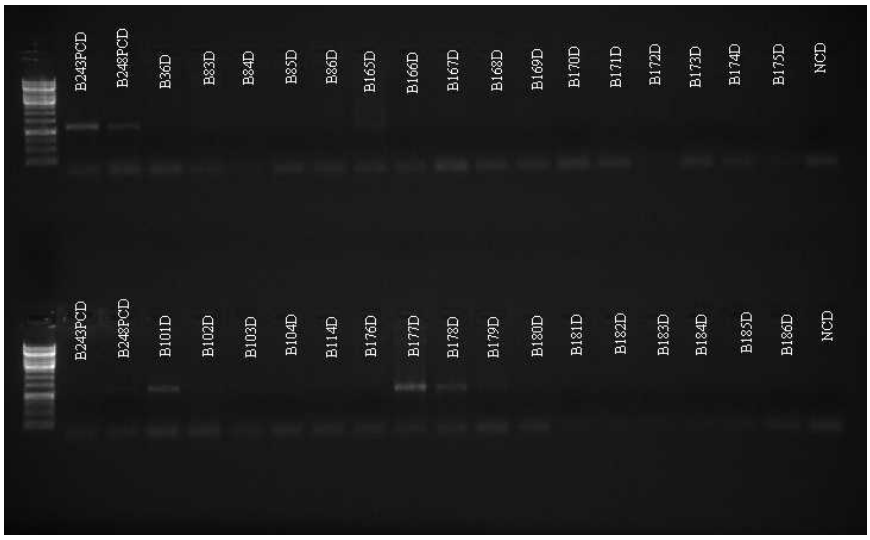

Figure C.9. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #9

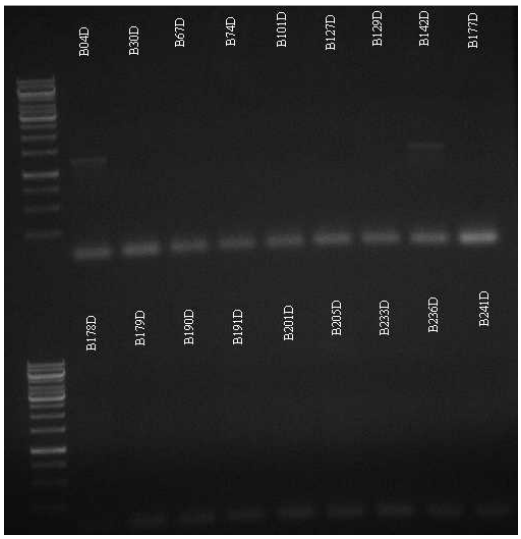

Figure C.10. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #10

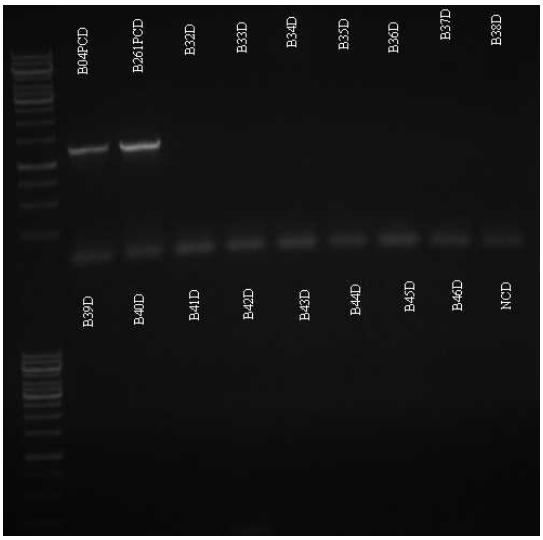

Figure C.11. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #11

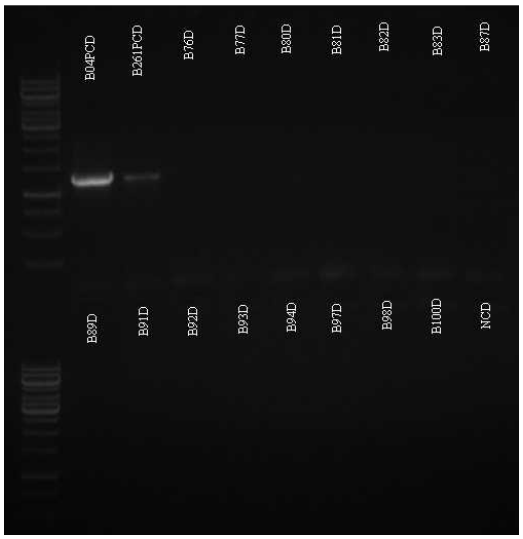

Figure C.12. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #12

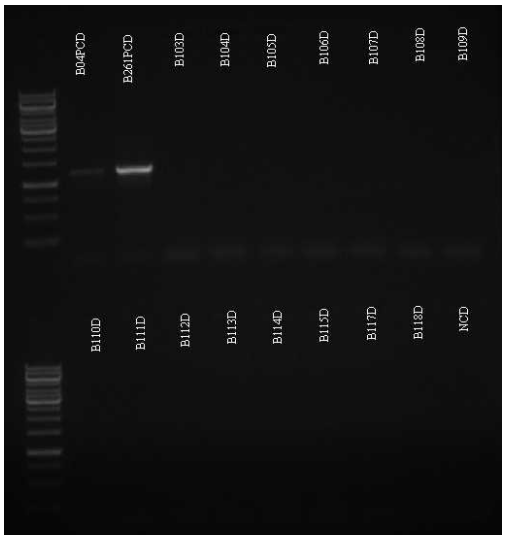

Figure C.13. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #13

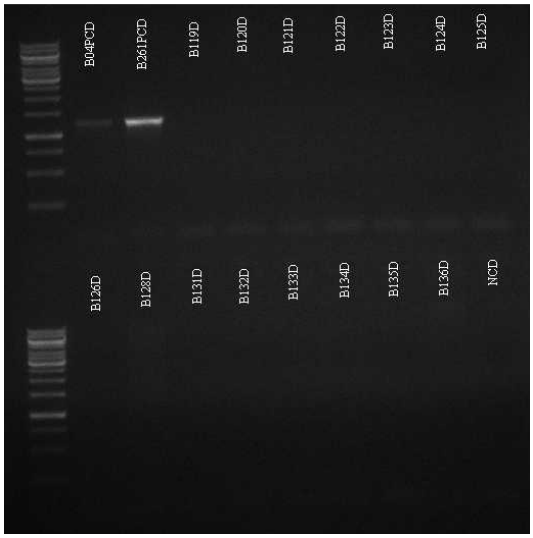

Figure C.14. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #14

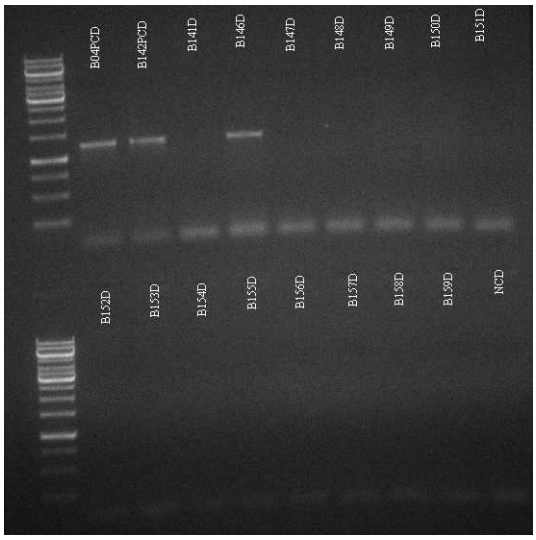

Figure C.15. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #15

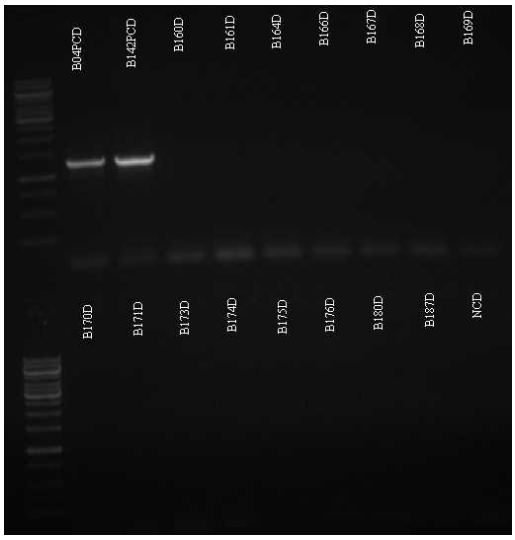

Figure C.16. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #16

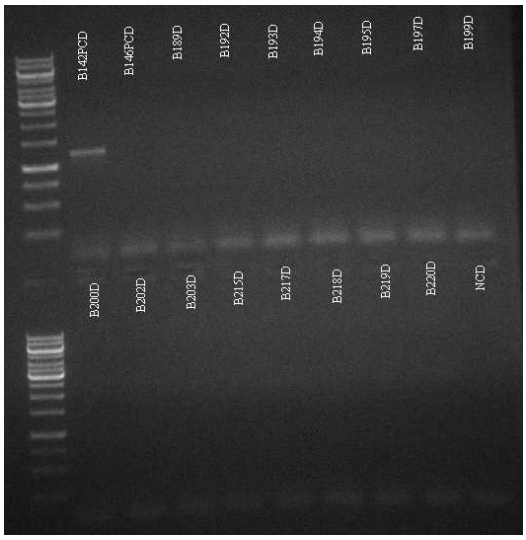

Figure C.17. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #17

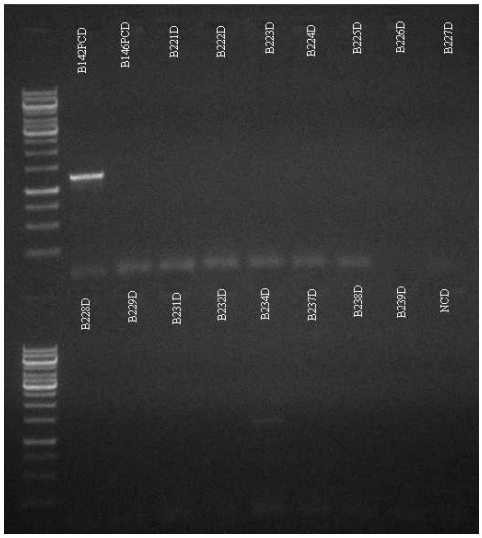

Figure C.18. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #18

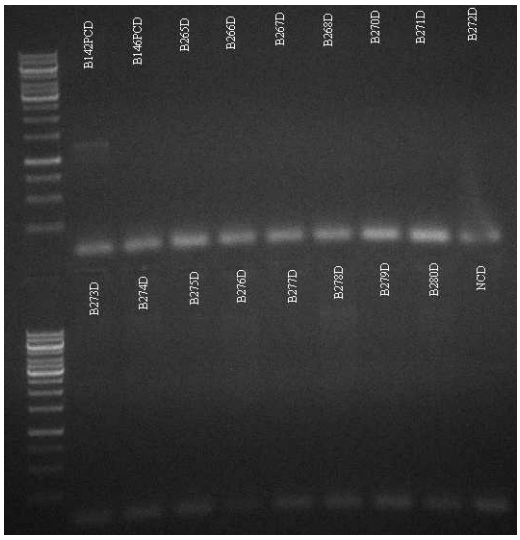

Figure C.19. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #19

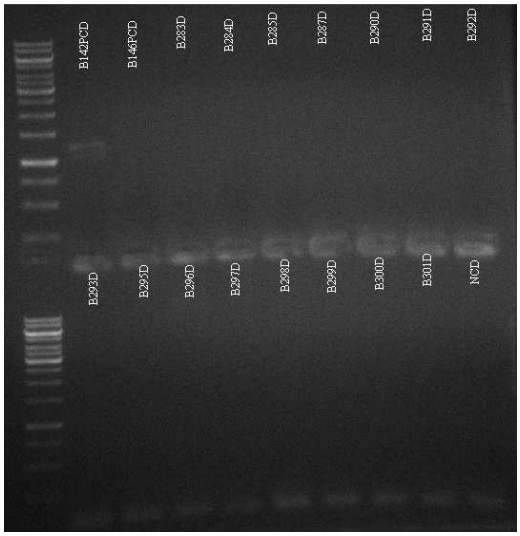

Figure C.20. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #20

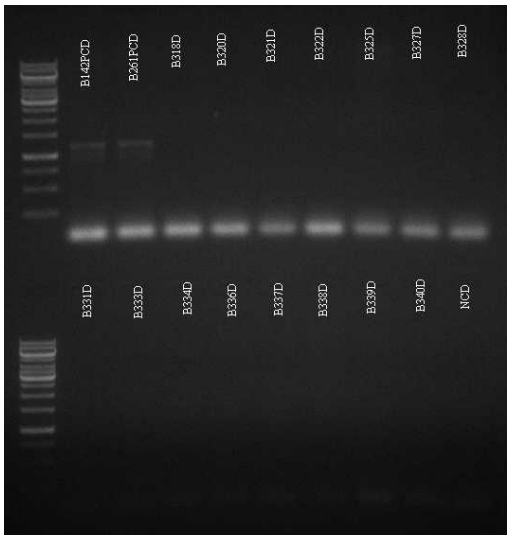

Figure C.21. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #21

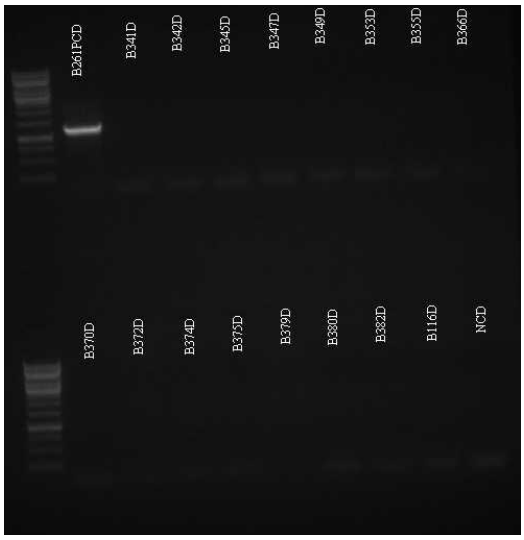

Figure C.22. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #22

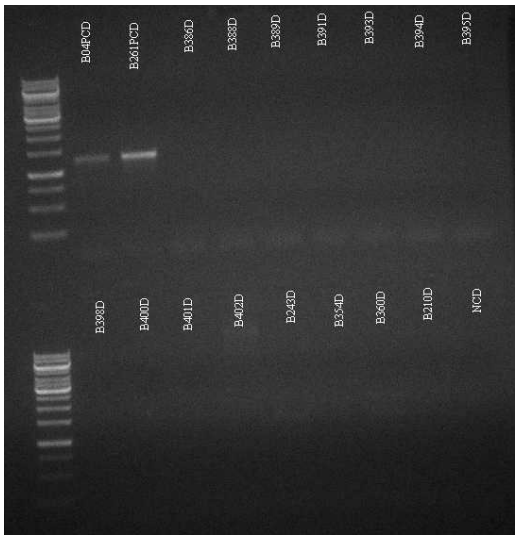

Figure C.23. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #23

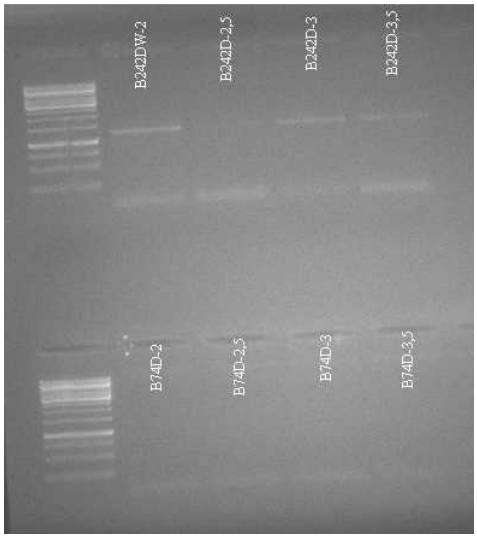

Figure C.24. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #24

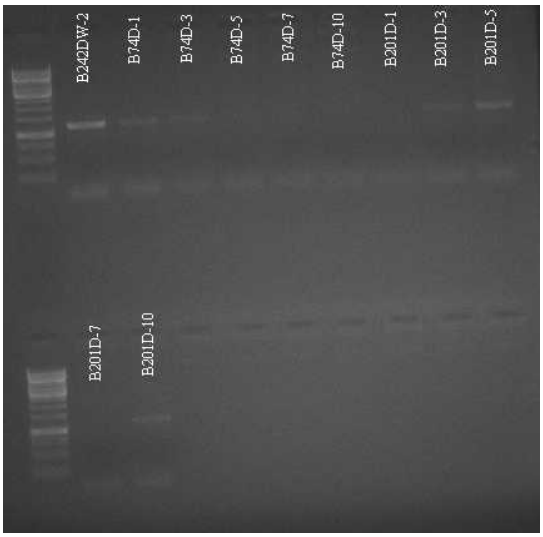

Figure C.25. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #25

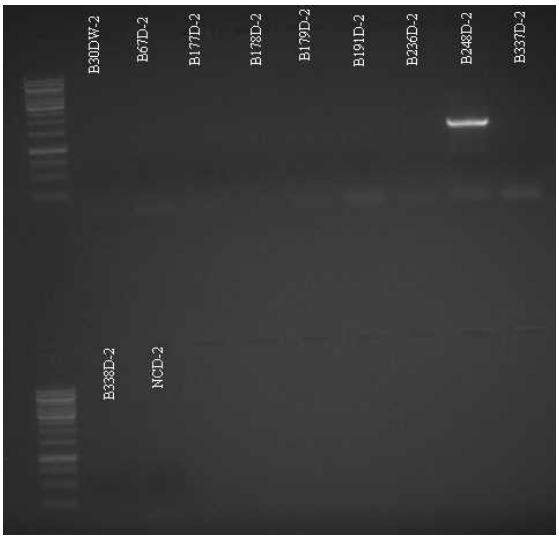

Figure C.26. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #26

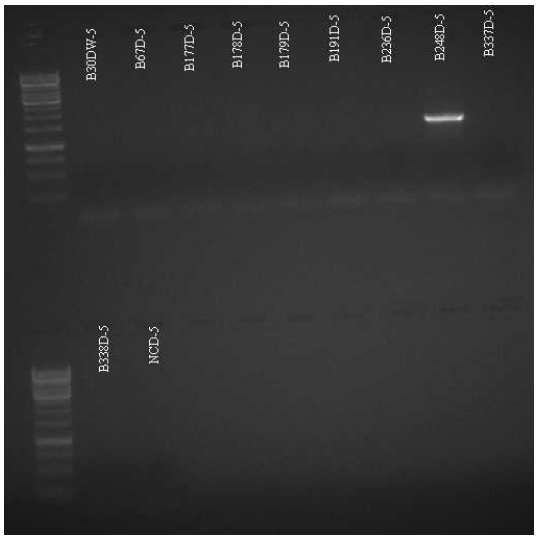

Figure C.27. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #27

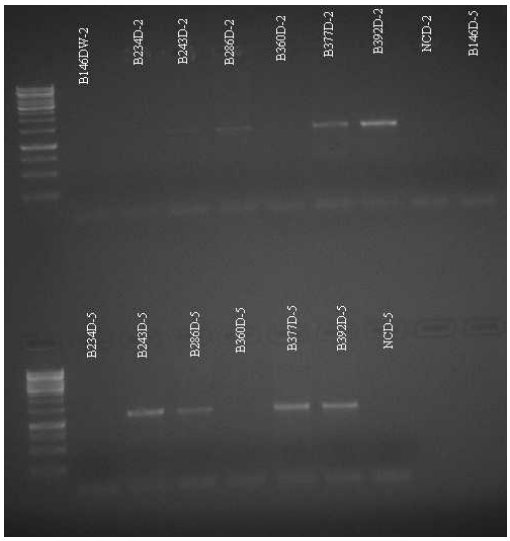

Figure C.28. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #28

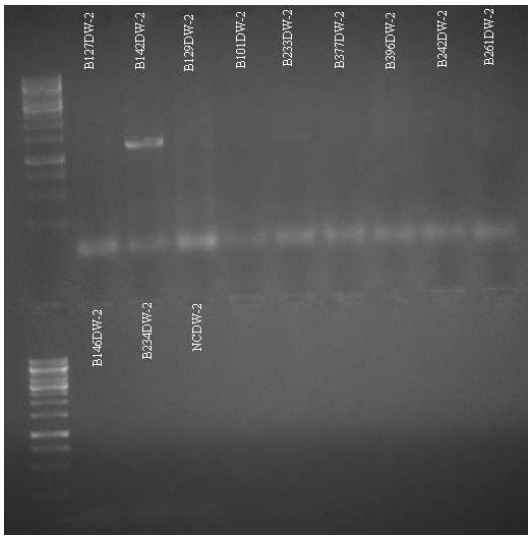

Figure C.29. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #29

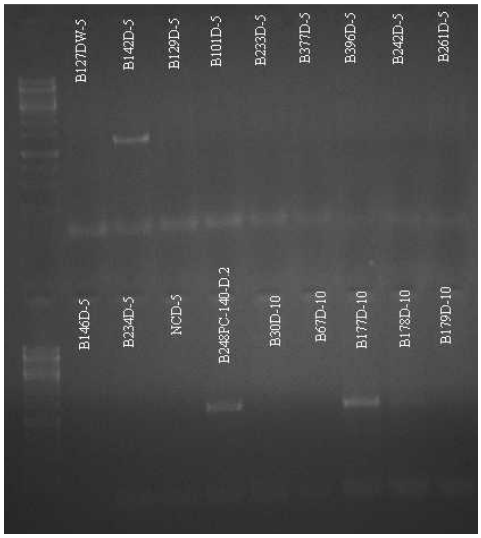

Figure C.30. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #30

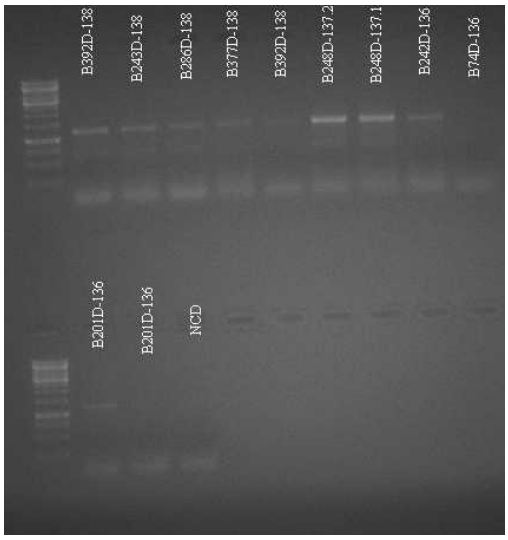

Figure C.31. Gel image of PCR reactions amplified with the primer pair DW2-DW4 #31

APPENDIX D: GEL IMAGES OF PCR REACTIONS AMPLIFIED WITH THE PRIMER PAIRS LEUCOF-LEUCOR AND DW2-DW4

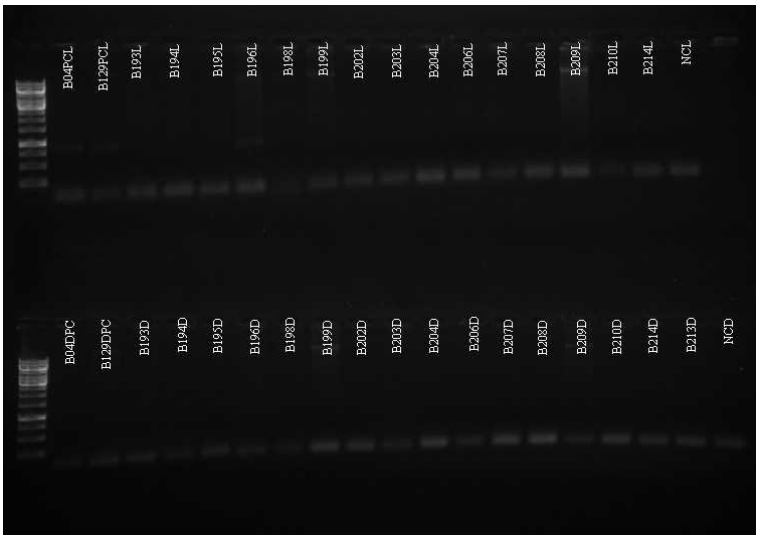

Figure D.1. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #1

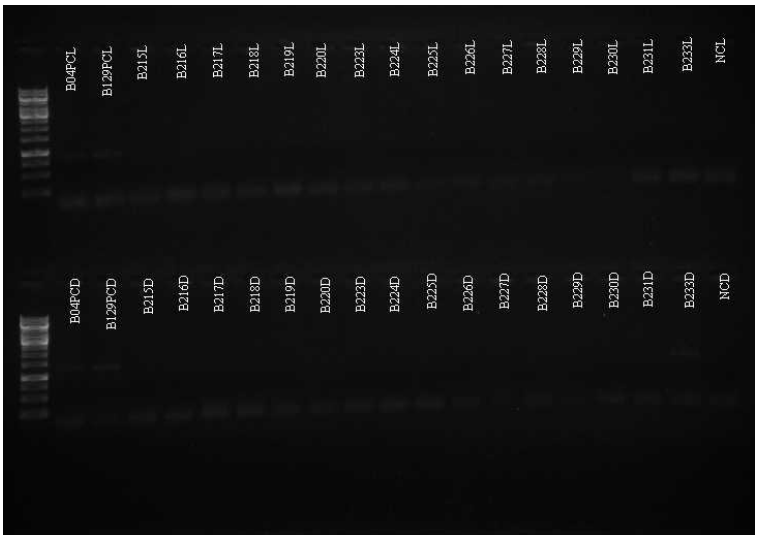

Figure D.2. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #2

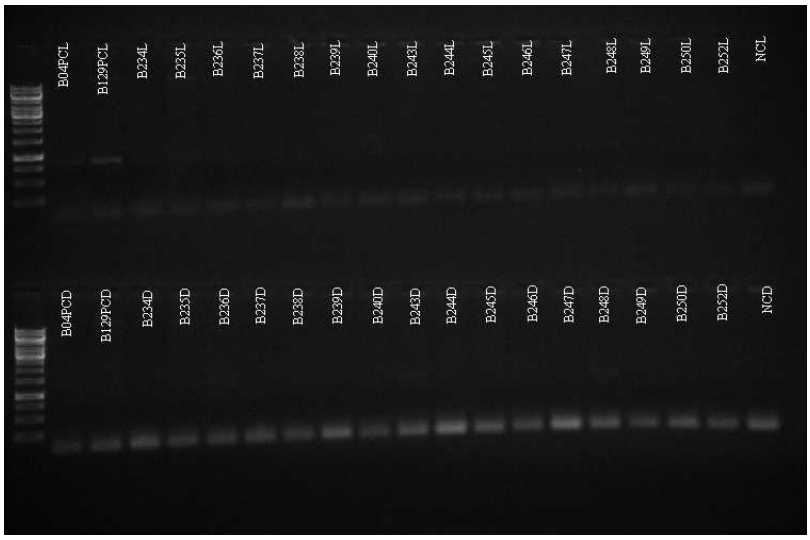

Figure D.3. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #3

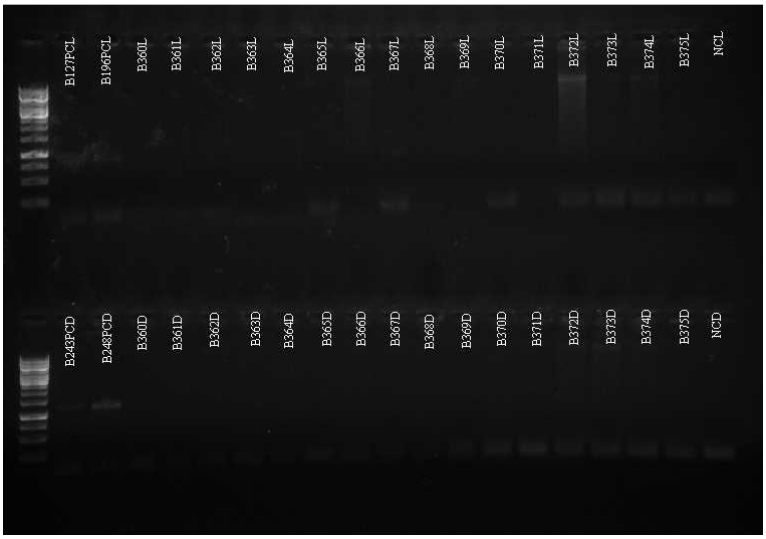

Figure D.4. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #4

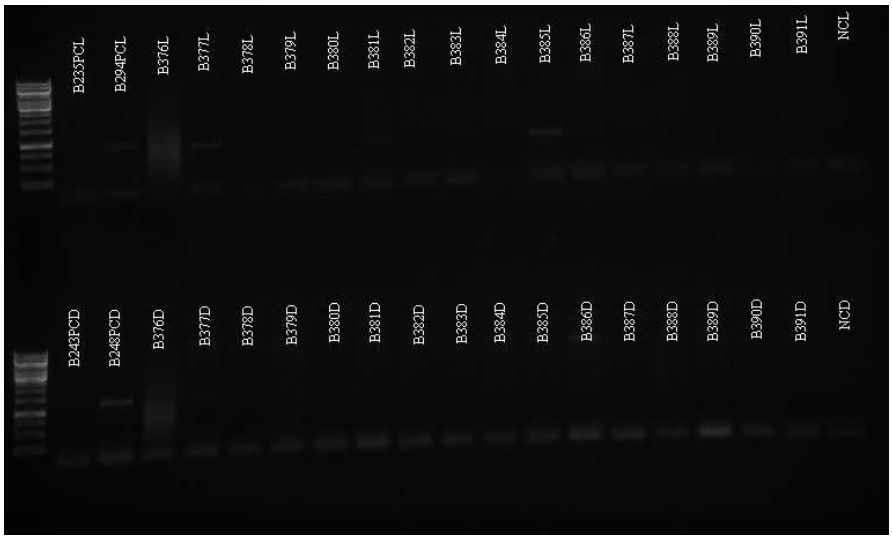

Figure D.5. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #5

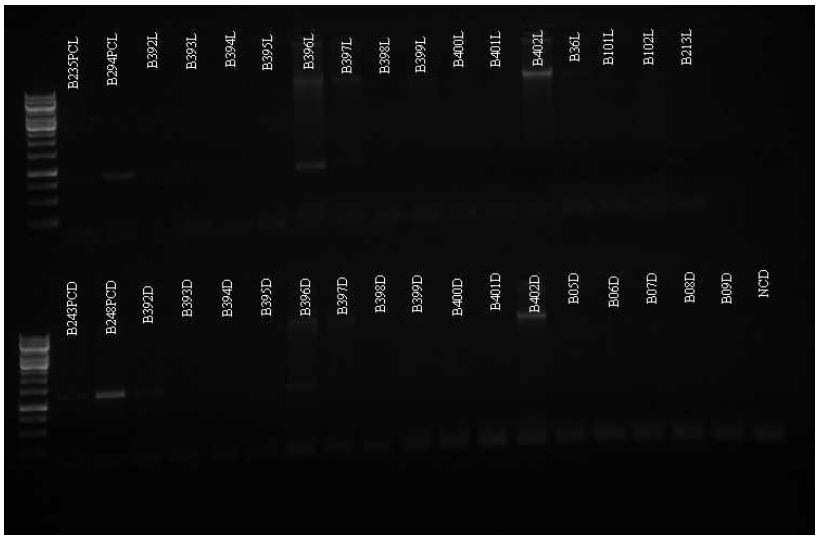

Figure D.6. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #6

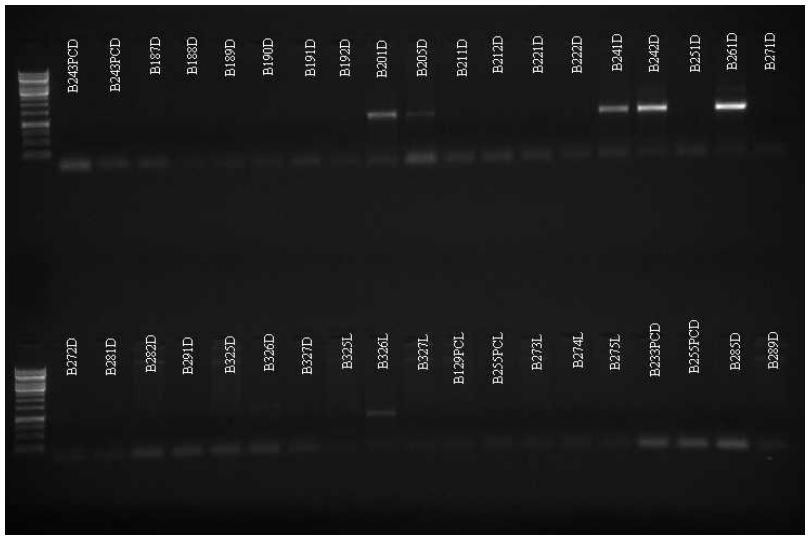

Figure D.7. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #7

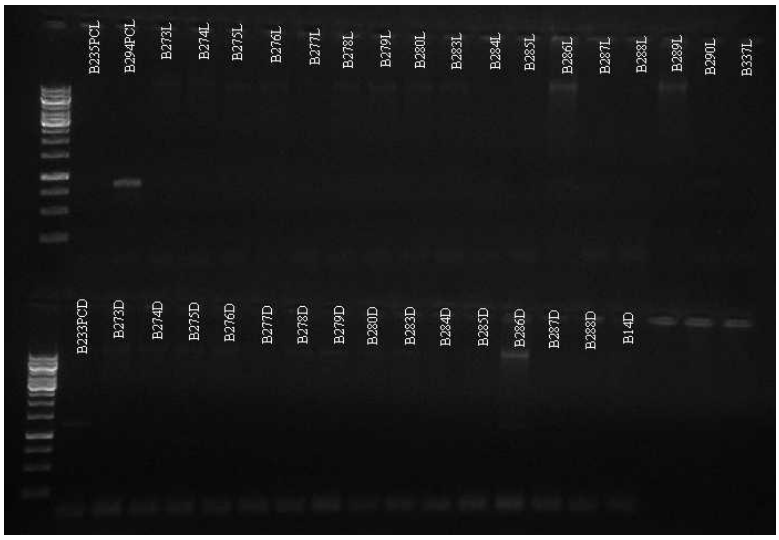

Figure D.8. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #8

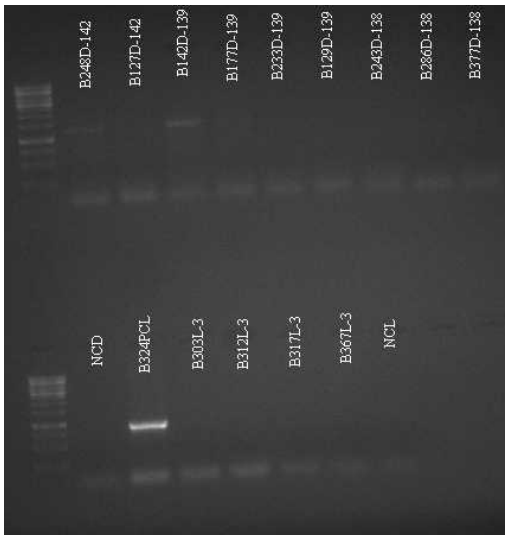

Figure D.9. Gel image of PCR reactions amplified with the primer pairs LeucoF-LeucoR and DW2-DW4 #9