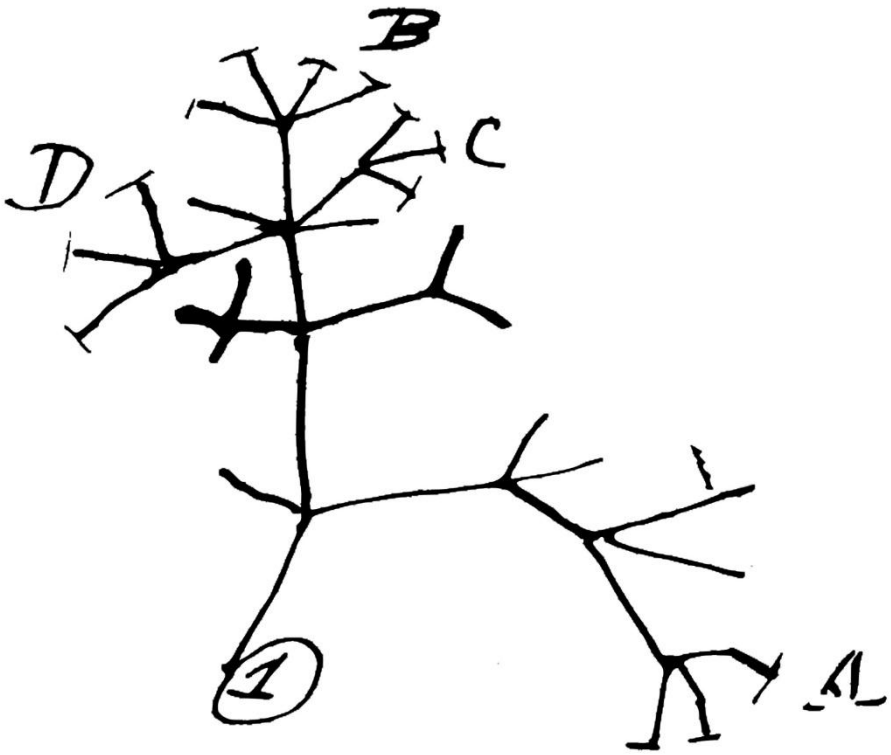
Integrative taxonomy for the *Motacilla alba* complex

Motacilla alba alba and *Motacilla alba yarrellii*



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Cover image: Darwin's Tree of Life

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Introduction

Taxonomy is central in biology; it determines if individual organisms are part of the same entities and helps the organization of those. Multiple disciplines provide valid information when trying to define new species, but a gap in communication between all and the fact that each perspective has a failure range difficult clarity in the decision-making process. Integrative taxonomy proposes combining multiple, complementary disciplines in order to achieve a more robust procedure (Steiner et al. 2010). Disagreement among disciplines can be solved by elucidating its evolutionary causes, and makes final resolutions more vigorous. This is the real challenge for future taxonomy, and will potentially benefit the field.

Integrative taxonomy uses genetics as an important information source as they give themselves some of the most precise resolutions when trying to discover undescribed species candidates. New technical improvements in sequencing short DNA fragments have made DNA taxonomy, also called barcoding, a large-scale, reliable discipline (Hebert et al. 2005). To resort to it, consensus of the scientific community is needed. Genes which allow repeatable amplification and sequencing and provide unequivocal resolution to organisms ID must be selected. The gene must be sufficiently variable to discriminate among species and to permit information without the risk of failure when analysing pooled samples. Alignment among distantly related taxa and sequencing with different protocols must be possible when using the gene. The C-terminal fragment of the mitochondrial gene for cytochrome oxidase, subunit 1 (COX1, COI) has demonstrated to fulfil these requirements and scientists advocate its use when sequencing and barcoding (Hebert et al. 2005). The use of one of COI's regions, COI-5P, is especially effective in arthropods and birds.

The White wagtail (*Motacilla alba*) complex is a group of subspecies which shows polymorphic characters, especially marked morphological and distribution differentiation, and its taxonomy is a controversial issue. This article focuses on the differences between two of the eleven *Motacilla alba* ssp.: the White wagtail (*Motacilla alba alba*), which breeds in most of the European continental countries, and the Pied wagtail (*Motacilla alba yarrellii*), the only

breeding *M. alba*. in the United Kingdom and Ireland. Up to date, most authors describe *Motacilla alba yarrellii* as another *Motacilla alba* subspecies (Gould, 1837), while some consider the Pied wagtail an independent species (*Motacilla yarrellii*).

The taxonomical status of the Pied wagtail is a recurrent discussion issue in the birding world, and, personally, I have been really familiar with it since I was young, being involved in different activities and projects. This interest into ornithology has leaded me to constantly be aware of any taxonomical changes among bird species, as they influence data collection in the field and during bird banding or counting. Moreover, especially during this last year, I have also been able to work in the genetics and evolution field. The connection of both ideas, along with the opportunity to take over a scientific research is what has conducted me to decide to take part in the process of studying the taxonomy of a particular species. After that, particular curiosity and interest into the Wagtail case together with the scientific controversies it causes among authors are the reasons why my research will be done around this complex and not another with a similar situation.

This project has two aims:

- Studying the divergences between both ssp. in order to determine the taxonomical status of the Pied wagtail (*Motacilla alba yarrellii*) and seeking for possible support to delineate it into an independent species.
- Testing the effectiveness of the COI-5P gene in taxonomy and its use between families, species and subspecies of birds.

About the first and main target of the work, distribution indicates possible sympatric breeding events between both ssp. of Wagtails, so one theory regarding the results involves the evolutionary paths of *Motacilla alba alba* and *yarrellii* being split recently. This would explain the evident morphological variances between both, while really marked genetic divergence may still take some time to appear.

To answer both of the questions, this report is divided into two main parts, which go through different fields used in integrative taxonomy. On the one hand, data about morphology, geography, behaviour and song are compared. I have reviewed scientific articles and books and personal knowledge and information from known people involved in the field has also been contrasted and included when necessary. On the other hand, the second section goes into genetics and analyses the COI-5P gene region of *ssp. Motacilla alba alba* and *Motacilla alba yarrellii* in order to find genetic variability between them by comparing sequences from both *wagtails.*

1. Theoretical background

1.1. The cell

Cells are the smallest structural and functional units of an organism; they are the basic elements which form all living things. Moreover, they carry out different functions which are essential for life, besides of containing the body's genetic hereditary material. Each part of the cell develops a different task, which is more specialized in organelles. There are two types of cells, according on how they store DNA.

1.1.1. Prokaryotic cells

Prokaryotic cells are those which **don't have a defined nucleus**. Their DNA is not enclosed within a nuclear membrane, but in the cytoplasm. Single-celled organisms are often prokaryotic cells. **Bacteria** and cyanobacteria are part of this group, and a clear example would be *Escherichia coli*. Prokaryotes were the first living things to evolve and are still the most common organisms today. In fact, archaeabacteria (ancient bacteria), treated as a different group form normal bacteria, are considered the oldest living organisms on Earth and are also prokaryotes (Gupta, 1998). They are found in the intestinal tracts of different mammals, such as cows or humans. Archaea are obligate anaerobes (Langworthy, 1982) and convert H2 and CO2 into methane. They divided into three phyla: the halophiles, the methanogens and the thermoacidophiles.

In contrast with the second type of cells, prokaryotic ones have defined parts. To start, they are surrounded by a **plasma membrane** and a **cell wall**, which keep their shape, protect the cell and regulate the exchange of substances between the interior and exterior sides of it. In addition, the whole cell is recovered by a solid **capsule**. To continue, prokaryotic cells have **cytoplasm**, which is composed mainly of water, salts and enzymes, and also contains the rest of organelles. The basic organelles are mesosomes, food stores and ribosomes. **Mesosomes** are associated with the respiration of the cell and are located on the infoldings of the plasma membrane, called cristae. Though, they are not present in all prokaryotic cells. **Food stores** can be of different sizes and colours because they can either be lipid globules or glycogen granules.

Ribosomes in prokaryotic cells are 70S type (Ben-Shem et. al., 2010), and their function is to translate the messenger RNA into protein. Furthermore, prokaryotic cells contain **plasmids**, which are moving gene carriers and are involved in DNA exchange. The **nucleoid region** is the area of cytoplasm where a single DNA molecule can be found. DNA is transferred from one cell to another inside plasmids and by pili.

Finally, flagellum and pili are the two parts in charge of the movement of the cell. The **pili** are attached to the cell and are used to attach to other bacterial cells and surfaces, besides enabling the exchange of substances from the exterior. **Flagellum** is uniquely used for locomotion. Prokaryotic cells move through liquids and the motion can involve the spin of the flagellum or the pili, which scrolls by pulling (Ken, Mark, 2008). Most microorganisms count on complex sensory systems which help them move to optimal environments.

1.1.2. Eukaryotic cells

Eukaryotic cells are more complex than prokaryotic, and have a nucleus where they store DNA. Animals, plants, fungi, and other groups such as protozoa are built up out of this type of cells. There are different kinds of eukaryotic cells: fibres and neuroglia are examples of **animal cells**, and parenchyma or sclerenchyma cells are types of **vegetal cells**.

There are some parts of this type of cells which are basic and can be found in all of them. First, the **nucleus** is the central unit of the eukaryotic cell. It contains and protects the genetic information. Surrounding the nucleus there is the nuclear membrane, which regulates the entry and output of substances within the nucleus and the rest of the cell. DNA in eukaryotic cells is organized in chromosomes and gets transferred to the rest of the cell by a process called the central dogma of biology. Thus, this process starts in the nucleus, where genes are copied and transcribed into messenger RNA. Next, mRNA is transported outside the nucleus and ribosomes translate it to proteins.

To continue with the basic structures, the **plasma membrane** also plays a crucial role in the functioning of the eukaryotic cell, as in the prokaryotic one. It is in charge of the communication with the environment and controls what gets

into and out of the cell. In contrast with the prokaryotic, eukaryotic cells have type 80S **ribosomes** which are larger than 70S type. These ribosomes contain more rRNA, although the functioning is exactly the same to the prokaryotic one (Verschoor, 1996). **Cytoplasm** and **cytoskeleton** are also present in eukaryotic cells and the organelles they contain vary depending on the type of cell. Organelles are responsible for producing ATP (adenosine triphosphate), which are molecules in charge of carrying energy (Timmis et. at. 2004).

Animal cells have **peroxisomes**, which metabolize the waste of the cell; **lysosomes**, which digest food, and a **Golgi apparatus**, which modifies proteins. Also, they count on **mitochondria**, which produce energy, and a **rough and smooth endoplasmic reticulum**, which make lipids and secretory and membrane proteins. **Centrioles** help the cell during the process of meiosis and mitosis, while **pili** are used to move and communicate with the exterior and **microvilli** increase the surface of the cell without increasing its volume. This improves the cell's absorption and secretion abilities. Finally, they have a small **vacuole** which is much larger in vegetal cells. Its function is to store and isolate products, and also water in the case of plant cells. Vegetal cells have some other significant differences with animal cells. To start with, they are surrounded by a solid **cell wall** and contain **chloroplasts**, responsible for the photosynthesis. Moreover, **plastids** are in charge of storing pigments and **leucoplasts** are used to store substances. To finish, **plasmodesmata** are channels bound to connecting different plant cells (Lucas et. al., 1993).

1.2. Molecular biology

The genome contains all the hereditary information in a cellular organism. DNA or **deoxyribonucleic acid** is stored in all cells; and is found basically in the nucleus, although it can also be present in mitochondria. In that case, we are talking about mitochondrial DNA (mtDNA). Even though DNA is the same in all cells, each gene is shown is concrete places, shaping the different traits of an organism. Therefore, **DNA determines which characteristics are to be activated or created**. Later, RNA (ribonucleic acid) carries out the transference of this genetic code, which allows the creation of proteins in the cell.

A repeated pattern of a **sugar** and a **phosphate group** forms the backbone of DNA. Also, each block **nucleotide**, the building of DNA, contains a **base** (Roberts, 1992). As their names indicate, deoxyribonucleic acid contains deoxyribose, while the sugar present in ribonucleic acid is ribose. Deoxyribose is ribose molecule which has lost an atom of oxygen:

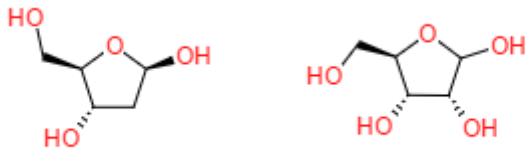


FIGURE 1: Deoxyribose and ribose molecules, respectively. Figure created using C*hemdoodle: chemical publishing software*.

The carbon atoms in these **sugars are numbered from 1 to 5**, starting always at the first carbon atom to the right of the oxygen (Biology discussion, 2013). Due to the presence of other ring compounds other than carbon, every number needs to written with a dash next to it. Hence, we number from 1' to 5'. The phosphate group is attached to the sugar molecule on the 5' carbon in the ring.

Finally, a base is needed to complete a nucleotide. Then, nucleotides will be arranged in strands forming a double helix structure. The four bases of DNA are **thymine** (T), **cytosine** (C), **adenine** (A), and **guanine** (G).

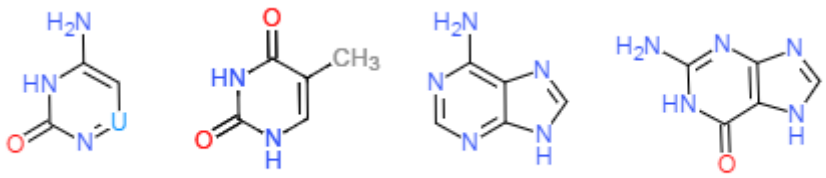


FIGURE 2: Cytosine, thymine, adenine and guanine molecules, respectively. Figure created using C*hemdoodle: chemical publishing software*.

Adenine and guanine are **purines**, which have two rings; while cytosine and thymine are **pyrimidines**, which have one ring. This is involved in the pairing of the nitrogenous bases, as a big base always attaches to a small one. Concretely, adenine pairs with thymine and guanine with cytosine. These

combinations, called **base pairs**, allow the most effective **hydrogen bounds** between the molecules, and also help keeping the same distance from the both of the DNA strands in the **double helix structure**. Bases act and are called as a base due to their possession of lone pairs of nitrogen, which act as electron donors (Liu, Barton, 2005). They attach to the 1' carbon atom carrying out a condensation reaction.

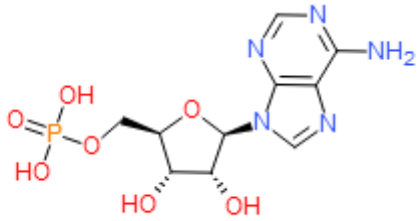


FIGURE 3: The complete structure of a nucleotide, with a base (adenine in this case) and the phosphate group attached. Figure created using *Chemdoodle: chemical publishing software*.

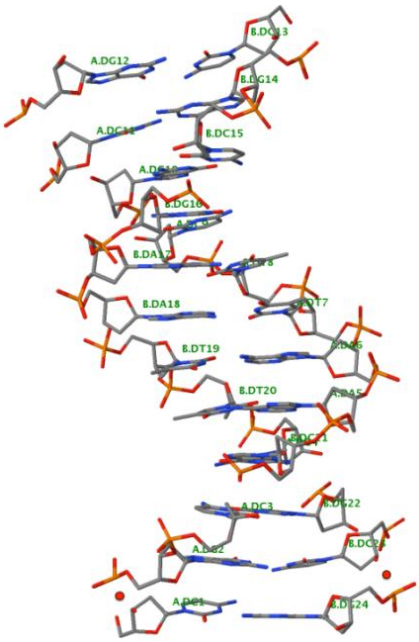


FIGURE 4: DNA double helix structure. Image retrieved from 3DNA: 3-Dimensional Acid Structures.

So DNA is formed by the union of nucleotides with different bases attached creating two long strands, which adopt a double helix structure. Nucleotides attach to each other by a condensation reaction: the 3' carbon atom of one sugar ring links to the phosphate group of the next nucleotide. The **sequence** the bases are found in the chain is what defines the different genes and, therefore, traits of the organism. This sequence is also what we use in **taxonomy**, because each species has a unique order of bases. Laboratory techniques such as the **PCR** (Bridge, Arora, 1998) help us find out which sequence defines each organism.

To finish up with the structure of DNA, the base pairs are a key factor. When joining the two DNA strands in the double helix structure, adenine needs to pair with thymine, and guanine with cytosine, as said previously. Both

of the strands run in a concrete order that we need to follow when talking about the genetic code. When analysing genes, sequences are always written from the 5’ end to the 3’ end.

One property of DNA which is worth highlighting is that it can **replicate** when cell division takes part. This allows the new cell have an exact copy of the DNA found in the old cell.

1.3. Semi-conservative replication

DNA carries out a complicated multi-enzyme process when replicating. The first step, carried out by helicase enzyme, consists on **unzipping the two strands of the double helix structure** so hydrogen bonds are broken between the base pairs. To separate the strands, helicase spins the DNA at about ten thousand RPM (Bujalowski et. al., 1994). This happens in different concrete regions all along the double helix called **replication forks**. Each of these areas is where the **synthesis of the new chains** will take place. Depending on its orientation, each strand receives a different name. The 3’ chain is called **leading strands**, while the 5’ one is the **lagging strand** (Chemguide, 2009).

When strand separation is finished, **new nucleotides are created** by the primase enzyme, which walks along the leading strand and later along the lagging one attaching the needed complementary bases. Next, a new enzyme called **exonuclease eliminates the primer** and the whole **new strand is sealed up** by DNA ligase.

DNA polymerase is the enzyme in charge of joining up the new nucleotides with the old ones, and two types are needed, due to the presence of two differently oriented strands. Thus, the lagging strand requires a more complicated copying process than the leading one due to its inverted orientation. To start, it is organized into fragments, called **Okazaki fragments**, which are later synthetized by a second polymerase enzyme oriented from 5’ to 3’ by the process described above (Magnusson, 1972).

The whole mechanism is called **semi-conservative replication** because half of both of the new double strands was a part of the old one, and the other half is new.

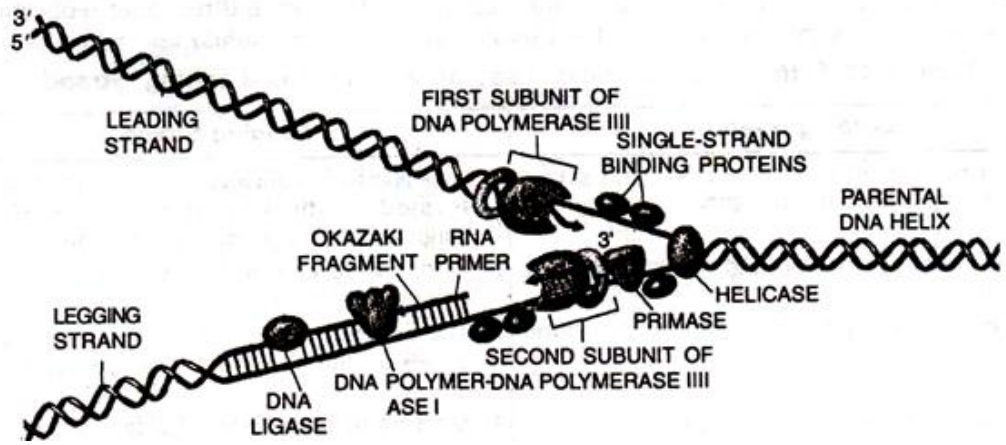


FIGURE 5: Diagram showing semi-conservative replication of DNA. Image from Biology discussion: DNA Replication

1.4. Transcription

The process of obtaining a protein sequence from the genetic code in DNA is called transcription. The first step of it is where **the information of the genes in DNA is transferred into RNA**. RNA is much shorter than DNA and contains the essential information that codifies for one single polypeptide chain. As stated before, RNA is built up of ribose and consists on a single chain instead of a double helix chain as DNA. Moreover, **it doesn't use the base thymine** (T), **but uracil** (U). This is due to the single strand structure of RNA. Thymine helps to attach one strand to the other in DNA, while RNA doesn't require it.

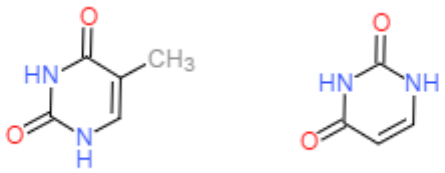


FIGURE 6: Difference between thymine (left molecule) and uracil (right molecule). Figure created using C*hemdoodle: chemical publishing software*.

The genetic information is found in only one of the two strands of DNA, the **coding strand**, which is read form 5' to 3'. The **non-coding strand** can also be called **template strand**, and is complementary to the coding one.

To first step of the transcription process is **selecting a gene and finding its start on the coding strand**; it is carried out by the enzyme called RNA

polymerase. This enzyme recognizes the right **promoter sequence** by detecting which end of the gene is closer to the 5' end of the strand and then wraps around it. Then, it **unwinds the double helix for a length of around 10 bases**, and **new nucleotides are added to this RNA chain starting at the 3' end**. After this, the enzyme moves along the gene zipping the DNA double chain behind it adding new nucleotides all the time, while **the RNA detaches from the template strand** (Genetics Generation, 2015).

Finally, the enzyme needs to find the end of the gene. The same way it recognizes upstream sequences, it can **find the downstream of a gene**, called the **termination sequence** of bases. Once the enzyme gets to this sequence, it detaches completely from the DNA chain and a new molecule of messenger RNA is finished. It will later get the genetic code to the cytoplasm of the cell.

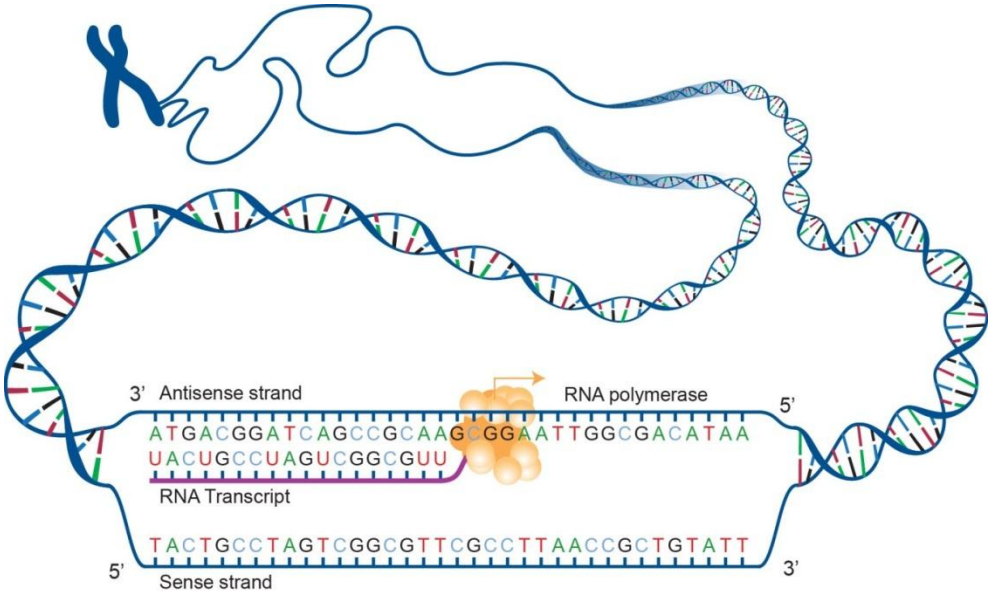


FIGURE 7: Diagram showing transcription from DNA to RNA. Image from Genetics Generation: DNA and proteins.

This code is based on only 20 different amino acids which codify for different proteins, and each of them is determined by three of the four bases of RNA. This results in 64 possible combinations, and each of them is called a **codon**. There are **three stop codons** (UAA, UAG and UGA) which mark the end of the chain (Ito, Namakura, 2000). Moreover, the **start codon** (AUG) signals the start of a new protein chain.

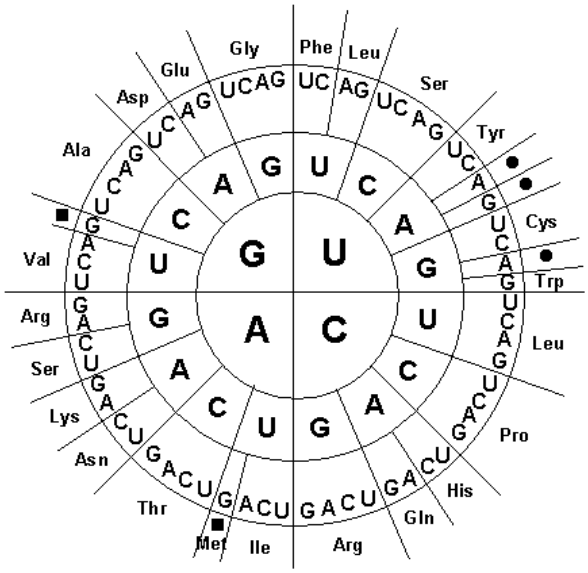


FIGURE 8: Genetic code read from the center (5') to the edge (3'), which shows what amino acids codify for each protein. Image from: Society For Biomedical Diabetes Research (SBDR).

To translate the genetic code into a protein chain, mRNA, which wouldn't interact with individual amino acids, needs to be converted into **tRNA** or **transfer RNA**. To do so, two structures are involved: ribosomes and another type of RNA, rRNA or ribosomal RNA.

To start with, **ribosomes** need to find the upstream of the start codon. They have a **large and a small subunit**, the second of those is responsible for **identifying the start point**. It attaches to the mRNA chain from the 5' end and moves along it until it finds a concrete pattern of bases where it is able to bind to. This sequence is found just before the needed start codon (SBDR, 2016).

The next step is where tRNA comes in. It is a short sequence of about 80 bases of mRNA, and the last base sequence is always CCA. **tRNA carries amino acids to the messenger RNA chain** and makes it possible to them to bind together. To make amino acids annex to it, the –OH group on the 3' carbon of the last ribose ring in a tRNA chain attaches to the –COOH group of an amino acid, forming an ester between them. At the bottom of the tRNA molecule there is the anti-codon, which is in charge of **pairing up each amino acid with the right tRNA molecule**. It does so using a shape recognition process carried out by enzymes.

Finally, the **message in mRNA** needs to be **decoded into a protein chain**. Messenger RNA is now attached to the upstream of the start codon, and the anti-codon of the tRNA carrying one amino acid pairs together with the complementary bases on this mRNA. When this happens, the ribosome is also totally bound to the system (Genetics Generation, 2015). Next, a repetitive process starts. **New complementary tRNA molecules with different amino**

acids bind to the chain, making the ribosome move along the mRNA to the new codon each time. At the same moment, peptide bonds are made between the amino acids, so the tRNA can break free and pick up the next amino acid.

At the end, the ribosome finds a stop codon, which doesn't code for amino acids, and unbounds from the protein chain.

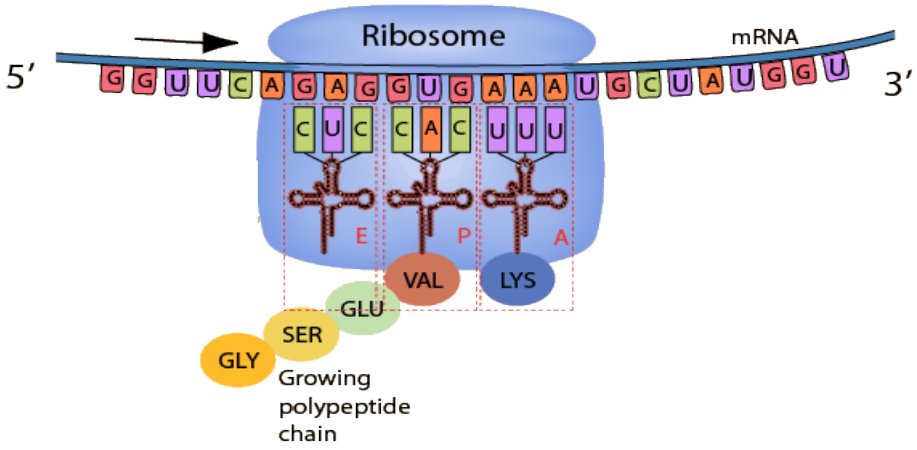


FIGURE 9: Diagram showing the process of the protein chain transcription. Image adapted from a figure found at hyperphysics website.

1.5. Evolutionary genetics

So the key for evolutionary success of a population lies on **genetic variability**, which is what defines **polymorphic characters**. The presence of diverse **phenotypes**, or appreciable characteristics of an individual, is a sign of this **genetic richness** and also indicates the existence of different **genotypes** or genetic information. This diversity is important for every group of organisms as it decides the **adaptation** and therefore, **survival capacity** of the body (Charlesworth et. al., 2009).

One way of expressing genetic variability is by expressing the data in terms of **allelic frequencies**. At least two alleles will define each gene, and consequently each genotype and phenotype. The most common genotypes in one population will define its genotypic and allelic frequency. Mating causes genetic exchange or **gene drift**, as some characters appear more attractive than others and help individuals select a mate. These individuals carrying the

most appealing characteristics will transfer their genes to the next generation. Given the condition that any factors would change allelic frequencies, the genotype of a population would remain exactly the same through time. This is explained by the **Hardy-Weinberg Law**, which predicts how gene frequencies are transmitted from one generation to another (Stern, 1943). Though, many factors influence gene transmission, such as **viability** or **fertility**. Also, **migration** and **mutation** create **gene flow** and variation among a population, and eventually the best adapted individuals will be the ones which survive. This concept of **natural selection** is what drives evolution according to Darwin, and has been confirmed to operate in the wild by thousands of papers (Leimu, Fischer, 2008).

Then, speciation is directed by mutation, migration and selection, but the most important factor is **genetic variability**. All mutations and other genetic changes do is create more **inheritable stable patterns of gene frequencies** within populations, enabling selection and evolution to work. So a **species** defines a group of individuals which can mate and offer **fertile offspring** (Intermediate genetics, 1997). As one population goes through changes it can ultimately diverge from the rest of the group creating a new one, by the called **anagenesis** or **phyletic evolution**. For another part, **cladogenesis** or **true speciation** describes the separation of a population in two species which co-exist, and is due to reproductive isolation (Charlesworth et. al., 2009). If a population is divided by a physical reproductive barrier, we talk about **allopatric speciation**; while when the cause of isolation is the migration of a subgroup of the original population into a new ecological niche, **parapatric speciation** takes place. Finally, **sympatric speciation** occurs when a subpopulation develops a more advantageous or different characteristic from the rest of the group, and that prevents inter-breeding.

In modern biology, the principal component in taxonomy and evolution is **random genetic drift**. **Sequencing** has enabled the detection of individual genotypes and different genetic patterns within populations, and databases such as GenBank provide vast amounts of information. This can make possible to detect minimum species divergence; and new technology has also made easier to observe **genetic markers**, or identifying factors, which has made a

difference in **gene mapping** even in non-model populations (Hayes, Goddard, 2001).

1.6. Mutations

Of course, any change in the replication or transcription of the genetic code, apart from radiation or particular chemicals, can cause an effect on the way genes express in an organism.

Each codon of a gene is formed by three bases, the first and second of which define the codification to proteins. Sometimes, one of the bases is replaced by another by mistake, causing a **substitution mutation**. On the one hand, any change on the first two bases of the codon would make a different protein (**missense mutation**) or even a stop codon (**nonsense mutation**) appear. That could have diverse effects depending on where the stop codon is formed. If it appears in the middle of the gene, it would make the protein too short, causing it to work improperly. Moreover, the creation of a different amino acid could also cause problems in the functioning of an enzyme, in the case of appearing next to its **active site** (Genetics Home Reference, 2013). On the other hand, a change in the third base of the codon wouldn't make any difference in the protein sequence. This type of changes are called **silent mutations**, and may have **a taxonomical meaning rather than evolutionary**.

More serious mutations happen when bases are inserted or deleted into the genetic code, as they break or add codons, and the whole sequence after them becomes completely meaningless. Then, the folding of the protein is also changed. Even though, if the deleted bases are part of a whole codon, for example, the rest of the chain would remain unchanged. When the message in the genetic code is no longer correctly parsed due to a deletion of insertion mutation, we talk about a **frameshift mutation**. Again, the importance of the change in the protein chain depends on where the mutation is found (Understanding evolution, 2016).

Mutations can cause a wide range of effects, from being completely unnoticeable on the phenotype of the organism to resulting into new features. If these are positive to the individual, evolution can take place, while if the effect

of the new characteristic is negative, it can provoke a disease or even the death of the organism. Of course, there are enzymes that fix possible mistakes during cell division to try to avoid this type of problems.

Finally, **phenotypes** are the type of variation that at least 1% of a population of organisms has, and is a potential source for future species separation.

1.7. The role of subspecies

So **hybridization**, **reproductive isolation**, **gene flow** and **directional selection** make taxonomy complicated, and one example are **polytypic species**. Those are species with distinct geographically separated populations that would interbreed if there was the opportunity (Wilson, 1953). This gives place to **subspecies**, considered low-key forms until the 1920s (Mallet, 2013). Even though, their importance was already remarkable, as the **climatic rules** of Gloger, Bermann and Allen were first studied through the analysis of subspecies (Mallet, 2013). Regardless though, the taxa was only employed when studying large museum or private collections (Philimore et. al., 2007). Recent advances in our comprehension of the genetics in speciation have enabled to recognize evolutionary paths which were before ignored and, therefore, to categorize **subspecies as a taxonomically meaningful status** (Gill, 2014), (Haig et. at., 2010).

Even though, subspecies are still controversial, as it is difficult to determine in what point populations showing different characters should be considered taxonomically distinct groups (Gaucher et. al., 1996). Moreover, when marked differences are exhibited by a subspecies, it should be considered where to draw the line for establishing a complete new species. Many studies have combined various information sources, including **genetics**, **ecology** or **morphology** to support the creation of new species out of subspecies (Mattiucci, 1997), (Sarver, 1998), (D'Amelio et. al., 2007). This is what has given birth to **integrative taxonomy**, which uses multiple disciplines to find reliable evidence for taxonomic decisions. Genetics has been one extremely relied field used in taxonomy as it provides useful data for our understanding of evolution and even ecology (Monaghan, Sartori, 2008).

1.8. Genetics in taxonomy

Evolutionary divergence gives places to a continuous process of differentiation between populations, subspecies and species, creating lots of genetic data to be considered. Taxonomy organizes the relationships and divergences between populations and establishes degrees of singleness for each of them. New advances in genetics provide better detail of how evolutionary paths are followed, so combining gene analysis to the traditional taxonomic fields, it is now possible to work and understand evolution below species level (Winker, 2016).

Avian speciation is especially slow because reproductive barriers, either postmating or premating, have a lower impact on populations than hybridization opportunities. Also, isolation factors are often associated with behavioural, nongenetic causes, such as song, which make the search for genetic divergences much more difficult (Grant, Grant, 1997). Regardless, the **Cytochrome c oxidase I** or **COX-1** gene has been proven to be able to distinguish between closely related species of most organisms, including birds. The gene is the first and most relevant of the three mtDNA subunits of **cytochrome c oxidase**, key enzyme which catalyses the terminal step of respiratory chain. The COX-1 region is formed by a bimetallic center, which contains a high-spin and a low-spin heme (**heme a3**, and **heme a**, respectively), and **copper B**. The reason why COX-1 is so variable relies in its components. Tthe composition of heme and copper, as well as **substrate** (substance on where the enzyme acts) type and affinity, vary according to **environmental** factors (Ludwig, Schatz, 1980), (Müller, Schläpfer, Azzi, 1988), (Gene Cards, 2016).

COI-5P is the standard region of the COX-1 gene for identification and sequencing purposes (Saunders, McDevit, 2012), and has been used in many different studies (Bucklin, Steinke, Blanco-Bercial, 2011), (Wilson, 2012). It is also the region used for **DNA barcoding**, used as a complement for molecular phylogenetics and population genetics, as it allows large-scale sequencing (Hajibabaeie et. al., 2007). This project will use the COI-5P region to search for possible variability between two subspecies (*Motacilla alba alba* and *Motacilla*

alba yarrellii) of the **White wagtail** (*Motacilla alba*). If *Motacilla alba yarrellii* has followed an evolutionary path different enough to delineate an independent species, we should be expecting a minimum of 2-3% genetic divergence from *Motacilla alba alba* (Hebert, et. al., 2004). Apart from the genetic analysis, other fields in where the Pied wagtail shows unique characteristics will also be researched and contrasted.

2. Empirical data analysis

2.1. Ecology and behaviour of the White Wagtail

The White wagtail (*Motacilla alba*), bird in the Passeriformes order, is widely distributed throughout all the **Palearctic climatic area** in exception of deserts and mountain plateaux. It also has a population located in **east Greenland** and **north-west Alaska**. The estimated population for Europe is between 8.000.000 and 24.000.000 individuals (BirdLife International, EBCC) and is not threatened nor has perspectives to be. The wide range of the species is due to its **adaptation capacity**.

2.1.1. Habitat

The following axes can be recognized when defining the ecological niche of both wagtail subspecies: **temperature**, **evapotranspiration** and **elevation**. During the reproductive period, it prefers **wet aquatic environments** as well as **pasture lands** and **humanized areas**, where it nests in **rock fissures** and **buildings**. The species shows marked anthropophilic habits when selecting a place where to nest. It fails to breed only in areas with low temperatures, especially ones with average annual temperature below 5°C or marked seasonal moisture deficit (Actual evapotranspiration - AET/ potential evapotranspiration - PET < 0·6). Evapotranspiration integrates evaporation and plant transpiration. (Huntley et al. 2007). Wagtails easily adapt to different environments, from sea level to high mountain habitats. The upper elevation limit they tolerate is 5000 meters, although breeding population spreads from sea level to approximately 2000-3000 meters. (BirdLife International, EBCC). It has predilection for wet environments, and is more likely to stay in the lower and mid-course of rivers with wide stream beds, slow flood and open banks (Peterson et al. 2006).

2.1.2. Seasonality

Individuals from both spp. (*Motacilla alba alba* and *Motacilla alba yarrellii*) are **present during the year** throughout all their distribution area, with slightly variable numbers due to **short migrations** from North to Southern Europe and

Africa and in reverse. (Migratory movements are described in Dispersal section).

2.1.3. Behaviour

A characteristic and not fully understood feature of the *Motacilla alba* behaviour is their constant **tail-wagging**. This movement has taken to different hypothesis regarding its aim: **flushing insects**, **showing submission** or **signalling a state of alertness to predators**. This last option is the one which seems most reliable, since Wagtails show a negative correlation between eating and wagging, and birds use to adopt a nonvigilant posture when pecking. Thus, the tail movement could be used to show predators their state of surveillance. This type of behaviour is also shown in other bird species, such as Wrens or Warblers (Robb, M. et al 2010).

White wagtails show an energetic and **undulating flight** which is **combined with vocalizations**, and **shake their heads** in an exaggerated way when walking or running (Svensson et al. 2001). They flock in large groups to roost. **Communal roosts** and the associated social behaviour can be explained by the need of finding a safe place where to sleep and also the protection from predators large groups offer. Even though, these reasons only cannot explain the wagtail's roosting habits, as they do not always choose the most sheltered available and do not sleep close together enough to influence the warmness of the roost. Regarding the protection theory, roosts do provide some security, but birds do not use it efficiently. Thus, it cannot be the only explanation for this behaviour. **Pre-roost gathering** gives place to another theory, which says that roosting is used as a way of exchanging information among individuals (Broom et al. 1976). This information may be about good food sites, so individuals which struggle to find feed themselves can follow others to good food sources (Ward, 1965).

2.2. Taxonomy

Currently, the *Motacilla alba* complex comprises **eleven recognized subspecies**, nine of which have a limited distribution and are endemic of the zones where they inhabit.

The two marked ssp. are the ones discussed in this project. Next to each ssp. name is the taxonomist or biologist who first defined it and also the year when the decision was formalized.

- ***alba*** (Liannaeus, 1758)
- ***yarrellii*** (Gould, 1837)
- *dukhunensis* (Sykes, 1832)
- *ocularis* (Swinhoe, 1860)
- *subpersonata* (Waldo, 1901)
- *persica* (Blandford, 1876)
- *personata* (Gould, 1861)
- *baicalensis* (Swinhoe, 1871)
- *lugens* (Glofer, 1829)
- *leucopsis* (Gould, 1838)
- *alboides* (Hodgson, 1836)

2.3. Distribution

The ***Motacilla alba alba*** is the most common wagtail, and is distributed throughout the **Western Palearctic** and some points of **Greenland** and **Alaska** (Huntley et al. 2007). It is present all over the Iberian Peninsula, especially in its northern half and Valencia. It is not regular in some areas of La Mancha, Badajoz and Andalucía (Atlas de las aves de España, 1997). Its population is stable and has not suffered significant changes regarding number or distribution during the last years.

In the **British islands**, including **Iceland** and the **Faroe** islands, there is the only isolated population of ***Motacilla alba yarrellii*** (Pied wagtail). It is resident and stable all over the UK, in exception of the north of Scotland, where it is absent during winter. The Pied Wagtail population is practically isolated reproductively, in exception from a few concrete **hybridization areas**, discussed later. [FIGURE 1]

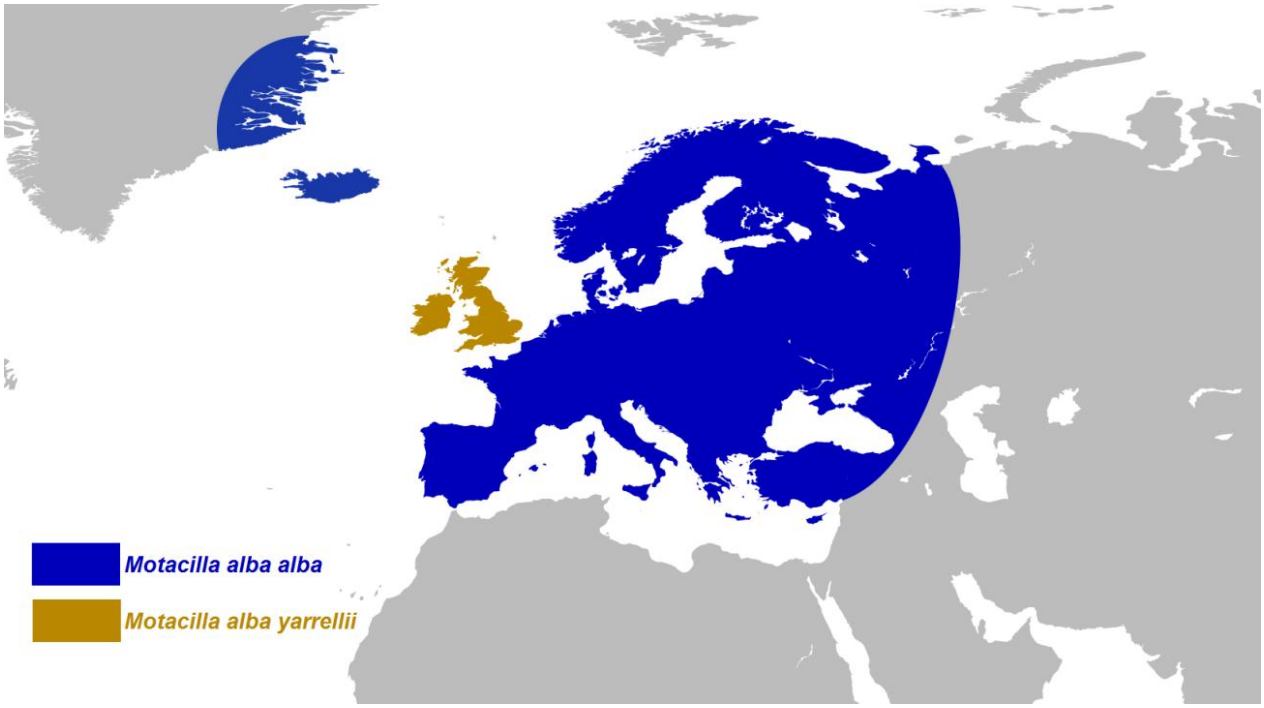


FIGURE 10: Distribution of *Motacilla alba alba* and *Motacilla alba yarrellii*

2.4. Dispersal

Motacilla alba alba and *yarrellii* do not show important migratory habits, but they clearly perform **diurnal seasonal movements** of over 200 km. Apparently, **partial-migratory habits** for bird species like the Wagtail are explained by the fact that there is a part of the total population which is migratory and another which has resident habits. These inter-species groups are conditioned by the weather in the residence area and the bird's individual migratory flexibility, as well as by the genes, as proven in a study on Blackcaps (Berthold, 1988). Juvenile individuals are more likely to migrate.

Alba subspecies is generally resident all over Europe, but there are a number of individuals which migrate during **breeding** and **wintering seasons**. Fall migration for Wagtails starts on August and ends over October. Spain receives an arrival of wintering contingents from Central and Northern Europe each winter, but the most important wintering areas for individuals from Northern and Western Europe, particularly Scandinavia, are Africa and South Sahara (Bird Migration, Thomas Alerstam). One recurrent but unclear route for Northern Europe individuals goes through the Baltic, and Poland has an especially migratory population of wagtails, which migrate through the Gulf of Gdañsk. Generally, the populations from colder zones tend to be more migratory and fly

to the South in large groups, while populations from Southern Europe are likely to make shorter, transhumant movements from high altitude habitats to lower ones (Bijlsma et al. 2001). Subspecies *Motacilla alba yarrellii* and *alba* follow slightly different migratory flyways. Ringing records show that alba has a **South-Eastern course**, while yarrellii tends to follow a **Western flyway**.

A route like this requires a percentage of energy the birds get from their fat body mass. Small passerines like Wagtails tend to lose 0.7% body mass per hour of flying. The Yellow wagtail (*Motacilla flava*) consumes 0.8% body weight per hour (Fry. et al 1972), data that could apply also to the White wagtail.

2.5. Hybridization areas

Migration takes individuals from *Motacilla alba yarrellii* and *Motacilla alba alba* subspecies to coincide occasionally in some areas, where they can **hybridize** and **successfully produce young individuals**. There is little evidence for these hybrids' breeding success capacities.

Paths followed during migration and hybridization zones indicate that **hybrids tend to migrate south-west** (Adriaens, Bosman & Elst, 2010). Even considering that it is hard to tell apart hybrids from pure individuals, there has been a few cases of proven reported **mixed pairs**. Individuals from the *Motacilla alba alba* subspecies are more numerous and the possibilities of seasonal movements to lead them to areas where *Motacilla alba yarrellii* breeds are higher. Alba subspecies breeds in Scotland and the Channel Islands, and individuals from Iceland migrate over England, but it does not seem to have created a hybridization zone with *Motacilla alba yarrellii*, fact that disconcerts experts. "*There is a genuinely low incidence of hybridisation between alba and yarrellii in Britain. Given the large numbers of alba that pass though Britain in spring, no 'hybrid zone' has become established*." (Richard Millington, email communication).

It is also remarkable that pure *alba-alba* pairings are present in zones where **both subspecies coexist**. *Motacilla alba yarrellii* breeds in really low number in some Northern-Eastern Europe. The highest breeding populations have been reported in North-Western France and the Netherlands, where the number of individuals does not exceed 30 birds (Bijlsma et al. 2001), (Van Dijk et al. 2002),

(Berthelot, 1994), (Dubois et al 2008). Most of these birds are paired with *Motacilla alba alba*. It is hard to determine how many hybridization cases have been occurred because of misidentifications, as most hybrids are young individuals, but mixed pairs are frequent in the zones stated above.

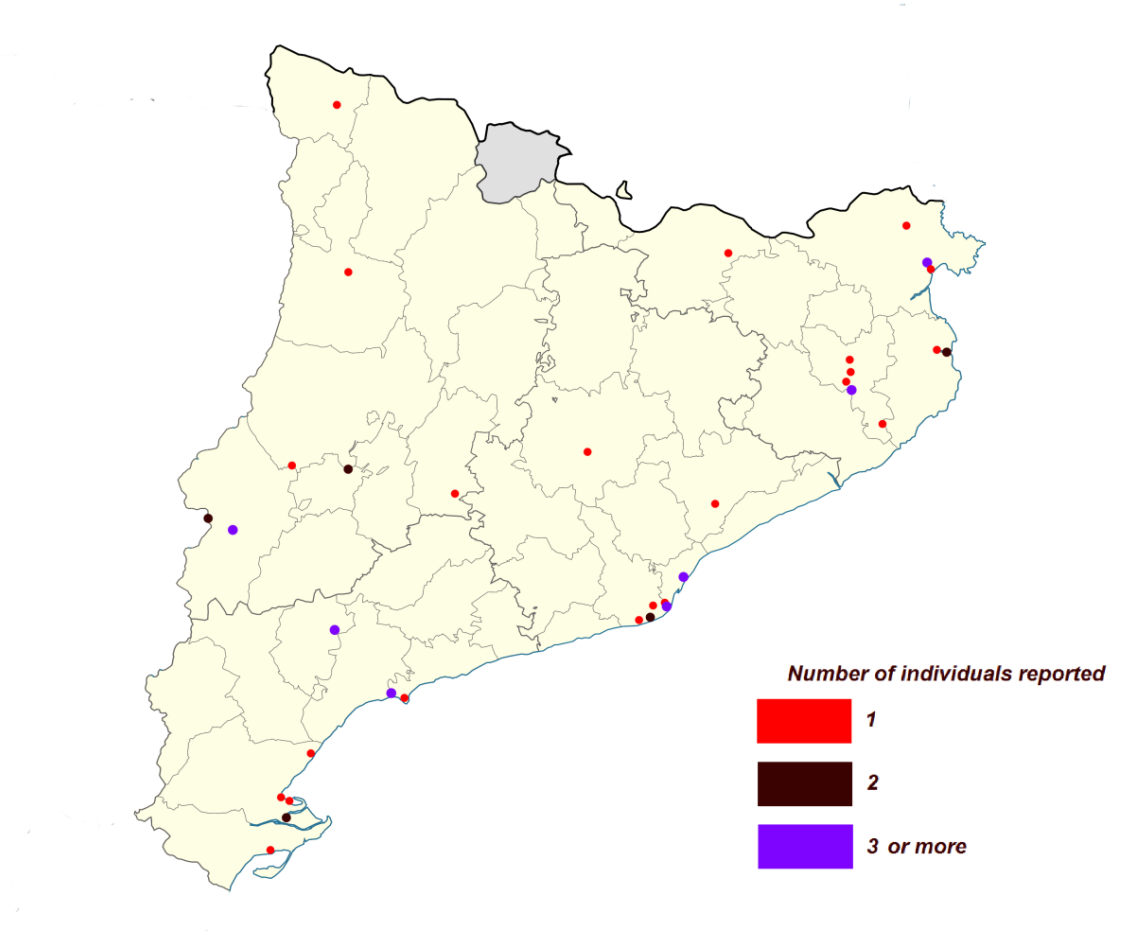


FIGURE 11: Reports of *Motacilla alba yarrellii* in Catalonia, outside of their destribution area.

2.6. Yarrellii reports in Catalonia

Pied wagtail individuals have been reported several times in Catalonia, highlighting the number of observations in the areas of the **Alt Empordà**, **Gironès**, **Baix Ebre**, **Baix Llobregat** and **Barcelonès**. Almost all citations are during **winter** (between December and April, stressing the months of January, February and March). The observations could be due to the **wintering migration** of *Motacilla alba yarrellii* individuals throughout Europe or to individuals being carried by **strong winds and storms**. A study at the meteorological historical data from the SMC (Servei Meteorològic de Catalunya)

does not reveal any special change in the average wind speed or precipitation accumulation in Catalonia during 2013, but there was an **explosive cyclogenesis** period that affected the whole Iberian Peninsula precisely during the last days of January, when the most individuals were reported. (ANNEX I) There have not been any cases of *Motacilla alba yarrellii* breeding in Catalonia, and **all observations were punctual**. [FIGURE 2]

2.7. Morphology

2.7.1. *Motacilla alba alba*, Liannaeus, 1758

FIGURE 12*: Motacilla alba alba* individual showing summer plumage. Photo by Arnau Solé.

Motacilla alba alba shows a **paler general look** than *Motacilla alba yarrellii*. In general terms, it has medium grey mantle and back and grey flanks, which can be paler or equal to the mantle colour. Individuals display black crown and nape, which differentiates from the grey mantle and does not merge in the sides of the neck. Greater coverts and *tertials* have dark black centres and moderately broad whitish edges.

Even though, these features are not enough for identification as plumage varies depending on the age of the individual and season of the year.

TABLE 1 Wing-span sizes (mm) of *Motacilla alba albalyarrellii* for adult and juvenile individuals.

	Ad.♂	Ad.♀	1Y♂	1Y♀
yarrellii	86-96	83-91	84-95	82-89
alba	84-94	81-89	87-94	

TABLE 1: Note that *Motacilla alba yarrellii* is slightly larger than *Motacilla alba alba*.

The two base forms are **autumn** and **spring displays** regarding season, and **adult** and **juvenile** individuals regarding age (Peterson et al. 2006), (Svensson et al. 1992), (Perrins, 1987). Males are larger than females and it is possible to sex them considering plumage and size only. (See TABLE 1 for sizes). Birds can be also sexed in accordance to their **incubation patch**, at least in spring and summer seasons (Svensson, 1992).

a) Spring and summer plumage

This plumage is shown by individuals from **April** to **July**. During this period of the year, ageing is complicated as the pre-breeding moult is not always defined. Sexing is also difficult because both males and females show really similar features. (Adriaens, Bosman, Elst, 2010). Even though, the following characteristics can make identification possible:

Adult ♂: to begin with, adult male individuals show **glossy black crown** and **grey mantle**. The **demarcation** between both colours in the nape is especially distinct. **Forehead is pure white**, making strong contrast with the crown. See also **glossy black throat and chin**, which never show any black spots mixed with the black. Furthermore, **clean white breast** and rather **grey** and **extent flanks** are displayed by adult birds. **General appearance** is **paler than in *Motacilla alba yarrellii*** (Hayman, Hume 2009). Moving on to feather details, greater coverts are **not completely moulted** and the contrast between old and new feathers is not clear. Therefore, a lot of experience is required to age correctly the bird. Birds with not **newly moulted inner coverts** are likely to be adults. Consider the moult in *tertials* too: the **outer tertial** should be **moulted**, while the **two inner one**s should show a rather **grey** colour. To finish, new, **contrasted primaries** also indicate adult birds (Svensson, 1992).

Adult ♀: adult females show the same characteristics as males, but some differences in the **head pattern** can be useful when sexing. To start, forehead shows usually less white than in males and sometimes shows **little black spots**. Also, the black of the **crown is often greyish**. **Demarcation** between grey mantle and crown is **rarely differentiated**, and the **throat shows white admixed with the black**. Even though, some individuals have completely black throat and chin.

Juvenile ♂: first, note rather or completely **crown with little or no black** admixed and **grey forehead**, showing no or little white. Secondly, **throat** usually shows **white admixed**, always more than in female adults. To really tell apart adults from juveniles, though, moult must be considered. **Greater coverts show two moult limits**: outer ones are usually brownish-grey and really worn, and make contrast with the new, fresh feathers. In between the newer and the

older feathers, there are usually some **middle-type** ones, which show not so recent but still unmistakable moult. For another thing, *tertials* and primaries are also **brownish** and look **worn**.

Juvenile ♀: when individuals have completely **grey head pattern** (apart from throat), they are sure to be females. In other cases it is really difficult to tell them apart from juvenile males without mistake (Svensson, 1992).

b) Autumn and winter plumage

Adult individuals moult from spring to fall form between **July** and **September**, depending on the nature of the bird and the weather. When sexing autumn plumage birds, ageing should be done first (Adriaens, Bosman, Elst, 2010).

Adult ♂: pale grey crown and **nape** which **merges with mantle and back**. **Forehead**, **throat** and **breas**t can show pure white in some individuals or have yellowish/greenish tonalities in others. Males are more likely to show **absolutely white feathers**. Moreover, a **black patch in breast** indicates adult. Medium to **dark grey rump** and **back** and **solid black crown** and **nape** indicate male individuals. Then, **flanks** vary from **greyish white** to **dark grey** and the colour is not extent under the whole wing. All greater coverts show the same coloration, which consists on a **blackish base tipped with grey** or grey-white edges in both webs. Also, the outer web is edged with brownish-grey. **Primary coverts** are glossy **black** or really dark grey, and the shape is rounded with smooth edges. *Tertials* are also a key element when ageing, and are uniform grey to shaft. Finally, legs and beak are darkish grey (Svensson, 1992).

Adult ♀: same as male, but forehead and nape tend to be grey or have little white mixed instead of being pure white. **Crown** should be **grey** in females.

Juvenile ♂: crown and nape should display some **black and greenish-yellow tinge** on the sides of the **head**, **throat** and **ear coverts** indicate first year (1Y) juveniles. Not much contrast between black and white in nape, crown and forehead is shown with *Motacilla alba alba* individuals. **Brownish-grey back** and **mantle** indicate juvenile, and some individuals show **white on forehead**. The throat should be white and the **breast** should show a **grey patch** instead of a black one as in adult form. Apart, a bird with a **pinkish base**

underneath the beak is likely to be juvenile, but some adults display it too. Individuals with a head pattern similar to adult with **extensive black nape** and **white forehead** should be males. Rump and flanks are similar to adult form. To age correctly *Motacilla alba alba* individuals in autumn, greater coverts are the most reliable feature. 1Y juveniles display **contrasted moult in the GC**, with **outer feathers showing brownish edges** and **inner being ones tipped grey or grey-white**. In adults, the colour is more uniform. Moult depends on the bird, though. Also consider ***tertials***, which should have **brownish-black centres** and be **edged white**, showing poor contrast. **Primaries** are sometimes a little **worn** too in juveniles, especially during October. The colour of the legs and beak is the same as in adults (Svensson, 1992).

Juvenile ♀: sexing can be done with **head pattern**. Females have a **less marked pattern than males**, showing no or little black.

2.7.2. *Motacilla alba yarrellii*, Gould, 1837

FIGURE 13: yarrellii individual photographed in Ireland. Photo by Arnau Solé.

Motacilla alba yarrellii individuals are larger than *alba* and have a **darker general appearance**. Their principal characteristic is their **pure black mantle**, which is the same colour as the crown and nape. Their **white breast** makes a lot of contrast with the **black throat**. Also, black from throat, nape and crown merges in the chin, making an unmistakable **head pattern**. The black and white **contrast** in all flight feathers is also remarkable, along with the dark grey flanks, rump and uppertail coverts (Peterson et al. 2006) (Svensson et al. 1992) (Livingstone, 1999) (Perrins, 1987).

Analysis of each of *Motacilla alba yarrellii*'s forms:

a) Spring and summer plumage

It is the most characteristic and easiest to identify form. Sexing with adult birds is really clear, but in juveniles differences are little.

Adult ♂: characteristic **glossy black upper parts** (from crown to upper tail coverts) and **breast**, which make contrast with **pure white forehead** and **underparts**. All **wing feathers** and coverts are usually **fresh** and show **dark black centres and white edges**. Note that any feathers show special **contrast**, as the moult is uniform. To finish, legs and beak are black, as in the *Motacilla alba alba* subspecies.

Adult ♀: females look similar to males, but the **black underparts have grey admixed**. In general, the colour varies from blackish-grey to black with dark grey spots. **Feathers** have also a **little lighter black than in males**, but never al light as in *Motacilla alba alba* birds (Svensson et al. 1992).

Juvenile ♂: to tell apart juveniles from adults, **moult** must be considered. First, **outer greater coverts** are **worn** and show no or little contrast, being **brownish-grey** with **not marked darker centres**. For another part, **inner coverts** are likely to be **worn and fresh**, so they make strong contrast with the outer ones. First summer birds are difficult to sex, but as in adults, the ones which have **black upperparts** should be males.

Juvenile ♀: just like juvenile males, but they are more likely to have **dark grey-olive upperparts**. Individual variations make sexing confusing, but no black in the upperparts always indicates female birds.

b) Autumn and winter plumage

Motacilla alba yarrellii shows exactly the **same form as in spring** in all ages and sexes, which makes it easier to tell when a bird is from the this subspecies. Even though, sexing in juveniles is challenging, and sometimes not possible.

Adult ♂: same form as in spring.

Adult ♀: same form as in spring.

Juvenile ♂: juveniles have **olive-grey admixed with the black in crown and nape**. The rest of the plumage is also the same as in spring.

Juvenile ♀: as in spring, a bird **lacking black in the upperparts** is likely to be a female. Even though, some males show this characteristic too.

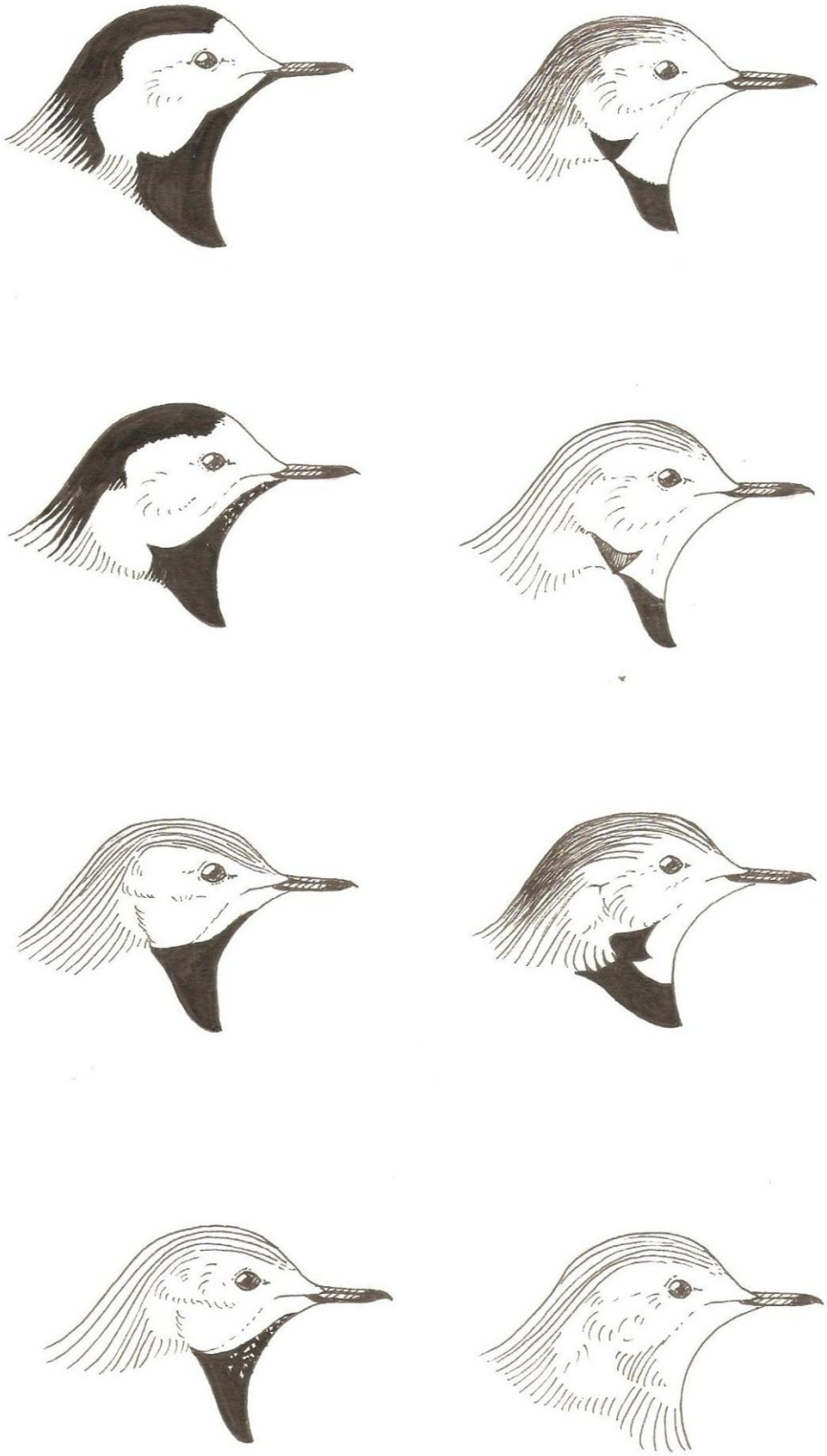


FIGURE 14: Head pattern identification chart for *Motacilla alba alba*, showing (from left to right starting at the top): ad. ♂ S, ad. ♂ W, ad. ♀ S, ad. ♀ W; juv. ♂ S, juv. ♂ W, juv. ♀ S, juv. ♀ W. Personal drawing.

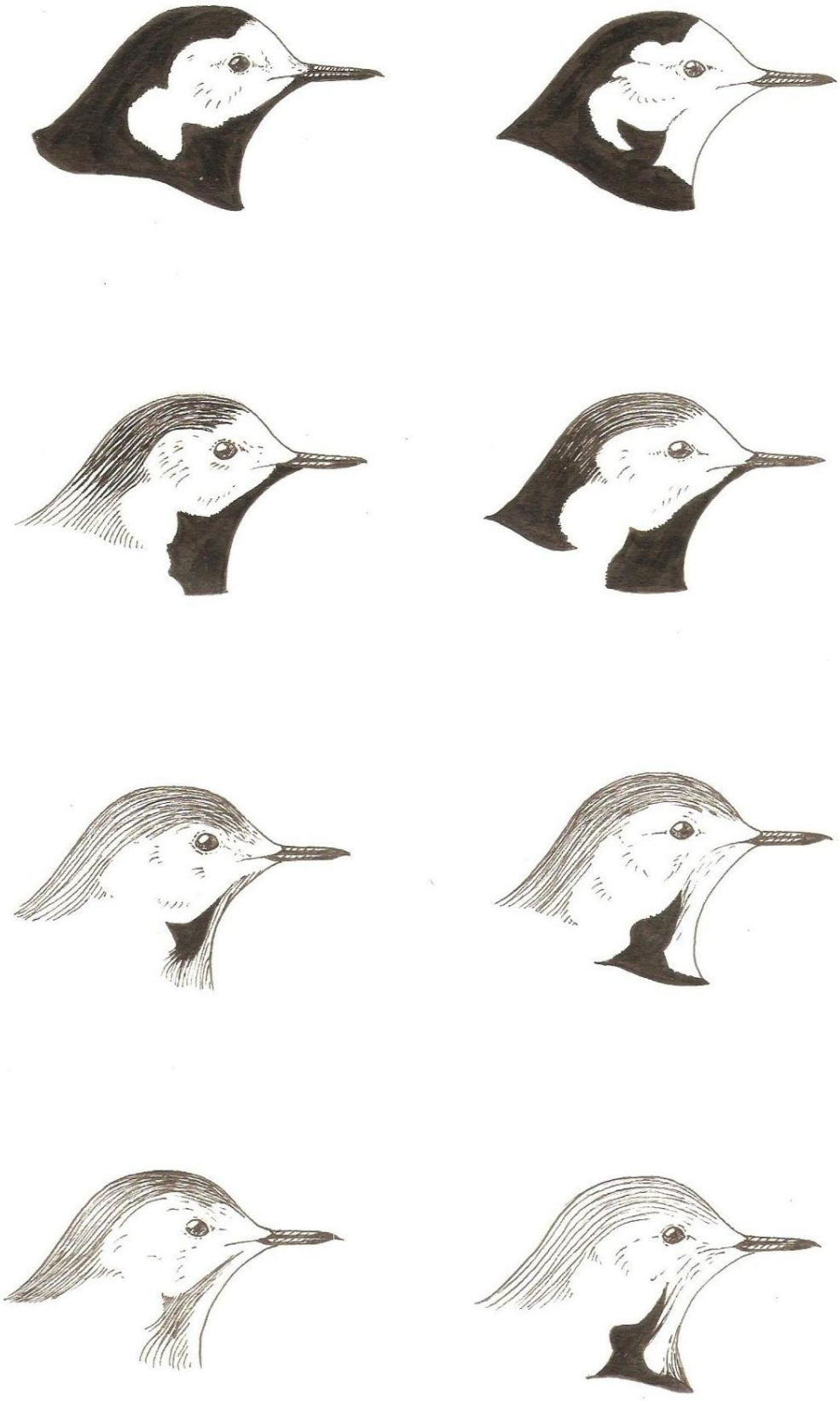


FIGURE 15: Head pattern identification chart for *Motacilla alba yarrellii*, showing (from left to right starting at the top): ad. ♂ S, ad. ♂ W, ad. ♀ S, ad. ♀ W; juv. ♂ S, juv. ♂ W, juv. ♀ S, juv. ♀ W. Personal drawing.

2.7.3. Subspecies diagnosis

The **four following features are especially useful and clear** when separating *Motacilla alba alba* and *yarrellii*. It is easier to identify correctly while banding or using individuals from collections, as details from wings and sizes are key characteristics.

(1) As seen above, the **tonality of the grey colour** of the upperparts is one of the key characters. In a recent paper, a code based on the Kodak grey scale which can help determine if a concrete grey value is typical from *Motacilla alba alba* or *Motacilla alba yarrellii* was established (Adriaens, P., Bosman, D., Elst, J., 2010). Values from 8 to 14 correspond to alba subspecies, while values from 13-18 relate to yarrellii. The areas that should be compared to the grey scale are the **mantle, scapulars, rump and flanks**. [FIGURE 3]

(2) Extent of flanks is also another reliable feature. In the *Motacilla alba alba* subspecies, flanks do not cover the whole space under the wing and leave a white space between two greyish patches. For their part, *Motacilla alba yarrellii* usually shows an entirely dark flank extent all over the belly.

(3) The **presence of dark spots** on the **belly** indicates *Motacilla alba yarrellii*.

(4) **Size of wing-span and tail** are important, as *Motacilla alba yarrellii* is clearly larger than *alba*. (See TABLE 1)

FIGURE 16: Kodak grey scale of upperparts for *Motacilla alba alba/ yarrellii* identification.

FIGURE 17: Image showing *alba* and *yarrellii* individuals compared (respectively), note the general tone difference, especially in flanks and back. *alba* photo by Arnau Solé; *yarrellii* from a personal trip to Newcastle.

2.8. Song and call

Generally, both taxa have **matching song and calls**, with the only exception of **one type of flight call**. Subspecies *Motacilla alba alba* and *yarrellii* have one matching flight call and one which differs clearly (Robb et al. 2010).

3. Molecular analysis: materials and methods

3.1. DNA extraction and quantification

For the molecular analysis, genomic DNA from *Motacilla alba alba* was extracted from two muscle samples, one from 1997 and the other from 2011, preserved in ethanol in the *Museu de Ciències Naturals de Barcelona.* The Speedtools® Tissue DNA Extraction kit (Biotools, Madrid, Spain) was used following the manufacturer's standard protocol for analysis of animal tissue samples. DNA isolation is done by breaking the cell membranes which enclose the genetic material by both chemical and physical methods. Later, DNA purification is done to discard the waste and avoid unwanted contamination of results.

The extracted DNA was quantified using Nanodrop® spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), used to evaluate if the quantity of DNA present in a sample is adequate for running a PCR.

3.2. Polymerase Chain Reaction (PCR)

PCR is an advanced laboratory technique which is used to produce millions of copies of a specific, previously chosen DNA segment. It is a relatively simple and economic process, and has eliminated the need to use bacteria when amplifying DNA.

Different components are required to carry out the PCR reaction, and each of them develops a specific job:

- **DNA template:** it contains the gene you want to copy. DNA must have been extracted from the original sample and can be from any live being, from animals and plants to bacteria and viruses, as they all contain DNA inside the nucleus of their cells. This DNA is found as a double stranded molecule. Depending on the area, DNA can carry out two functions: containing genes (genetic information) or controlling the expression of these genes. In the regions with genetic information, one string is the coding string and the other one the non-coding one. This second string is actually our template.

- **Polymerase:** it is the heat resistant enzyme used in PCRs to synthesize the new strands of DNA complementary to the target sequence by adding single nucleotides. The most used one is Taq DNA polymerase, which is extracted from *Thermis aquatic*. Pfu DNA polymerase from *Pyrococcus furiosus* is also used because it offers a higher fidelity when copying DNA.
- **Primers:** they are short strings of 15-30 nucleotides that are complementary to the initial part of the target sequence, so they can attach there by base pairing. The polymerase begins synthesizing new DNA from the end of the primer. To carry out the reaction, two primers are needed (forward and reverse primers). One of them would attach to the sense and the other one to the anti-sense strand of the sequence.
- **Nucleotides (dNTPs, deoxynucleotide triphosphates)**: they are single units of the four nitrogenous bases in DNA (adenine, thymine, cytosine and guanine) which are reorganized by the enzyme to create new DNA strands.
- **MgCl2:** divalent ions are the important part of the compound, as polymerase enzymes require them for their efficiency. Mg2+ concentration can make the PCR results vary, and are an essential part of the PCR mix. If the concentration is too low, enzymes may not attach and therefore, no product will be obtained. For another part, if too much Mg2+ is added, specificity can decrease and the product will not be right.
- **KCl:** as seen before, the DNA structure is based the repetition of a pattern formed by a phosphate group and a sugar group. The KCl in the PCR reaction is used to neutralize the negative charge on the DNA backbone, helping the annealing stage to develop correctly. What the KCl does is reducing the repulsion between the primers and the DNA strand, which facilitates the binding of the primers. The standard concentration of KCl used is 50mM, and it is important and should be reviewed according to the length of the DNA target. If too much is used, the separation of long DNA double-strands becomes slower. Thus, for long chains of DNA consisting on 20-30kb, a 10-40% reduction of the KCl concentration leads to the most efficient results (Cheng et al., 1995).

Even though, in short products, doubling the KCl concentration improves the reaction.

- **Buffer:** buffers are used to create the ideal background conditions for the reaction to take place. They keep pH in between the range which is more profitable.

Distilled water is added to the mix to help get a specific volume for all samples.

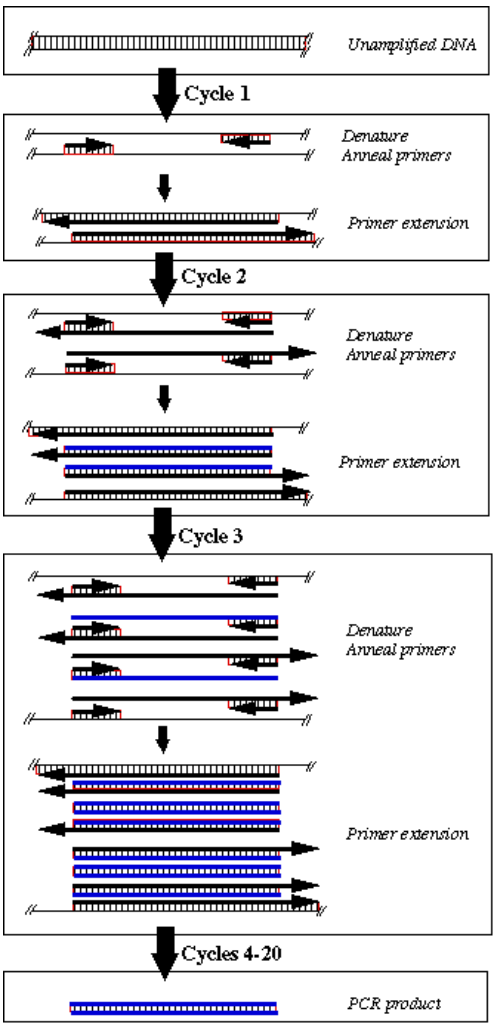


FIGURE 18: diagram showing the process DNA goes through when running a PCR. See all three steps described. Image from San Diego State University: College of Science, Microbial Genetics page,

When running a PCR, reaction mixtures go through three stages: **denaturation**, **annealing** and **extension**. Each of them matches to drastic temperature changes. Once the reactants are mixed together in a vial and placed in the PCR Machine, the first stage starts. The sample temperature is brought up to the about **90°C** for **thirty seconds**, though the time and heat conditions may vary according to the expected results (Rychlik, Spencer, Rhoads, 1990). When heated, the hydrogen bonds between the strands break down and they are therefore separated. Then, in stage 2, the mix is cooled down to the annealing temperature, which is around **60°C**, so the primers can anneal to the strands. This stage lasts **20-50 seconds**. Finally, extension is carried out by heating the sample up again to **75°C** for about **a minute**. The temperature creates the most favourable environment for DNA polymerase to act, adding the bases and building identical DNA fragments to the original strand.

All three steps conform a cycle, and they can be repeated about thirty times, creating about 1 billion copies of the target DNA region in only a few hours. To select a gene, primers which will attach to that concrete region are designed.

3.3. PCR amplification

Four different PCR experiments were done in order to test the results with different primers and conditions. In PCR 1 and 2 Folmer primers LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') were used (Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994). The expected fragment size for these primers and the needed one is 710 nt. In PCR 3 we used primers L14987 (5' CAT CTC CGC CTG ATG AAA CT 3') and H15685 (5' TGC TGG AGT GAA GTT TTC TGG 3'). They should amplify and we were expecting a 420 nt fragment size. In PCR 4, we used primers L15562 (5' CCC ATT TCA CC CCT ATT TCA 3') and H16025 (5' CTA GAG CTC CGA TAA TGG GGA 3'), whose expected fragment size is 680 nt, which was the expected size for the study too (Gómez-Díaz et al. 2006). We used GoTaq® Flexi DNA Polymerase (Promega, Fitchburg, Wisconsin, USA) to the manufacturer's specifications. Products from PCRs 1-2 were run on a 1% agarose gel, and products from the 3-4 were run on a 2% agarose gel. DNA concentrations were 77.5 ng/µL for the 1997 sample and 53 ng/µL for the 2011 one in all PCR experiments unless specification.

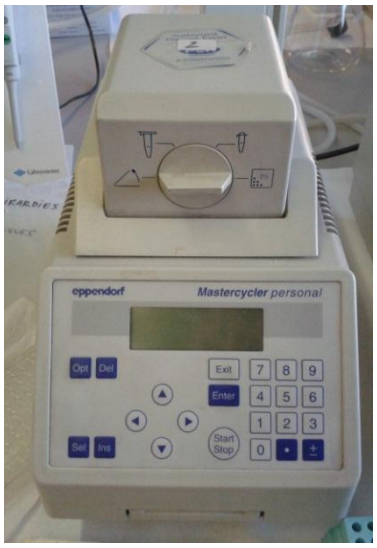


FIGURE 19: PCR machine from the UB laboratory.

FIGURE 20: UB lab where experiments were done.

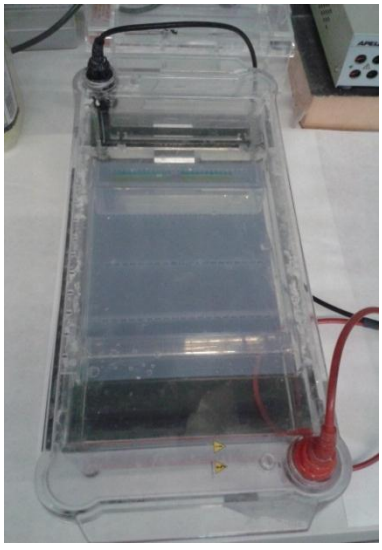


FIGURE 21: Agarose gel used for checking results.

PCR 1 We ran 10 samples with DNA and a negative control without DNA. There were 5 samples from the 1997 moisture and 5 from the 2011 one. The reactions were carried out under the following conditions: 5 min at 95°, 35 cycles of [30s at 95°, 45s at the annealing temperature, 45°, 45s at 72°], and a final extension period of 7 min at 72°. This PCR did not work correctly as the samples turned out to be contaminated.

PCR 2 In PCR we run 10 more samples and another negative control without DNA. As we hadn't gotten marked enough bands for the 2011 sample, we also varied the temperature gradient for all samples and the DNA concentration for 2011 samples. Specifications for each sample concentration and annealing temperature can be found in TABLE 4. The PCR conditions were the following: 5 min at 95°, 35 cycles of [30s at 95°, 45s at the annealing temperature (see TABLE 4), 1 min at 72°], and a final extension period of 7 min at 72°. Results weren't positive as we got two DNA bands instead of just one at the correct bp number.

PCR 3 For this PCR, we changed our primers and used L14987 and H15685 (Gómez-Díaz et al. 2006). We run four samples (details at TABLE 5), a positive *Puffinus puffinus* DNA and a negative control without DNA. We used the two temperatures and concentrations that had given better results in PCR 2 for samples from both years. Also, primer concentration was reduced to 10 µl in order to avoid getting two bands. MgCl2 quantity was also reduced so primers would not attach in more than one region. The reaction was carried under the following temperatures: 4 min at 94°, 40 cycles of [45s at 94°, 45s at the annealing temperature (see TABLE 5), 1 min at 72°], and a final extension period of 5 min at 72°. The bands for this PCR were not marked enough to sequence.

PCR 4 Four samples (details at TABLE 6), a positive *Puffinus puffinus* DNA sample and a negative control were run. The program was the same as in PCR 3. Primers used were L15562 and H16025 (Gómez-Díaz et al. 2006). Primers were different for this PCR and they ended up not attaching.

After each PCR, results were analysed with the aim to check for mistakes and fix them. To see if the product obtained is correct, DNA molecules are run in an

agarose matrix gel, where an electric field is applied and forces biomolecules to move through the gel while distributing them according to length. Gel images are found in ANNEX III for each PCR. The whole extraction and PCR process was carried out at the UB molecular genetics laboratory.

4. Discussion

As the amplification of the COI-5P gene in the laboratory was not possible, GenBank sequences were used for the analysis of the region. I compared a total of 29 sequences, including 27 *Motacilla alba alba* vouchers, one *Motacilla alba yarrellii* sequence and a general Passeriformes sample to detect any possible differences. First, all sequences were analysed in BioEdit, using the ClustalW alignment method.

The first look revealed that *Motacilla alba yarrellii* shows exactly the same nucleotide sequence as some *Motacilla alba alba* individuals, with **0-1% genetic divergence for the COI-5P gene**, giving **no genetic support for species separation**. Even though, **some intraspecific divergences appeared in the *Motacilla alba alba* taxon** (shown in TABLE 7 and 8; ANNEX IV) and are discussed below. Theories regarding these changes referred to **polymorphisms** or **non-significant changes**. To start, differences between defined species of passerines for any of the given nucleotide sequence showed a 2-10% percentage of divergence.

	% query coverage	**Identity %**	**GenBank accession number**
Motacilla grandis	100%	98%	JF499146.1
Motacilla cinerea	100%	97%	JF957024.1
Motacilla flava	100%	96%	GQ482204.1
Anthus hellmayri	99%	91%	FJ027128.1
Emberiza schoeniclus	99%	90%	GU571379.1
Spizella breweri	99%	90%	DQ434133.1

TABLE 2: Selected high-covered BLAST significant alignments with *Motacilla alba alba* nucleotide sequence (GenBank KBPBR032-06), showing clear extraspecific divergence.

Other *Motacilla* recognized species such as the Japanese wagtail (*Motacilla grandis*), the Grey wagtail (*Motacilla cinerea*) and the Western yellow wagtail (*Motacilla flava*) appeared to diverge in **2-4% of the nucleotides**. The closest approach to the *Motacilla alba* taxon occurred with the Japanese wagtail. Hellmayr's pipit (*Anthus hellmayri*) showed 9% divergence from the original

Motacilla sequence. Pipits are phylogenetically close to wagtails, and that connection is even visible when referring to different ecozones. Hellmayr's pipit is a resident of the Tropic of California region, while the White wagtail is a basically Western Palearctic species. More distant passerines like the Common reed bunting (*Emberiza schoeniclus*) and Brewer's sparrow (*Spizella breweri*) showed a higher percentage of differences, bringing the distance with the Emberizidae family to 10%.

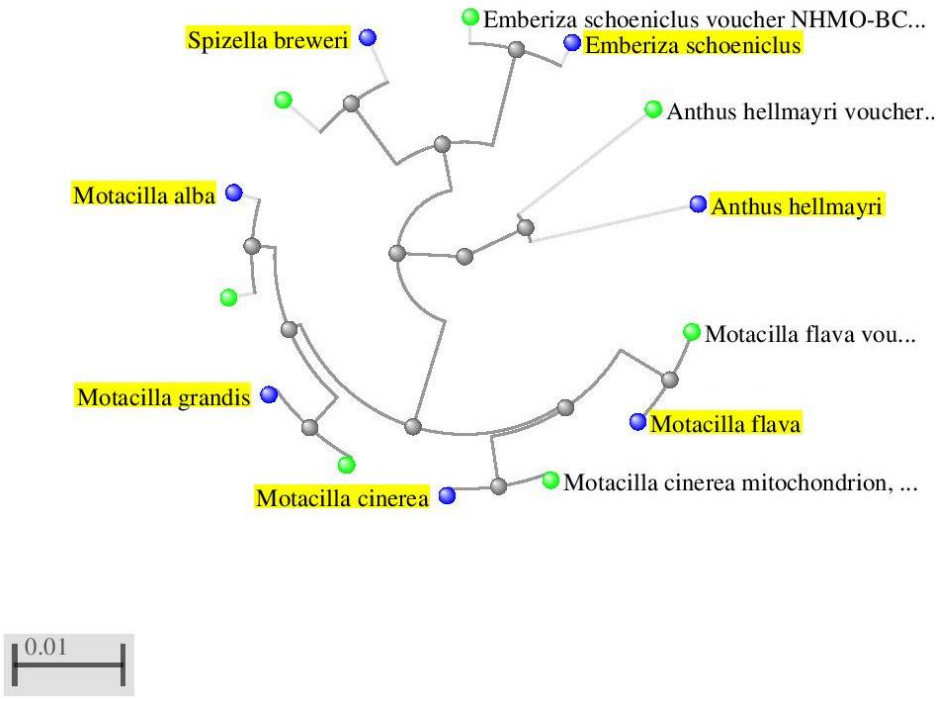


FIGURE 22: Phylogenetic tree for the species in the table, clearly showing the distance between them. As seen before, the Japanese wagtail (*Motacilla grandis*) shows the highest similarity with the White wagtail (*Motacilla alba alba*)

Once the extraspecific divergence percentages were revised, it was fair to state that the **differences found between the *Motacilla alba alba*** sequences were **not significant** enough to consider the possibility of a separated species having been found. **Thirteen different nucleotide sequences were found**, each of them showing **at least one different base** from the others (TABLE 7, 8; ANNEX IV).

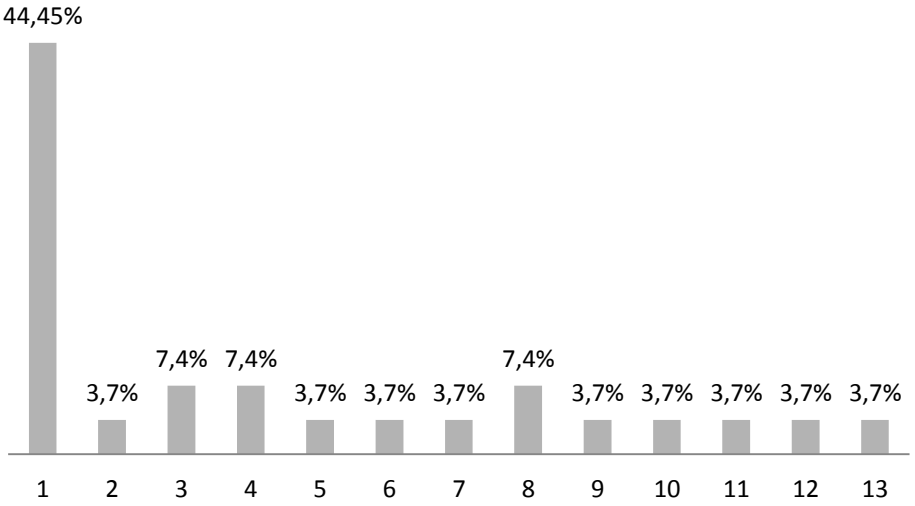
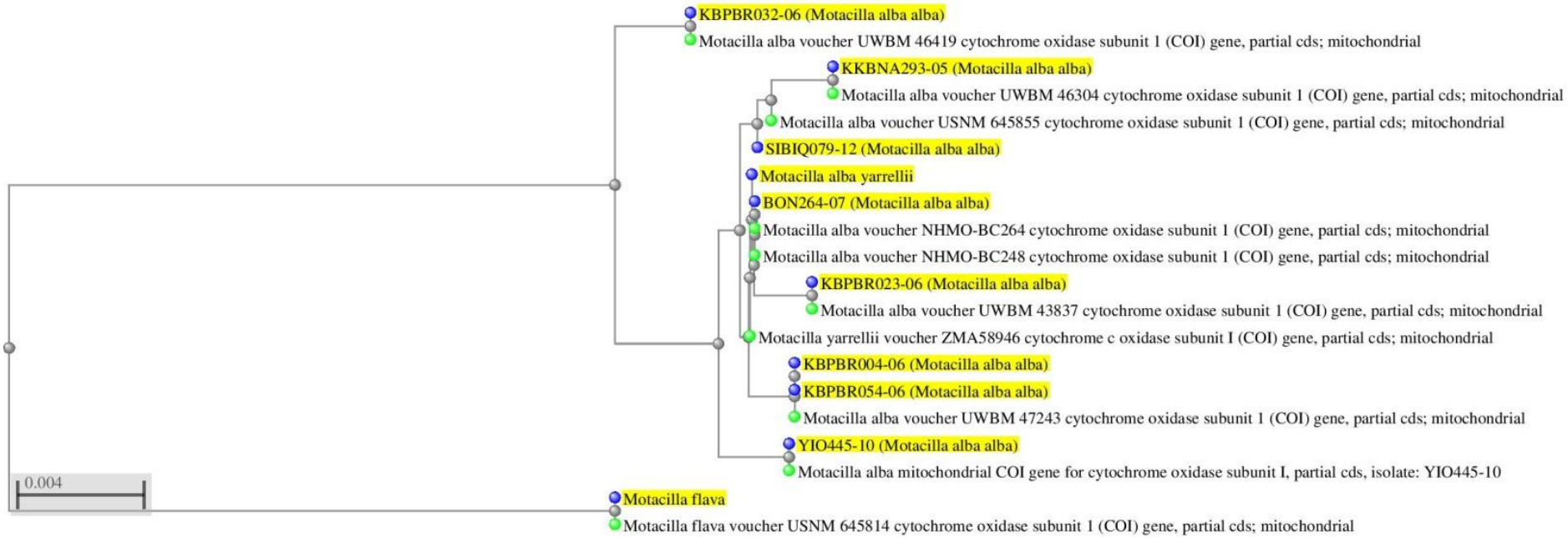


FIGURE 23: Intraspecific variation in the *Motacilla alba alba* subspecies. Each sequence is shown with its percentage of appearance in between the whole analysed group of vouchers. Each column has a model nucleotide order which all sequences in the group follow (see TABLE 9 in ANNEX V).

Analysis of the position of the divergent bases between the *Motacilla alba alba* subspecies revealed they were all the **third bases of the codon**. This gives **no evolutionary or functional value to the differences**, as the last nucleotide of the codon doesn't influence the codification to proteins of the genetic code. Thus, the protein sequence, which determines the characteristics of an individual, remains the same. This would explain such a high presence of different nucleotides in between the same subspecies. As the *Motacilla alba yarrellii* also showed the same sequence, a **species separation remains not possible with the COI-5P gene**.

A phylogenetic tree of all the sequences considered in the study could strengthen the idea of the Pied Wagtail not being a separated species, while one *Motacilla alba alba* sequence seemed to diverge from the rest (KBPBR032-06). This is also explained by the fact that nucleotide sequence differences are not representative of the species.



FIGRUE 24: Phylogenetic tree showing *Motacilla alba yarrellii* as a clear part of the *Motacilla alba alba* group, with the Western yellow wagtail (*Motacilla flava*) for comparison.

Even though, **the tree is clear about the position of the Pied wagtail** (*Motacilla alba yarrellii*) as a subspecies of the *Motacilla alba*, just as the White wagtail (*Motacilla alba alba*). The difference between the *alba* and the *flava* group shows the distance between actual different species.

Regarding the poor genetic divergences for the Pied wagtail, it can be stated that **the COI-5P gene is not variable enough in birds to work among subspecies**. Another gene, perhaps associated with the **distinctive characters** of the object of study should be chosen for such precise taxonomy purposes. In the case of the *Motacilla alba yarrellii*, I suggest genes associated with **pigmentation**, as the main differences with the *Motacilla alba alba* are morphological. **Also, the whole Cyt-b gene has proven to be useful when comparing among genres and families** (Castresana, J., 2001).

Going back to the **COI-5P** results, it **could only find differences which don't change the protein order**. Due to this outcome, it was interesting to blast the protein sequence and see if it was possible to detect differences between other passerines. The result was that **the protein sequence** of the COI-5P really **can't distinguish between related species**. To get the results, the translation from nucleotides to proteins needs to be done from a vertebrate mitochondrial genetic code.

	% query coverage	**Identity %**	**GenBank accession number**
Phoenicurus phoenicurus	99%	100%	AKG06322.1
Acrocephalus palustris	99%	100%	ADE23378.1
Passer montanus	99%	100%	AAT95514.1
Erithacus rubecula	99%	100%	ADE23544.1
Hippolais icterina	99%	100%	ACV45209.1
Phylloscopus coronatus	99%	100%	BAO78355.1
Saltator maximus	99%	100%	AET08148.1

TABLE 3: Selected BLAST significant alignments with *Motacilla alba alba* protein sequence (GenBank KBPBR032-06). Differences with other passerines are 0% from the protein sequence.

Thus, it can be stated **that the COI-5P gene can only be used to identify species from the nucleotide sequence, while the protein sequence doesn't vary in between the same family.**

Finally, about the *Motacilla alba yarrellii* situation, two scenarios would be possible:

(1) The first one would be the existence of a **single gene pool** (one species), with **phenotypic polymorphisms** between populations, the strongest part of which are morphological. This theory would explain the **lack of genetic divergence**. Distribution shows that *Motacilla alba yarrellii* is partially isolated from *Motacilla alba alba* so this would explain the phenotypic differences the Pied wagtail shows.

(2) The second theory would advocate the existence of **two different gene pools** (two species) with **recent genetic drift**. Due to recent **sympatric speciation**, genes could have not evolved enough to detect differences in the COI region. Also, **gene flow between both pools could have been heterogeneous**, so **discrepancy between genetics and phenotypic traits would be even bigger**.

Conclusions

In conclusion, both objectives of proposed for the project have been accomplished, as solid conclusions have been obtained.

- Regarding the taxonomic status of the *Motacilla alba yarrellii*, genetic support for separating the ssp. from *Motacilla alba alba* and the rest of the *Motacilla alba* complex was not found in the COI-5P region.

Some base changes could be detected, but their position in the third base of the codon gives no evolutionary meaning to the differences. However, phenotypic characters are shown by both taxons, especially in morphology. These differences could be explained by two theories, including one discussed when predicting results for the project. The first one would advocate the presence of phenotypic polymorphisms between two distinct groups among one single species, while the already presented before would refer to two separated species with recent and possibly heterogeneous gene flow between them. This situation has also been found in other bird species, such as Lesser (*Carduelis flammea cabaret*) and Common (*Carduelis flammea flammea*) redpolls (Ottvall et. al., 2002).

- Respecting the use of barcodes in taxonomy, the COI-5P gene doesn't show enough variation for working among subspecies, and appears to only be able to recognize species from the nucleotide sequence. In contrast, the protein sequence doesn't show any or few changes among family.

This can be explained by the fact that variation in between populations appears in the third base of the codon, which doesn't codify for a protein but still represents a change in the nucleotide sequence. This type of variation was found in the *Motacilla* taxon among the *Motacilla alba alba* vouchers and in major frequency between close passerines of the White wagtail such as *Anthus* and *Emberiza*. As the COI-5P clearly isn't variable enough to work among subspecies, one future study in the field I propose would be analysing more concrete genes associated with particular characteristics for the targeted subspecies. In the case of the Pied wagtail (*Motacilla alba yarrellii*) and the

White wagtail (*Motacilla alba alba*), a pigmentation related gene could be studied in order to find possible genetic differences that have been missed here. Nevertheless, species separation should not be based only in genetics, and empirical data collected in this paper shows clear differences between both Wagtails.

Even though they could be overcome, some difficulties were encountered during the whole project, involving access to samples and sequencing. British and Irish museums were contacted in order to ask for Pied wagtail (*Motacilla alba yarrellii*) skin or muscle samples for sequencing, including the Natural History Museum in London and the Cambridge University Museum of Zoology, among many others. Petitions were signed in two of the cases, although final permission was not granted so sequences had to be obtained from GenBank. *Motacilla alba alba* samples, on the other hand, could be obtained from the Museu de Ciències Naturals de Barcelona without any complications. Also, when carrying out PCR experiments in the laboratory, different incidences described before were found and the final result could not be obtained. Even though, the mistakes in every case could be identified and the experience allowed me to totally understand the laboratory processes necessary for obtaining sequences from samples. Eventually, research into GenBank was required for the sequence comparison part of the project.

It is fair to say that results obtained for the *Motacilla alba* subspecies studied are in accordance with the information found and with the situation of other species. The whole project can summarize the controversial issue of the *Motacilla alba* subspecies *Motacilla alba alba* and *Motacilla alba yarrellii*, which seem to have followed common evolutionary paths until the present day and their taxonomical status can't, therefore, be reconsidered. Time should be able to make genetic divergence appear so it is possible that both subspecies can actually be split in the future.

Finally and from my point of view, integrative taxonomy can make the most reliable decisions about species separation because it is based in collecting and contrasting data from multiple sources. Now, as explained at the beginning of the project, genetics is a really important part of it. The perspective I could

obtain from the whole research is that genetic support should not only be one of the areas considered in integrative taxonomy, but the principal source of information for making decisions. Even though any of the fields should be forgotten, I personally think genetics offers the clearest results. It is unlikely to depend on environmental or ecological causes, while morphological differences, for example, are physically evident but can depend on really small or insignificant changes in the genome which do not necessarily mean evolutionary divergence. This will also be accentuated in the near future, as innovations in genetics are present every day and will allow more detailed studies. The combination of these new methods and the traditional uses of morphology, ecology and others will make a real change in the way we see taxonomy. Many bird species are found in an ambiguous situation, but as classification will be improved better comprehension of detailed evolutionary paths is clearly to come.

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Annexes

ANNEX I: Observation records from *Ornitho.cat*

20 March 2015 - Girona (GIR) - Miquel Angel Fuentes
16 February 2015 - L'Ametlla de Mar (BEB) - Miquel Casas
15 February 2015 - Llagostera (GIR) - Carlos Alvarez-Cros
14 December 2013 - Girona (GIR) - Guillem Saguer Parés
17 November 2013 - Riu Ondara a Hostalets (SEA) - Òscar Pérez Petrus
17 March 2013 - La Pobla de Segur (PJU) - Víctor Sanz Sánchez
16 March 2013 - Cambrils (BCA) - Alex Mascarell Llosa (2 individuals)
13 March 2013 - Campllong (GIR) - Miquel Àngel Fuentes
13 March 2013 - La Roca del Vallès (VOR) - Jordi Ponce
10 March 2013 - Pals (BEM) - Raül Calderon
10 March 2013 - Campllong (GIR) - Guillem Saguer Parés
9 March 2013 - Torres de Segre (SEI) - Oriol Cortes i Joan Estrada Bonell (2-3 indv.)
6 March 2013 - Pla de Llobregar (BLL) - Raúl Bastida Vives
5 March 2013 - Camprodon (RIP) - Joan Vidal Nogué
3 March 2013 - Castelló d'Empúries (AEM) - Guillem Saguer Parés
1 March 2013 - Fornells de la Selva (GIR) - Miquel Àngel Fuentes
27 February 2013 - L'Ampolla (BEB) - Manolo Sánchez Blanc
26 February 2013 - Parc de la Ciutadella (BAR) - Jana Marco
26 February 2013 - Deltebre (BEB) - Manolo Sánchez Blanch (2 indv.)
25 February 2013 - Deltebre (BEB) - Alex Mascarell Llosa
24 February 2013 - Castelló d'Empúries (AEM) - Gerard Dalmau (>6 indv.)
24 February 2013 - Parc Diagonal de Mar, Barcelona (BAR) - Anònim
24 February 2013 - Cornudella de Montsant (PRI) - Santi Borràs
23 February 2013 - Manresa (BAG) - Marc Illa Llobet
23 February 2013 - Prat del Llobregat (BLL) - Raúl Bastida Vives
21 February 2013 - Pals (BEM) - Fran Trabalon
12 February 2013 - Vilanova de la Barca (SEI) - Joan Estrada Bonell
3 February 2013 - Cambrils (BCA) - Àlex Mascarell Llosa
3 February 2013 - Torroella de Montgrí - Francesc Dalmau
27 January 2013 - Viladecans (BLL) - Josep Ramoneda i Marc Illa
22 January 2013 - Barcelona (BAR) - Ricardo Ramos
22 January 2013 - Sant Boi de Llobregat (BLL) - Anònim
22 January 2013 - Viladecans (BLL) - Anònim (2 indv.)
13 January 2013 - Pantà de Camelis, Terres de Segre (SEI) - Francesc Moncasí Salvia (2 ex.)
6 April 2012 - Naut Aran (VAR) - Aleix Comas
14 March 2012 - Mercabarna, Barcelona (BAR) - Raúl Bastida Vives
6 March 2012 - Estany d'Ivars i Vila-Sana, Ivars d'Urgell (PUR) - Sergi Sales
27 February 2012 - Delfià, Rabós (AEM) - Daniel Roca
14 March 2011 - Castelló d'Empúries (AEM) - Gabri de Jesús
24 February 2011 - Amposta (MON) - Xavier Bayer
28 December 2010 - Ivars d'Urgell (PUR) - Guillem Saguer Parés
18 January 2009 - Vila-Seca i Salou (TAR) - Institut Català d'Ornitologia

AEM: Alt Empordà	BAG: Bages
BCA: Baix Camp	BEB: Baix Ebre
BEM: Baix Empordà	BLL: Baix Llobregat
BAR: Barcelonès	GAX: Garrotxa
GIR: Gironès	MON: Montsià
PJU: Pallars Jussà	PUR: Pla d'Urgell
PRI: Priorat	RIP: Ripollès
SEA: Segarra	SEI: Segrià
VAR: Val d'Aran	

ANNEX II: specific information for PCR samples

PCR 2- TABLE 4

Sample identification	Annealing temperature	DNA concentration
197Ma-A (1997)	46.7°	$77.5\ \mathrm{ng/\mu L}$
197Ma-B (1997)	48°	$77.5\ \mathrm{ng/\mu L}$
111Ma-A (2011)	46.7°	$53\ \mathrm{ng/\mu L}\ (i)$
111Ma-B (2011)	48°	$53\ \mathrm{ng/\mu L}\ (i)$
111Ma-C (2011)	46.7°	$2i$
111Ma-D (2011)	48°	$2i$
111Ma-E (2011)	46.7°	$1/2i$
111Ma-F (2011)	48°	$1/2i$
111Ma-G (2011)	46.7°	$1/10i$
111Ma-H (2011)	48°	$1/10i$

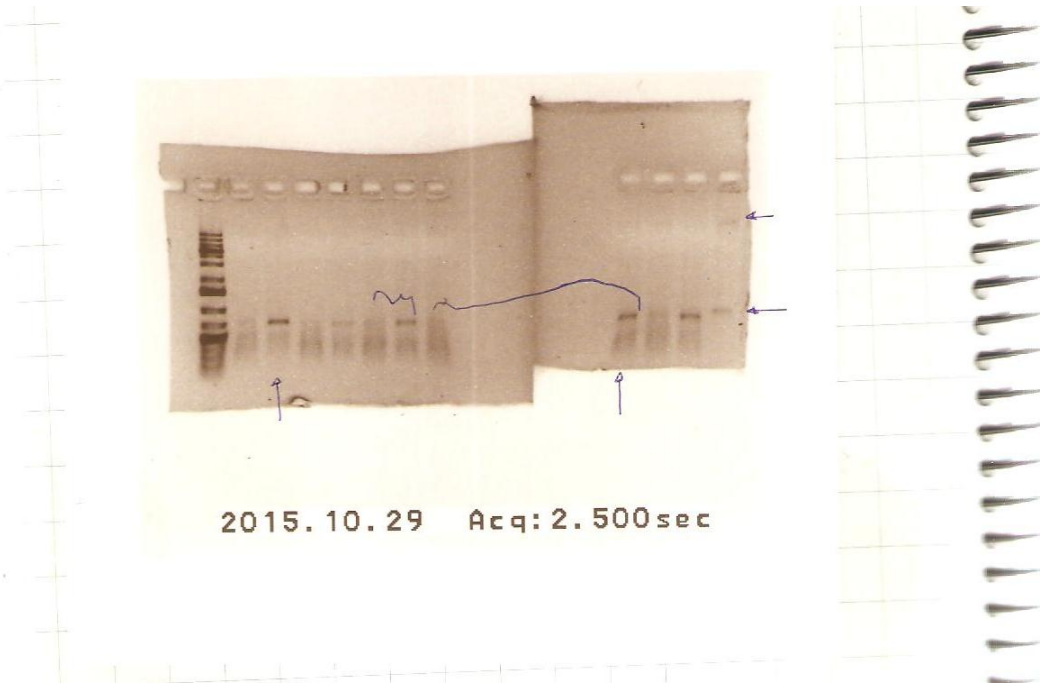
PCR 3- TABLE 5

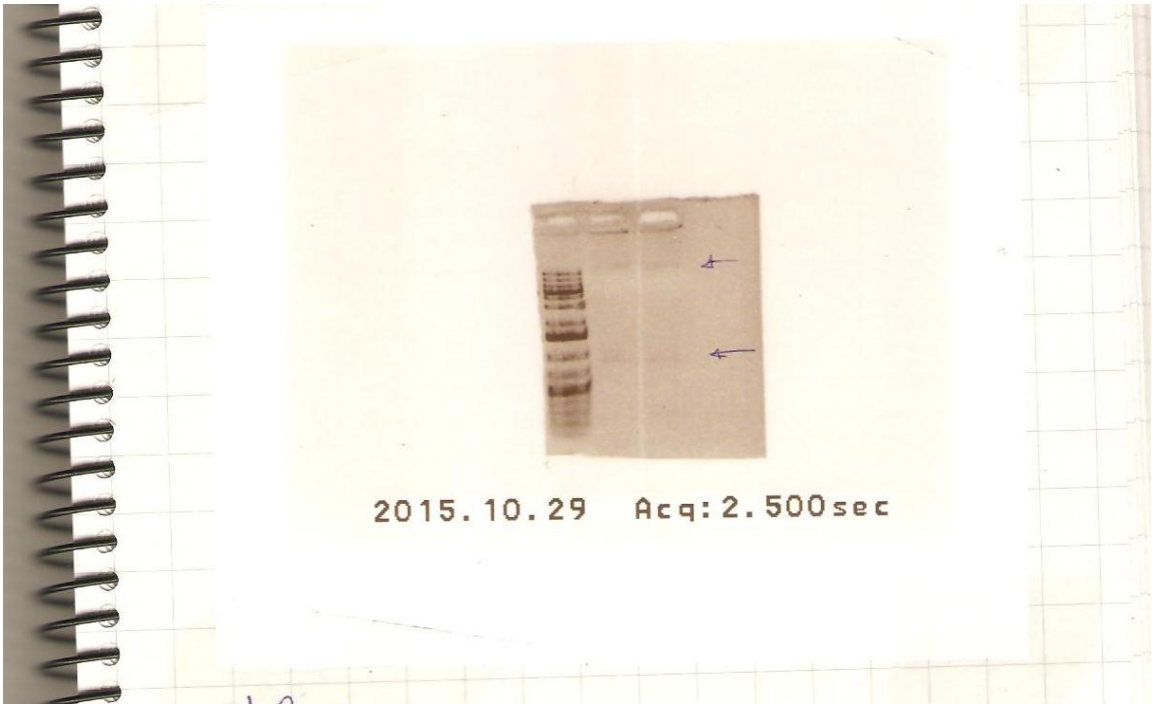
Sample identification	Annealing temperature	DNA concentration
497-A (1997)	59°	$77.5\ \mathrm{ng/\mu L}$
497-B (1997)	57°	$77.5\ \mathrm{ng/\mu L}$
411-A (2011)	59°	$53\ \mathrm{ng/\mu L}$
411-B (2011)	57°	$53\ \mathrm{ng/\mu L}$

PCR 4- TABLE 6

Sample identification		Annealing temperature	DNA concentration
597-A (1997)		59°	$77.5\ \mathrm{ng/\mu L}$
597-B (1997)		57°	$77.5\ \mathrm{ng/\mu L}$
511-A (2011)		59°	$53\ \mathrm{ng/\mu L}$
511-B (2011)	57°	$53\ \mathrm{ng/\mu L}$	

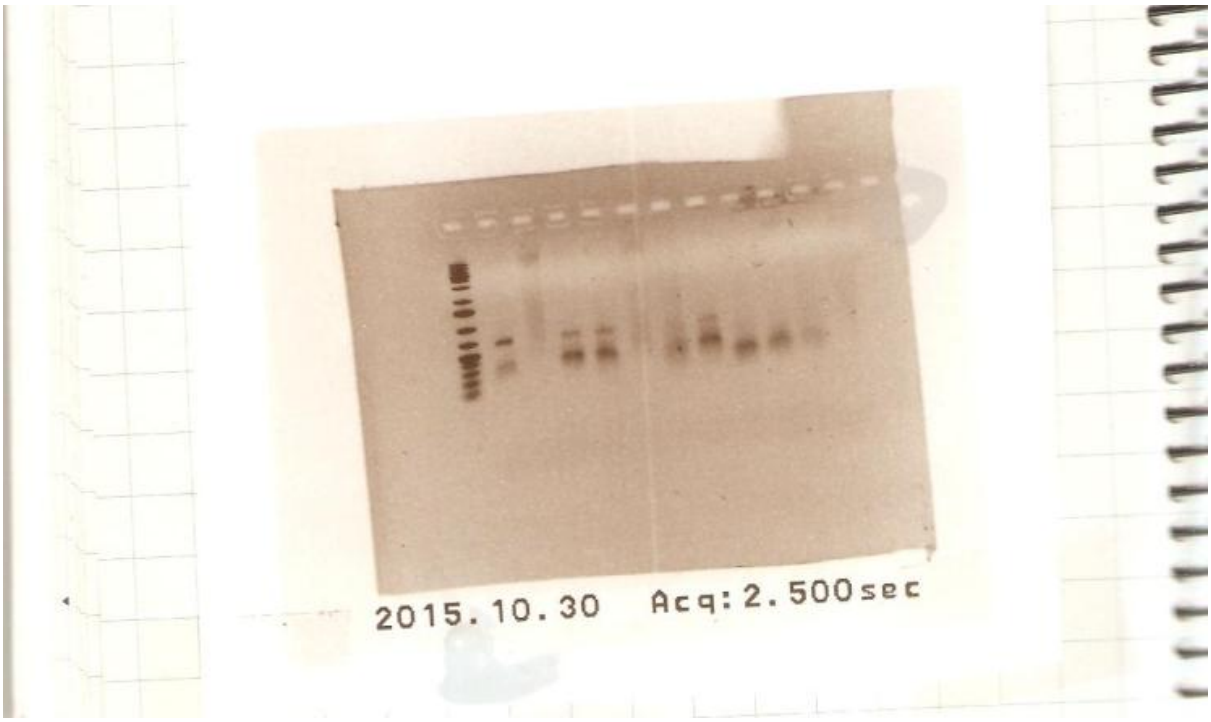
ANNEX III: Gel images for PCR experiments

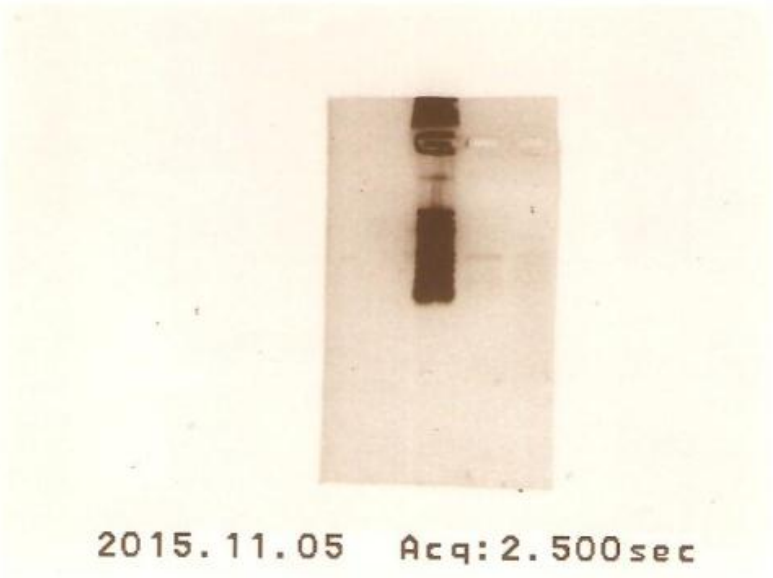




Gel images for PCR 1: PCR results for all ten samples, 5 from 1997 and 5 from 2011. Sample order: 100 bp ladder, 111Ma-A, 197Ma-A, 111Ma-B, 197Ma-B, 111Ma-C, 197Ma-C, 111Ma-D, 197Ma-D, 111Ma-E, 197Ma-E, negative control without DNA. Results: two PCR products for each sample; 600-700 bp and 500 bp.

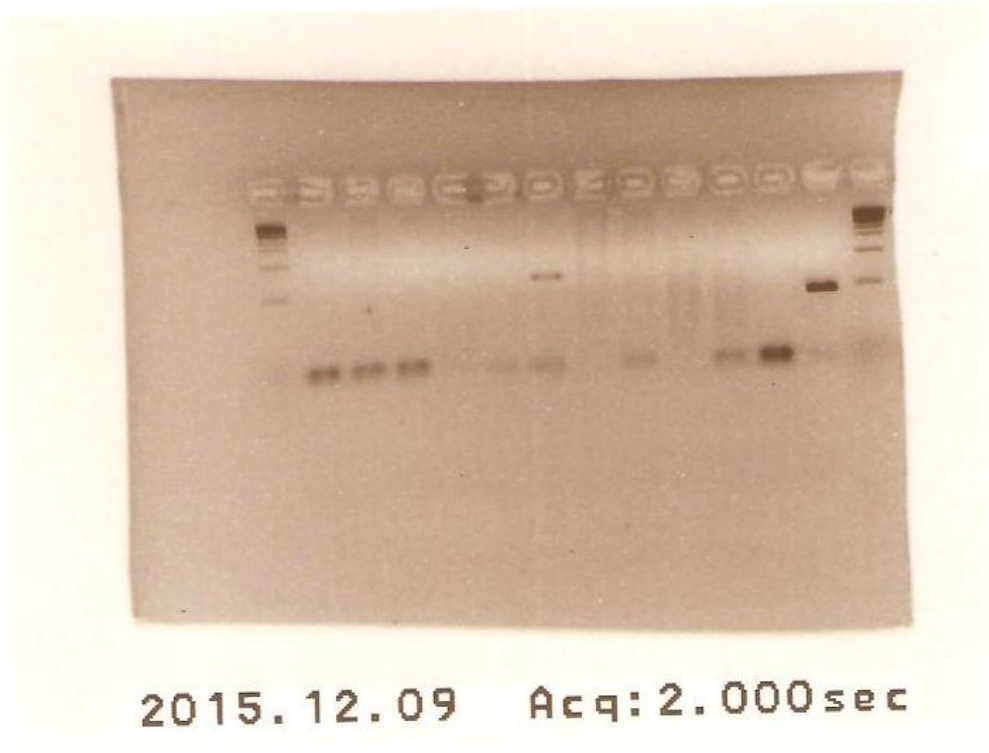
Purification: of the most marked two samples (197Ma-A and 111Ma-C). Sample order: 100 bp ladder, 197Ma-A, 111Ma-C. Results: not marked enough bands.





Gel images for PCR 2: PCR results for all ten samples, 2 from 1997 and 8 from 2011. Sample order: 100 bp ladder, 197Ma-A, 197Ma-B, 111Ma-A, 111Ma-B, 111Ma-C, 111Ma-D, 111Ma-E, 111Ma-F, 111Ma-G, 111Ma-H, negative control without DNA. Results: again, two PCR products for each sample; 600 bp and 300-400 bp.

Purification: of the most marked two samples (197Ma-A and 111Ma-C). Sample order: 100 bp ladder, 197Ma-A, 111Ma-A. Results: not marked enough bands.



Gel images for PCR experiments 3 and 4: four samples for each PCR, two from 1997 and 2 from 2011. Sample order: 1 kb ladder, 497-A, 497-B, 411-A, 411-B, negative control without DNA (1), *Puffinus puffinus* DNA positive control sample (1), negative control without DNA (2), 597-A, 597-B, 511-A, 511-B, *Puffinus puffinus* DNA positive control sample (2), 1 kb ladder. Results: 500 bp band for all samples, no PCR product for the negative controls, 1000 bp band for the positive controls.

ANNEX IV: Variation tables for the analysed sequences (GenBank)

TABLE 7

	Position								
GenBank sequence number and scpecies	#39 bp	#113 pb	#225 bp	#267 bp	#315 bp	#381 bp	#417 bp	#483 bp	#522 bp
BON264-07|Motacilla alba	**C**	**G**	**G**	**T**	**G**	**C**	**C**	**T**	**T**
BISE429-08|Mot. alb.	-	.	.	.	.	.	.	.	.
BON248-07|Mot. alb.	.	.	.	.	.	.	.	.	.
BJNSM476-10|Mot. alb.	-	.	.	.	.	.	.	.	.
GBIR1567-09|Mot. alb.	-	.	.	.	A	.	.	C	.
KBPBR002-06|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPBR004-06|Mot. alb.	-	.	.	.	.	T	.	.	.
KBPBR011-06|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPBR018-06|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPBR023-06|Mot. alb.	-	_	.	C	.	.	.	.	.
KBPBR032-06|Mot. alb.	-	.	A	C	.	.	T	C	.
KBPBR054-06|Mot. alb.	-	.	.	.	.	T	.	.	.
KBPZM138-07|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPZM140-07|Mot. alb.	-	.	.	.	.	.	.	.	.
MIUTH007-13|Mot. alb.	-	.	.	.	.	.	.	.	.
SIBIQ049-10|Mot. alb.	-	.	.	.	.	.	.	.	.
SIBIQ079-12|Mot. alb.	A	.	.	.	.	.	.	.	.
YIO219-10|Mot. alb.	-	.	.	.	.	.	.	.	.
YIO445-10|Mot. alb.	-	A	.	.	.	.	.	.	.
BISE139-08|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPBR015-06|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPBR050-06|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPZM136-07|Mot. alb.	-	.	.	.	.	.	.	.	.
KBPZM139-07|Mot. alb.	-	A	.	.	.	.	.	.	.
KKBNA293-05|Mot. alb.	-	.	A	.	.	.	.	.	.
SIBJP098-10|Mot. alb.	-	.	.	.	.	T	.	.	.
YIO254-10|Mot. alb.	-	.	.	.	.	.	.	.	C
GBIR5536-15|Motacilla yarrellii	-	.	.	.	.	.	.	.	.
KFIP026-07|Passeriformes|COI-5P	.	.	.	.	.	.	.	.	.

TABLE 8

	Position								
Sequence	#557 bp	#585 bp	#669 bp	#708 bp	#720 bp	#723 bp	#747 bp	#748 bp	#749 bp
BON264-07|Motacilla alba	**A**	**A**	**T**	**C**	**C**	**A**	**T**	**C**	**T**
BISE429-08|Mot. alb.	·	·	·	·	-	-	-	-	-
BON248-07|Mot. alb.	·	·	·	·	·	·	·	·	·
BJNSM476-10|Mot. alb.	·	·	·	·	·	·	-	-	-
GBIR1567-09|Mot. alb.	G	·	·	·	-	-	-	-	-
KBPBR002-06|Mot. alb.	·	·	·	·	·	·	-	-	-
KBPBR004-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPBR011-06|Mot. alb.	·	·	·	A	·	·	·	·	·
KBPBR018-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPBR023-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPBR032-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPBR054-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPZM138-07|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPZM140-07|Mot. alb.	·	·	·	·	·	·	·	·	·
MIUTH007-13|Mot. alb.	·	·	·	·	T	-	-	-	-
SIBIQ049-10|Mot. alb.	·	·	·	·	T	C	-	-	-
SIBIQ079-12|Mot. alb.	·	·	·	·	-	-	-	-	-
YIO219-10|Mot. alb.	·	·	·	·	·	·	·	·	-
YIO445-10|Mot. alb.	·	G	·	·	·	·	·	·	·
BISE139-08|Mot. alb.	·	·	·	A	-	-	-	-	-
KBPBR015-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPBR050-06|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPZM136-07|Mot. alb.	·	·	·	·	·	·	·	·	·
KBPZM139-07|Mot. alb.	·	G	C	·	·	·	·	·	·
KKBNA293-05|Mot. alb.	·	·	·	·	T	C	-	-	-
SIBJP098-10|Mot. alb.	·	·	·	·	·	·	C	-	-
YIO254-10|Mot. alb.	·	·	·	·	·	·	-	T	C
GBIR5536-15|Motacilla yarrellii	·	·	·	·	·	·	·	-	-
KFIP026-07|Passeriformes|COI-5P	·	·	·	·	·	·	·	·	·

ANNEX V: Model sequence for each number, TABLE 9 information

Sequence number in the graphic	Model nucleotide order (GenBank sequence identification)
1	BON264-07
2	GBIR1567-09
3	KBPBR004-06
4	KPBR011-06
5	KBPBR023-06
6	KBPBR032-06
7	MIUTH007-13
8	SIBIQ049-10
9	SIBIQ079-12
10	YIO445-10
11	KBP2M139-07
12	SIBJP098-10
13	YIO254-10