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GENETIC DIVERSITY OF MEDITERRANEAN AUTOCHTHONOUS CHICKEN BREEDS

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GENETIC DIVERSITY OF MEDITERRANEAN AUTOCHTHONOUS CHICKEN BREEDS

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CONTENTS

ACRONYMS

ARA	Italian breeding association
AMOVA	Analysis of molecular variance
AVIANDIV	Development of Strategy and Application of Molecular Tools to Assess Biodiversity in Chicken Genetic Resources
Bp	Base pair
Chr.	Chromosomes
All.	Alleles detected
DAD-IS	Global Databank for Farm Animal Genetic Resource
DNA	Deoxyribonucleic acid
EAAP	European Federation of Animal Science
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricolture Organization of the United Nations
FAOSTAT	Statistic Division of FAO
FCA	Factorial correspondence analysis
FIAV	Federation of Italian Poultry Association
F_{IS} (*f*)	within-population inbreeding coefficients
F_{IT}	overall population inbreeding coefficient
F_{ST} (*Theta*)	between-population inbreeding coefficients
K	Numer of clusters
MCMC	Markov chain Monte Carlo interactions
min	Minutes
mtDNA	Mitochondrial DNA
Na	Number of alleles observed
PCR	Polymerase chain reaction
sec	Seconds
SNP	Single-nucleotide polymorphism
SSR	Simple sequence repeats

SUMMARY

Local breeds can be considered a part of the history of some human populations as well as important materials from a scientific point of view. The characterisation and inventory of animal genetic resources and routine monitoring of population for variability are fundamental to breed improvement strategies and programmes and for conservation programmes. Molecular genetics provide us with very remarkable tools to analyse the variation between and within breeds. Different approaches have been developed to understand the different aspects that contribute to breed differentiation.

The thesis is made up in three contributes. The objective of the first part (Chapter 3) was to investigate genetic variation and to analyze population structure in two Italian breeds (Ancona and Livorno) as potential valuable genetic variability source. Blood samples from 131 individuals were collected and genotyped through a thirty microsatellites-based analysis. All the observed descriptive statistical indexes suggested a heterozygosity deficiency and an inbreeding level (mean observed heterozygosity = 0.46, mean expected heterozygosity = 0.53, F_{IS} in Ancona and Livorno = 0.251 and 0.086). The tree from inter-individual DAS distance using Neighbour-Joining algorithm and the FCA analysis showed a higher internal variability in Livorno than in Ancona. STRUCTURE analysis showed the genetic uniqueness of the breeds and the presence of sub-groups in Ancona originating from a possible genetic isolation.

In Chapter 4, the genetic characterization of five Italian chicken breeds (Ancona, Livornese bianca, Modenese, Romagnola and Valdarnese bianca) was described, including their remote genetic origins, the differentiation among them and their present level of biodiversity. The first aim of this study is to investigate the maternal genetic origin of five Italian local chicken breeds based on mitochondrial DNA (mtDNA) information. The second topic was to assess the genetic diversity and population structure of these chicken breeds, and to quantify the genetic relationships among them by using 27 microsatellite markers. To achieve these targets, a 506 bp fragment of the D-loop region was sequenced in 50 chickens of the five breeds. Eighteen variable sites were observed which defined 12 haplotypes. They were assigned to three clades and probably two maternal

lineages. Results indicated that 90% of the haplotypes are related to clade E, which has been described previously to originate from the Indian subcontinent. For the microsatellite analysis, 137 individual blood samples from of the five Italian breeds were collected. A total of 147 alleles were detected at 27 microsatellite loci. The five Italian breeds showed a slightly higher inbreeding index (F_{IS} = 0.08) when compared to commercial populations used as reference. Structure analysis showed a separation of the Italian breeds from these reference populations; a further sub-clustering allowed to discriminate between the five Italian breeds.

Aim of the third study was to investigate the genetic diversity and relationship among sixteen Mediterranean chicken populations using sequencing mitochondrial DNA and a panel of 27 microsatellite markers (Chapter5). A 506 bp fragment of the mtDNA D-loop region was sequenced in 160 DNA samples. Twenty-five variable sites, that defined 21 haplotypes, were observed and assigned to three clades and probably three maternal lineages. The major haplotype (E1) was present in the Mediterranean populations, originates from the Indian subcontinent. Different sequences were included in haplogroup A and B that are distributed in South China and Japan. For the microsatellite analysis, 465 individual blood samples from of the sixteen Mediterranean chicken populations were collected. The results indicated that about 22% of the total variability originated from variation between the Mediterranean populations as previously reported in other European chicken breeds. Structure analysis exhibited extensive genetic admixture in many studied populations. In conclusion, suitable conservation measures should be implemented for these breeds in order to minimize inbreeding and uncontrolled crossbreeding. A special care is required for the conservation and preservation of these potentially vulnerable breeds.

RIASSUNTO

Le razze locali possono essere considerate parte della storia di molte popolazioni umane, così come materiale importante dal punto di vista scientifico. La catalogazione, la caratterizzazione e il controllo di routine della variabilità delle risorse genetiche animali sono pratiche fondamentali nelle strategie di miglioramento genetico e nei programmi di conservazione. La genetica molecolare ci fornisce importanti strumenti per analizzare la variabilità genetica tra e all'interno delle razze. Numerosi approcci sono stati sviluppati e utilizzati per comprendere i diversi aspetti che contribuiscono alla differenziazione delle razze.

Questa tesi è costituita da tre contributi scientifici.

L'obiettivo del primo (Capitolo 3) è stato quello di studiare la variabilità e analizzare la struttura di popolazione di due razze avicole Italiane (Ancona e Livorno), poiché possono essere considerate una fonte preziosa di variabilità genetica. Sono stati raccolti campioni di sangue da 131 animali e genotipati mediante l'utilizzo di un panel di 30 marcatori microsatelliti. Gli indici genetici calcolati suggeriscono un deficit di eterozigosità e un certo livello di consanguineità (eterozigosità media osservata = 0,46; eterozigosità media attesa = 0,53; F_{IS} in Ancona e Livorno = 0,251 e 0,086). L'albero delle distance inter-individuali DAS, elaborato mediante l'algoritmo Neighbour-Jouning, e l'analisi FCA, hanno evidenziato una elevata variabilità interna in Livorno rispetto alla razza Ancona. L'analisi mediante il software STRUCTURE ha evidenziato l'unicità genetica delle due razze oggetto di studio e la presenza di subgruppi nella razza Ancona, derivanti da un possibile isolamento genetico.

Nel quarto capitolo, è descritta la caratterizzazione genetica di cinque razze avicole italiane (Ancona, Livornese bianca, Modenese, Romagnola e Valdarnese Bianca), incluse le loro origini, la loro differenziazione e il loro attuale livello di variabilità genetica. Il primo obiettivo di tale studio è di indagare l'origine genetica di queste cinque razze di pollo italiane sulla base delle informazioni provenienti dal DNA mitocondriale (mtDNA). Il secondo obiettivo è stato quello di valutare la variabilità genetica, la struttura di popolazione e le loro relazioni genetiche mediante l'utilizzo di 27 marcatori molecolari microsatelliti.

Al fine di raggiungere tali obiettivi, è stato sequenziato un frammento di 506 bp della regione D-loop del DNA mitocondriale di 50 animali delle cinque razze avicole oggetto di studio. Sono stati individuati diciotto siti di variabilità che hanno definito 12 aplotipi. Questi ultimi sono stati assegnati a tre aplogruppi, probabilmente attribuiti a due linee materne. I risultati hanno mostrato che il 90% degli aplotipi ricade nell'aplogruppo E, originario del subcontinente Indiano come descritto in precedenza da altri autori. Per l'analisi microsatellitare, 137 singoli campioni di sangue sono stati raccolti nelle cinque razze italiane oggetto di studio. Un totale di 147 alleli è stato rilevato in 27 marcatori microsatelliti. Le cinque razze Italiane hanno mostrato un livello di consanguineità leggermente superiore (F_{IS} = 0,08) rispetto alle popolazioni commerciali utilizzate come razze di riferimento. L'analisi con il software STRUCTURE ha rilevato una chiara separazione delle cinque razze Italiane da queste popolazioni riferimento; una seconda analisi delle sole razze oggetto di studio ha permesso di discriminare le singole razze italiane.

Scopo del terzo studio è stato quello di descrivere la variabilità genetica e le relazioni tra sedici popolazioni avicole allevate nel bacino del Mediterraneo, mediante il sequenziamento della regione D-loop del DNA mitocondriale e l'utilizzo di un panel di 27 marcatori molecolari microsatelliti (Capitolo 5). Un frammento di 506 bp del D-loop mitocondriale è stato sequenziato in 160 campioni di DNA. Sono stati osservati 25 siti di variabilità e 21 aplotipi che definiscono tre aplogruppi e probabilmente tre linee materne. Il principale aplotipo, individuato nelle popolazioni del Mediterraneo, è rappresentato dall'E1 derivante dal subcontinente Indiano. Altre sequenze sono incluse negli aplogruppi A e B, i quali originano dal sud della Cina e dal Giappone. Per l'analisi microsatellitare, sono stai racconti 465 campioni di sangue. I risultati indicano che circa il 22% della variabilità totale origina da variazioni che intercorrono tra le popolazioni oggetto di studio. L'analisi di STRUCTURE ha rilevato un'ampia mescolanza genetica in molte delle popolazioni studiate.

In conclusione, adeguate misure di conservazione dovrebbero essere attuate al fine di minimizzare fenomeni di consanguineità e d'incrocio incontrollato nelle razze studiate. Particolare attenzione, pertanto, è richiesta al fine di conservare e salvaguardare queste razze potenzialmente vulnerabili.

1st CHAPTER

INTRODUCTION

1. ANIMAL GENETIC RESOURCES

The general term "biodiversity" is the contracted form of biological diversity. Biodiversity is indeed commonly used to describe all form of life, including all species and genetic variants within species and all ecosystems that contain and sustain diverse forms of life (CAST, 1999). On the other hand, agricultural biodiversity refers to the diversity of the cultivated plants and domestic farm animals utilized by man for the supply of food and other goods and services.

The capacity of agro-ecosystems to retain, improve productivity and adapt to changing circumstances remain crucial and vital to global food security. On a worldwide scale, animal biodiversity is defined as the variability among organisms of different or same species with respect to the environment in which they live, giving special attention to genetic biodiversity (Lehman and Tilman. 2000). For the livestock sector, animal genetic diversity is a resource to be drawn upon to select stocks and develop new breeds. More generally, different livestock populations have to provide society with a greater range of option to meet future demands, so the management of the world's agricultural biodiversity has become an important aspect to the international community (FAO, 2007).

The livestock sector, in particular, forms a substantial and essential component of agriculture output by producing high quality food. In developed countries the higher standard of living is generally accompanied with a greater consumption of animal products: meat, milk and eggs. In contrast, livestock in developing countries is an important component in the earning of livelihoods of some 70% of the world's poor rural people (Hoffmann and Scherf, 2005). The challenges for livestock production systems in effluent societies are food quality and safety to safeguard the human health, animal welfare in intensive systems and the sustainable use of resources.

The utilisation of farm animal genetic resources contributes to meet the different challenges in developed and developing countries. Between 1995 and 2004 global animal production increased (milk, 15 %; egg, 35 %; meat, 25 %) as reported by Rosati *et al.* (2005). The growth in production is predominantly realised in

countries with a rapidly growing livestock sector like Brazil, China, Mexico, Thailand and several East European countries (The World Bank, 2005). In the analysis of 148 country reports by Oldenbroek, (2006), it is evident that differences do exist between continents in the evolution of livestock production systems. In particular, in Europe the introduction of environmental and production restrictions has increased production cost, decreased the self-sufficiency and induced changes in livestock systems. A substantial amount of land is no longer used for agriculture and is surrendered back to nature. Less intensive systems like organic farming have been introduced and are growing in importance. At the same time a significant number of part-time farmers and hobbyists keep farm animals in rural areas (FAO, 2007).

1.1 AIMS OF THE THESIS

The major aim of this thesis is to characterize genetic diversity of some autochthonous chicken breeds in the Mediterranean area.

The specific goals were to:

- evaluate the genetic diversity within and between five Italian local chicken breeds by way different molecular markers (microsatellites and mitochondrial DNA);
- investigate the phylogenetic origin in some Mediterranean chicken breeds by mitochondrial DNA analysis;
- evaluate the residual genetic variability of these breeds by microsatellites marker.

These topics are original and have not been investigated before for the breeds under study. Generated information will increase knowledge and be useful both to the poultry breeder and also to the scientific community at large.

The overall goal of the following paragraphs (from 2 to 4) is to review information available on chicken genetic resources.

2. LIVESTOCK GENETIC RESOURCES IN THE MEDITERRANEAN AREA

The Mediterranean basin is one of the oldest and historically one of the most important cradles of agriculture. As the natural crossroad of three continents, the Mediterranean has played a dominant and permanent role in the development of florid civilizations. The region is characterised with a geophysical environment that is not exactly conducive for the development of modern practical agriculture. The arid and semiarid conditions so typical of the southern part created extremely difficult conditions, in spite of which human and animal populations have succeeded and flourished (Boyazoglu and Flamant, 1990). The variance of culture, climate, vegetation, land-use, socio-economic reality and food habits, have all come together to shape agriculture production systems in general and, in particular, the livestock farming systems.

2.1 RISK STATUS CLASSIFICATION

The risk status classification is an important element is defining the genetic sustainability of livestock breeds or populations. It is an indicator and informs stakeholders on whether and how urgent, genetic conservation actions need to be taken. Different classification has been used by the European Federation of Animal Science (EAAP, 1998) or FAO (Scherf, 2000) to describe the various degree of risk, but the most widely reported is the one provided by FAO thought the Global Databank for Farm Animal Genetic Resources (DAD-IS).

The risk status is classified into categories according to the number of available breeding males and females, the inbreeding rate (estimated from the effective population size), or population dynamics like increasing or decreasing population size. A framework to harmonise risk categories across institutions has been proposed (Gandini *et al.*, 2005).

DAD-IS monitors and classifies the world's breeds into the following risk categories:

- EXTINCT: the case when it is no longer possible to recreate a population of the breed. Extinction is absolute when there are no breeding males (semen), breeding females (oocytes), nor embryos remaining.
- CRITICAL: a breed is categorized as critical if:
    - the total number of breeding females is less than or equal to 100 or the total number of breeding males is less than or equal to five
    - the total breeding animals is less than or equal to 120 and decreasing and the percentage of females being bred to males of the same breed is below 80.
- ENDANGERED: a breed is categorized as endangered if:
    - the total number of breeding females is greater than 100 and less than or equal to 1 000 or the total number of breeding males is less than or equal to 20 and greater than five;
    - the overall population size is greater than 80 and less than 100 and increasing and the percentage of females being bred to males of the same breed is above 80;
    - the overall population size is greater than 1,000 and less than or equal to 1,200 decreasing and the percentage of females being bred to males of the same breed is below 80.
- CRITICAL-MAINTAINED or ENDAGERED-MAINTAINED: these categories identify specific populations for which active conservation programmes are in place or populations are maintained by commercial companies or research institutions.
- NOT A RISK: a breed is categorized as not a risk if none of the above definitions apply and the total number of breeding females and males are greater than 1,000 and 20, respectively or the population size is greater than 1 200 and the overall population size is increasing.

3. GALLUS GALLUS DOMESTICUS AND ORIGIN

The origin of the domestic chicken (*Gallus gallus domesticus L.*) has been debated ever since Darwin (Darwin, 1868). Archaeological remains of domestic chicken are found in 16 Neolithic sites along the Yellow River in Northeast China as well as in

the Indus Valley. Because some of these remains date back to 8,000 years ago, domestication must have been undertaken at least since that time.

Previous molecular studies suggested a single domestic origin in Southeast Asia (Thailand) (Fumihito *et al.*, 1995; 1996). At least six distinct maternal genetic lineages have now been identified (Liu *et al.*, 2006), suggesting more than one domestication centre. Four living species of genus Gallus are known: red junglefowl (*Gallus gallus*), La Fayette's junglefowl (*Gallus lafayettei*), gray junglefowl (*Gallus sonnerati*), and green junglefowl (*Gallus varius*), that differ by their morphological aspect and geographical distribution in Asia.

Gallus gallus is the closest to domestic chickens by its morphological traits and gives fertile offspring after crossing with domestic chickens, whereas matings between domestic chickens and any of the other three wild species yields very poor hatchability and chick survival. This Red junglefowl exhibits a strong sexual dimorphism with males having red fleshy wattles. This chicken is most widely distributed over the Southeast Asia.

Gallus lafayettei morphologically resembles the red junglefowl, with an orange brown breast feathers, having purple spot on the top of the neck and a yellow spot on the comb. It is found in Sri Lanka

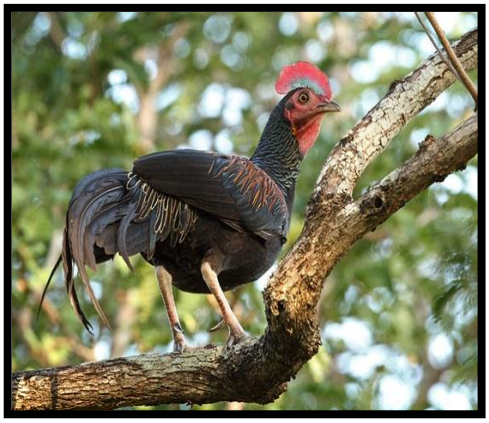
Gallus sonnerati has grey plumage and can be found distributed from southwest to central India.

Gallus varius is found only on the island of Java and its immediate vicinity of Bali and Lombok. It is characterized by several peculiarities including a wattle with three different colours (red, yellow and blue) and plumage with a greenish tinge.

Gallus gallus (Animal diversity web, University of Michigan ©)

Gallus lafayettei (www.hlasek.com – lubomir hlasek ©)

Gallus sonnerati (Nidhin G. Poothully ©)



Gallus varius (Lars Petersson ©)

According to archaeological findings and literature from the early 20th century, it is thought that chickens reached Europe along two main trading routes: a northern route through China and Russia and a southern route through Persia and Greece (Crawford, 1990). An alternative scenario reports that the two routes started from Iran, one via the Mediterranean Sea, and the other via the Black Sea. The Mediterranean type of chicken is considered to be the most ancestral type of the

European domestic chickens. Records from Greek mythology support the fact that chicken reached Greece by 700 BC. Poultry keeping was well developed under the Romans, who utilised chickens as a food source and also used them for leisure, religion and divination (Tixier-Boichard *et al*., 2011).

3.1 AVIAN SPECIES AND CHICKEN BREEDS

Chickens represent an important category (63% of all avian breeds) and the oldest type of poultry.

Chicken breeds are divided according to type and are classified into: layers (used exclusively for eggs production), broilers (production of chicken meat), dual-purpose breeds (meat and eggs), fighting breeds and ornamental breeds. The most important breeds in the development of modern egg laying strains including the White Leghorn, New Hampshire and Plymouth Rock were developed only in the second half of the nineteenth century

In the developed countries, commercial synthetic strains dominate the production of meat and eggs, while local breeds are marginalised and restricted to the hobby sector. In the developing countries local breeds still play an major important role; and in some cases make up 70-80 % of the (national ?) chicken population (Guèye, 2005; FAO, 2006). Chicken types found in the hobby sector may look very different from each other, but that does not necessarily mean they are genetically very diverse (Hoffmann *et al.*, 2005). The same may be true for some of the indigenous breeds in developing countries (FAO, 2006).

In Europe, the Leghorn is the most widespread breed and it is also an important contributor to the modern day commercial egg laying strains. White Leghorns originated from the Italian common chickens that reached the United States of America in the 1820's, where they were selected for egg production. The breed was imported back into Europe after the First World War. The second most common European breed is the British Sussex breed.

3.2 STATUS OF AVIAN GENETIC RESOURCES

Europe has the highest number of indigenous avian breeds (851), followed by Asia (408), Africa (146) and Latin America regions (138). Near Middle East, North America and Southwest Pacific regions have the lowest number (Table 1).

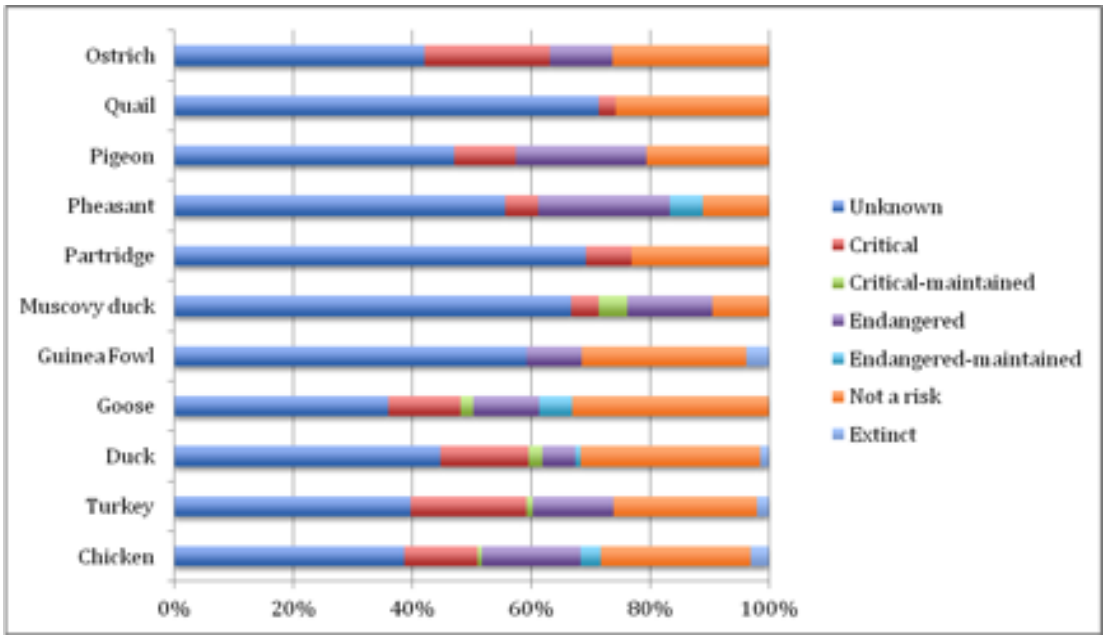
Table 1. Avian species: number of reported indigenous breeds

Species	Africa	Asia	Europe and Causasus	Latin America and Caribbean	Near and Middle East	North America	Southwest Pacific	World
Chicken	89	243	608	84	24	12	17	1077
Duck	14	76	62	22	4	1	7	186
Turkey	11	11	29	11	3	11	2	78
Goose	10	39	100	5	2	0	2	158
Muscovy duck	7	10	10	3	1	0	3	34
Partridge	2	8	3	0	0	0	0	13
Pheasant	0	7	5	6	0	0	0	18
Pigeon	7	12	30	7	8	1	2	67
Ostrich	6	2	4	0	0	0	1	13
Total	146	408	851	138	42	25	34	1644

Extinct breeds are excluded

In April 2010 FAO (DAD-IS) reported that the total global breeds amounts to 8 075 In a worldwide context, 9% of all avian breeds are classified as extinct, 7% critical, 1% critical maintained, 9% endangered, 3 % endangered maintained, 35 % not a risk and for the remaining 36% the situation is unknown because no information is available (FAOSTAT). Among the avian species, chicken has the highest number of breeds at risk on a global trend. This is partly related to the large number of chicken breeds in the world, but the proportion of breeds at risk (Figure 1) is also high in chickens (33 %). The regions with the highest proportion of their animal breeds classified as at risk are Europe and the Caucasus (28 % of mammalian breeds and 49 % of avian breeds), and North America (20 % of mammalian breeds and 79 % of avian breeds) (FAO, 2007). Europe also has the highest number of transboundary chicken breeds, defined as breeds that occur in more than one country.

Figure 1: Risk status of world's avian breeds. Percentage (chart) and absolute (table) figures by species (January, 2006).

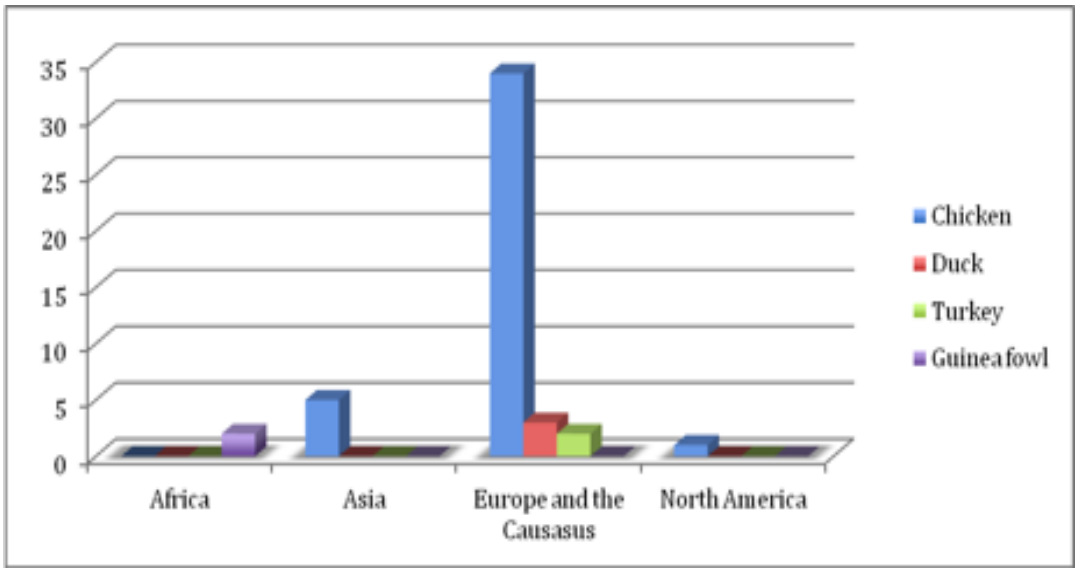


Source: FAO (2007)

Status	Chicken	Turkey	Duck	Goose	Guinea Fowl	Muscovy duck	Partridge	Pheasant	Pigeon	Quail	Ostrich	Total
Unknown	493	41	96	65	32	14	9	10	32	25	8	825
Critical	156	20	32	22	0	1	1	1	7	1	4	245
Critical-maintained	9	1	5	4	0	1	0	0	0	0	0	20
Endangered	212	14	12	20	5	3	0	4	15	0	2	287
Endangered-maintained	42	0	2	10	0	0	0	1	0	0	0	55
Not a risk	321	25	65	60	15	2	3	2	14	9	5	521
Extinct	40	2	3	0	2	0	0	0	0	0	0	47
Total	1273	103	215	181	54	21	13	18	68	35	19	2000

Forty chicken breeds are already declared extinct over the last 100 years (Figure 2), 34 of which are in Europe.

Figure 2: Number of extinct avian breeds

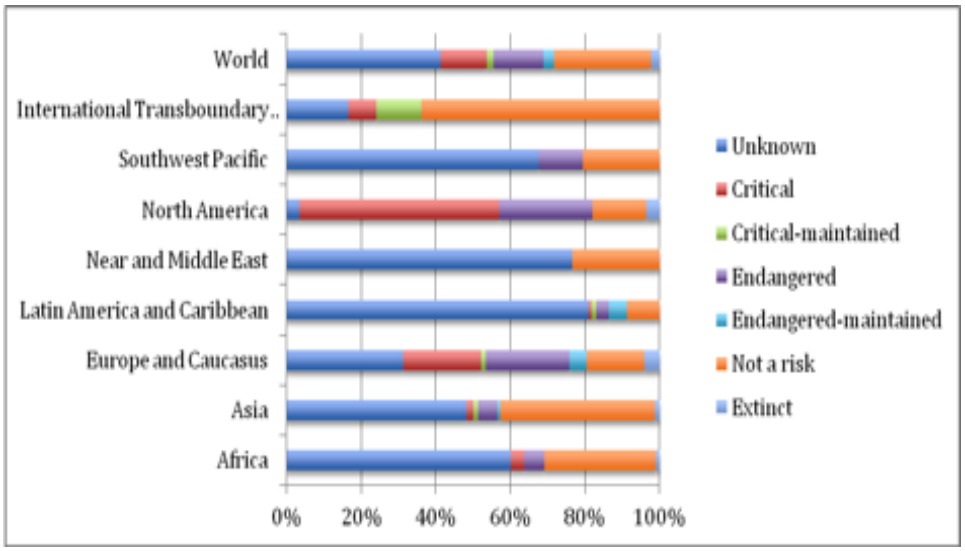


Source: FAO (2007)

Species	Africa	Asia	Europe and the Causasus	North America	World
Chicken	**0**	**5**	**34**	**1**	**40**
Duck	**0**	**0**	**3**	**0**	**3**
Turkey	**0**	**0**	**2**	**0**	**2**
Guinea fowl	**2**	**0**	**0**	**0**	**2**
Total	**2**	**5**	**39**	**1**	**47**

Figure 3 shows the distribution of avian breeds at risk by region. The regions with the highest proportion of their breeds classified as being at risk are Europe and the Caucasus (49 %), and North America (79 %). Europe and North America are two regions that have developed highly specialized livestock industries.

Figure 3: Risk status of world's avian breeds. Percentage (chart) and absolute (table) figures by region (January 2006).



Source: FAO (2007)

Status	Africa	Asia	Europe and Caucasus	Latin America and Caribbean	Near and Middle East	North America	Southwest Pacific	International Transboundary breeds	World
Unknown	113	214	305	120	33	1	23	26	835
Critical	7	8	204	1	0	15	0	12	247
Critical-maintained	0	6	12	2	0	0	0	19	39
Endangered	10	23	220	5	0	7	4	0	269
Endangered-maintained	0	3	45	7	0	0	0	0	55
Not a risk	56	184	151	13	10	4	7	100	525
Extinct	2	5	39	0	0	1	0	0	47
Total	188	443	976	148	43	28	34	157	2017

3.3 POULTRY SECTOR IN THE WORLD

The poultry sector continues to grow and industrialize in many parts of the world. The interest in poultry and poultry products has grown tremendously over the last 20 years;. almost every country has a poultry industry of some sort. Japan is steadily increasing its domestic production of both broilers and eggs. Countries of the former Soviet Union always produced poultry and eggs and are still continuing to improve their output to meet the increasing domestic demand. China, the Middle East and Africa are all areas where over the last few years the demand for poultry has increased dramatically.

In the period 2000 and 2010 poultry meat and egg production have shown remarkable dynamics. The trade volume of poultry meat varied between regions such that Asia and Africa recorded annual gains of around 4.5% during the decade, while less than 4 % was registered in the other continents. In Europe, there was a remarkable difference between countries within and outside of the European Union, in particular Russia and Ukraine. In the Europe community, the growth was less than 20 % as the total production climbed from 8.2 million tonnes to 9.7 million tonnes. In the non-EU countries a shift from 1.2 million tonnes to 4.1 million tonnes was observed (FAOSTAT).

Generally speaking, poultry production falls in one of two main production systems: the commercial production system that generally utilises the modern synthetic strains and the village system that employs different chicken breeds. Commercial patented strains purposely developed to fit into intensive production systems are used in the commercial system, while under the village system the more typical local breeds are popular.

3.4 CONSERVATION STRATEGIES OF LOCAL CHICKEN GENETIC RESOURCES

The interest and awareness in livestock conservation has gradually increased over the last 25 years due to the drastic reduction of local populations that have been replaced by the expansion of more productive types (Hall, 1995). The global use of

highly productive animal has resulted in the gradual erosion of genetic diversity in most species. Poultry genetic resources are the most endangered (FAO, 2007).
A thorough understanding of the extent and the nature of genetic diversity among and within breeds and populations is an essential prerequisite for the effective management and utilization of genetic resources in chicken as well as in other farm animals (Weigend *et al.*, 2004). From the FAO database, it is estimated that about 25 % of all chicken breeds are included in some sort of conservation initiatives, but there is no information on the efficiency of these programmes. According to FAO country reports, only 15% of countries have poultry conservation programmes.

An important step in the genetic resources sustainable management is the establishment of conservations measures. Theoretically, three types of conservation measures can be implemented: *in situ* conservation, *ex situ in vivo* conservation and *ex situ* in vitro conservation. The distinction between the different conservation programmes can be rather vague, and Country Reports, do not usually make a clear distinction between the various types.
Geerlings *et al.*, 2002 proposes only two approaches to conservation of animal biodiversity namely: 1) *in vivo*, that includes both *in situ* and *ex situ in vivo*, and 2) in vitro that include *ex situ*. *Ex situ* refers to conservation approaches outside of a breed's natural habitat, such as in zoos and gen banks. In situ is the conservation of the breed within its ecosystem and natural habitats. It involves the maintenance and recovery of viable populations in their natural surroundings where they have developed their distinctive properties (Geerlings *et al.*, 2002). In situ conservation programmes must include identification and registration of animals and monitoring of populations and population size. The majority of Country Reports indicate the presence of *in vivo* conservation measures, while only 37 % indicate the presence of in vitro conservation. Various governmental, non-governmental and private organizations try to preserve genetic diversity of livestock *in situ*. In the case of poultry, maintaining an *in situ* population of the non-commercial breeds is largely dependent on the enthusiasm of local show people and hobby farmers. In the developed world, many people keep minor chickens breeds as a hobby and hence providing an opportunity for the *in vivo* conservation. In addition to *in situ*

conservation, gene banks are being established for the *ex situ* conservation (Woelders *et al.*, 2006).

Many gene banks are present in Japan, India, the Nordic countries, France, the Netherlands, Poland, the Czech Republic and Hungary, while in other countries, the establishment of gene banks is being planned. The technology to store semen from all the livestock species and embryos of cattle, sheep and goats is easily available and widely utilised. Sometimes tissue DNA samples are also collected for the main species. In developing countries the implementation of in vitro conservation measures, is usually limited to the storage of semen from some local cattle and sheep breeds at private or governmental institutions. On the other hand only a few gene banks store poultry and horse semen. In Europe and North America many universities and research institutes try to conserve locally developed breeds of chicken that are no longer used by the industry. For chickens in vitro conservation of semen is a recent development. Cryoconservation is a proven technology and is an important complement to in vivo breed conservation (Woelders *et al*., 2006).
There are several reasons for the conservation of genetic diversity in farm animals such as: rare or local breeds fulfil specific requirements with respect to local terrain or climate or may produce typical regional products. Efforts to conserve genetic diversity of farm animals include measures to stimulate the inclusion of indigenous and rare breeds by farmers. In many developing countries conservation programmes are necessary and these programmes should be encouraged and supported through external technical and financial assistance. Effective prioritization of breeds for conservation programmes is facilitated by phenotypic and genetic characterization and by knowledge of the size and structure of the population. The FAO definition of animal genetic resources eligible for conservation includes animal populations with economic potential, scientific use and cultural interest. There are several reasons why the implementation of conservation measures for a particular breed might be considered important: genetic uniqueness; high degree of endangerment; traits of economic and scientific importance; ecological, historical and cultural value. Conservation and development of local breeds is important because of their contribution to the

livelihoods of farmers and biodiversity as well as their social and cultural importance (FAO, 2007).

4. ASSESSMENT OF BIODIVERSITY

Information on genetic diversity is important in optimizing both conservation and utilization strategies. In recent years, genetics conservation for the preservation of species and breeds has received increasing attention. In genetics conservations, knowledge of the degree of kinship between individuals is of particularly importance in the selection of breeding programs aimed at reducing incidence of Sire-daughter / Dam –son matings in order to minimize inbreeding and the loss of genetic variation (Frankhman *et al.*, 2002). The erosion of genetic variation reduces the ability of a given population to adapt to environmental changes and decreases also its chances for long-term survival. Inbreeding decreased genetic diversity, depresses reproductive performance and increases the risk of the breed becoming extinct (Saccheri *et al.*, 1998). Genetic studies can reduce the risk of extinction and can help to develop a population management program that minimize the negative effects associated with inbreeding. Preservation of population biodiversity is crucial as to minimize the loss of genetic variation as a consequence of inbreeding (Russello and Amato, 2004). The evaluation of genetic diversity within and between different populations has been undertaken by using several DNA marker techniques. Molecular methods play an important role in estimating the genetic diversity among individuals by comparing the genotypes at a number of polymorphic loci (Avise, 2004). Development in fundamental DNA technology contributed to a burst of applications in multiple research areas, including the study of genetic variation and diversity in chickens (Weigend *et al.*, 2004).

The molecular markers are indispensable for determining the genetic variability and biodiversity with high level of reproducibility. These molecular tools are available to study the genetic structure of a wide range of populations, to quantify the genetic variability at the genome level, to reconstruct scenarios for population history, and to propose new management programmes (Erhardt and Weimann, 2007). In particular, they have been used successfully in population genetics for:

measuring local gene flow and migration, assigning individuals to their most likely population of origin, measuring effective population size through the generations comparison of allele frequencies and detection past demographic bottlenecks through allele frequency distortions (Jehle and Arntzen, 2002). The most recent progress in the characterization of poultry resources has been based on the use of molecular markers; the European project AVIANDIV (1998–2000) provided the first large-scale study of genetic diversity in domestic chickens using microsatellite markers (Hillel *et al.*, 2003). These markers are classified in two types: mitochondrial and nuclear markers. Several types of molecular markers, including mitochondrial DNA (mtDNA) and nuclear DNA markers are available but none of them can be regarded as optimal for all applications. In genetic diversity studies, the most frequently used markers are single nucleotide polymorphisms (SNP), microsatellites and mtDNA.

Single nucleotide polymorphisms (SNP)

In recent years, SNP have been described as being a potential a very promising class of molecular markers for biodiversity studies. These single nucleotide polymorphisms (SNPs) are a major focus in human studies as third generation genetic marker (Collins *et al.*, 1997; Wang *et al.*, 1998), and they are also used as alternative to microsatellites in genetic diversity studies. Being biallelic markers, SNPs have rather low information content since they are the most common form of genetic variation in the genome. As their name implies, SNPs are single base changes or nucleotide variations that can occur in genes (promoter, exons, or introns) or between genes (intergenic regions). The SNPs within the coding sequences are categorized as either synonymous (does not result in an amino acid change) or non-synonymous (results in an amino acid change). Non-synonymous SNP are of interest due to their potential effect on protein expression and, ultimately contributes to new phenotypes. In contrast, synonymous SNP probably have minimal effects on gene expression; exceptions might be those nucleotides that are important in DNA-protein interactions in the promoter and other genomic regions or those nucleotides that are involved in RNA stability. Both synonymous and non-synonymous SNPs are excellent genetic markers for mapping studies.

The SNP are not a new feature in chicken research. The importance of SNP in chickens was demonstrated by several investigators who focused on linkage mapping of genes, association studies using candidate gene approaches, and evolutionary analyses (Fotouhi *et al.*, 1993; Kuhnlein *et al.*, 1997; Liu *et al.*, 2001; Smith *et al.*, 2001, 2002; Zhou *et al.*, 2001; Lamont *et al.*, 2002, and others too numerous to mention). The SNP in expressed regions of the genes are of particular interest because they can potentially impact protein function and the phenotype of an individual. Recent information in literature has revealed that microsatellite markers are useful in determining heterozygosity and estimating genetic distances among closely related species (Chen *et al.*, 2004), and also for the estimation of cumulative power of discrimination of any population including the avian species.

Microsatellites(SSR)

Microsatellites or Simple sequence repeats (SSR) are the most popular markers in studies of livestock genetic characterization (Sunnuck, 2001). They are also known as short tandem repeats and consist of a stretch of DNA with few nucleotides, generally 1-5 base pair (bp) long, located in both coding and non-coding regions. Microsatellites are co-dominant markers and are highly polymorphic and abundant. They can be amplified by Polymerase Chain Reaction (PCR**)**, rendering them highly versatile markers for molecular fingerprinting. They are hypervariable and often show tens of allele at a locus that differ from each other in the number of repeats. FAO (2004) has recommended that diversity in chickens and other livestock should be assessed using a set of microsatellite loci.

Mitochondrial DNA (mtDNA)

Mitochondrial DNA is a circular molecule of 16 785 bp in size (Desjardins and Morais, 1990), also known as mtDNA polymorphism and has been extensively used in phylogenetic and genetic diversity analyses. This technique is gaining an more increasingly important role as a genetic marker in population studies.

Mitochondrial DNA contains a non-coding region named control region (D-Loop). The length of the D-Loop region is approximately 1 kilo base pairs (kb) and can be amplified by PCR. Sequence analysis of this fragment has been used to measure molecular diversity and to identify conservation units for better management of

the species (Onuma *et al.*, 2006). The polymorphisms in the sequence of the hyper variable region of the D-loop of mtDNA have contributed to the identification of the wild ancestors of domestic species and understanding of the evolution of livestock domestication. Ideal markers should have co-dominant expression and found in readily accessible tissue. High degree of polymorphism and random distribution throughout the genome makes markers more informative (Weigend and Romanov, 2001; Bruford *et al.*, 2003). However, a survey on genetic diversity studies revealed that microsatellites are the most preferred markers in the study of chickens and other livestock species (Baumung *et al.*, 2004)

In conclusion, mtDNA studies provide a valuable preliminary description of the population structure and demographic history, but nuclear marker (like microsatellites) would provide valuable information to complete the analysis (Saillant *et al.*, 2004; Bowie *et al.*, 2009; Kvist *et al.*, 2011).

5. GENETIC DIVERSITY MEASURES

Within livestock species, the genetic diversity is most obviously expressed in the wide spectrum of animal types. Breeds are defined as populations within a species of which the members can be determined by a set of characteristics particular to the breed (FAO, 1998). The FAO definition assumes that in the phenotypes of characteristics or traits, there is a clear distinction between populations. Conservation efforts should be as efficient as possible, securing a the retention of maximum amount of genetic diversity with the available resources. To this end, breeds at risk need to be evaluated to determine the potential amount of genetic diversity. Evaluation is very much dependent on the rationale for conservation (Ruane, 1999) and may require balancing diversity within and between populations.

Recently, there has been a major shift from the differentiation of poultry breeds according to morphological and feather colouring characteristics, to scientific differentiation based on measurements at the molecular level (FAO, 2008) . The use of molecular markers can provide quantified criteria for assessing genetic diversity, either within or between populations. However, although these markers can be used to study kinship between populations, provide information on

evolution of populations, detection of introgressions and contribute to the genetic definition of a breed's entity, the molecular markers do not provide information on phenotypes and special adaptive traits.

Appropriate sampling is critical when molecular characterization is utilised to make comparisons between breeds. A minimum group of 30 to 50 unrelated individuals is required to derive unbiased conclusions. The determination of the chicken genome in 2004 (Hillier *et al.*, 2004) has facilitated the use of molecular markers for chicken breed/ecotype characterization. Although genome knowledge is not as thorough in other poultry species, linkage maps are available for ducks, quails and turkeys, and reference to the chicken genome is generally an efficient approach for studying gene order and gene structure. The availability of molecular markers is therefore not a limiting factor in most poultry species. Highly polymorphic microsatellite markers are preferred because they provide much information for a limited number of loci; most studies use between 20 and 30 markers. Molecular tools for the study of genetic diversity using single nucleotide polymorphisms are likely to be developed further in the future.

Genetic diversity within breed can be estimated by the number of alleles, the expected heterozigosity (Frankham *et al.*, 2002) and marker estimated kinships within a breed (Eding and Meuwissen, 2001). Genetic diversity between breeds can be studied by various measures, but the most important parameter for assessing diversity between breeds is the genetic differentiation or fixation indices which reveal the partitioning of genetic diversity (Wright, 1969). A wide range of studies for the assessment of genetic diversity were conducted using genetic distances (Nei, 1972; Reynolds *et al.*, 1983). Bayesian clustering approaches have been suggested for admixture analysis of different populations (Pritchard et al., 2000). This approach has already been used in the study of population structure of various farm animals (Rosemberg *et al.*, 2001; Granevitz *et al.*, 2009; Leroy *et al.*, 2009; Lasagna *et al.*, 2011). As reported by Tixier-Boichard, Bordas and Rognon (2008), studies in chickens using microsatellite markers have shown large variations in heterozygosity, ranging from 28 % for a fancy breed to 67 % for a village population, but the average value (of about 50 %) is rather lower than that observed in domestic mammals. The highest levels of within-population diversity were found in wild ancestor species, unselected local populations, a few

standardized breeds kept in large populations, and some commercial broiler lines. A range of values were obtained for European some fancy breeds, reflecting the variability of population history within this type of population. Expected values for heterozygosity in the commercial lines range from 50 to 63 % in broilers, 45 to 50 % for brown-egg layers, and about 40 % for white-egg layers (Hillel *et al.*, 2003).

In the study of mtDNA D-loop, the haplotype network analysis clusters individuals based on haplotypes present and indicates how different haplotypes originate from those in other individual. The median networks of haplotypes were generated by partitioning the groups of haplotypes to portray mtDNA relationships and infer about population expansion and domestication events (Bandelt *et al.*, 1995). The ancient haplotypes can be distinguished from contemporary ones due to their higher frequencies and central positions surrounded by derived haplotypes in a star like topology (MacHugh and Bradley, 2001).

Applying haplotype network analysis, Liu *et al.* (2006) and Oka *et al.* (2007) concluded multiple and independent domestication events in South China, Southeast Asia and the Indian subcontinent. These studies suggest that there is a significant reservoir of genetic diversity within local breeds of chicken.

6. AN ITALIAN CONSERVATION CASE STUDY: CONSERVATION AND VALORISATION OF VALDARNESE BIANCA BREED

In the Tuscan region of Italy, the interest in developing measures for the conservation of indigenous chicken breeds is due to historical, social and economic reasons. The autochthonous breeds are an important resource of gene pools for future breeding and research purposes.

The Valdarnese bianca breed has recently been incorporated into a conservation and valorisation program. This breed can be considered as the only traditional Italian meat-type chicken, birds having white feathers with dark yellow shanks and a single comb. The weights of the cock and the hens range from 3.0 to 3.5 Kg and from 2.5 to 3.0 Kg respectively. This breed is characterized by very slow juvenile feathering, white eggshells, average egg weight of 50 g, and an average egg production of 135 per year. Pullets usually come into lay by the age of 7 months.

This breed reaches slaughter weight of ~2 kg in approximately 120 days.The Valdarnese, also referred to as Valdarnese Bianca, Valdarno Bianca or Pollo del Valdarno, is a breed of large white chicken from the upper Valdarno, the valley of the Arno river, in Tuscany, central Italy. It became virtually extinct in the 20th century, but the population is recovering. It is a different breed from the Valdarno chicken, which originates in the lower part of the Valdarno, and is black. The first description of the white chickens of the Valdarno is by Licciardelli in 1899 (Licciardelli, 1899). Pochini (1900, 1905) recommends the Valdarno breed above all others as suitable for both small and large-scale rearing, for its rapid growth and the maternal instinct of the hens, but notes that it requires space and does not adapt well to close confinement. He illustrates four colour varieties, black, white, buff and cuckoo, and notes that the black and the white are the most common (Pochini, 1900). The Valdarno breed was also described by Faelli (Faelli, 1905).

Various examples of Valdarnese bianca chickens were exhibited in Cremona and Varese in the 1950s. In the following years, the Valdarnese became the subject of extended and heated discussions on its authenticity with critics insisting that its high productivity was only due to hybrid vigour. The breed had enough strong willed supporters that a breed association was formed in 1955. Studies of the qualities of the breed by Quilici, director of the Stazione Sperimentale di Pollicoltura (experimental poultry-breeding station) of Rovigo from 1957 led to the first scientific description of the breed.

The collapse in the 1960's of traditional agricultural sharecropping production systems caused a rapid decline in breed numbers, aggravated both by the growth of intensive methods of poultry-farming and by competition from the White Leghorn which were available as day old chicks incubated in northern Italy. The breed association was dissolved in 1964, and the Valdarnese continued to decline through the later part of the 20th century until it had virtually disappeared (Zanon, 2012). The risk of extinction of the breed was recognised in the 1990s, and the "Conservatorio delle Razze Avicole in Pericolo di Estinzione" (Conservation centre for avian breeds in danger of extinction) of the Veneto region began a repopulation programme (Gualtieri, 2006). When the Conservatorio closed in 2001, the remaining breeding stock was transferred to the Valdarno area. This flock formed the basis for a project for the recovery and protection of the breed launched by the

Agenzia Regionale per lo Sviluppo e l'Innovazione nel Settore Agricolo-Forestale (ARSIA), a part of the Tuscan regional administration for agriculture.

The Valdarnese is not included in the official standard of the Federazione Italiana Associazioni Avicole, the federation of Italian poultry associations, which is the national authority overseeing poultry breeding in Italy (FIAV, 1996). The breed is, however, recognised by the Regione Toscana, the regional administration of Tuscany, which publishes the breed standard. A breed register is held by the Associazione Provinciale Allevatori, or provincial breeders' association, of Arezzo (Gualtieri, 2006).

Although the Breeders Association of the Arezzo (now ARA-Tuscany) province has since 2005 established and maintains a herd book, breed numbers have remained low. A study published in 2007 used a figure of approximately 1200 for the total breeding stock, of which approximately 300 were cocks (Spalona *et al.*, 2007). Until 2009, the genotyping of the individual animals (from 10 farms) for marker assisted conservation scheme was carried out using microsatellite technique (Strillacci *et al.*, 2009).

An example of conservation flock in Arezzo province (Italy)

RATIONALE

Conservation programs should be considered as an international responsibility, and conservation activities must be focused on maximizing the effective number of individuals contributing to the gene pool, as this helps in preventing erosion of animal genetic diversity as a result of inbreeding. Appropriate breeding and conservation strategies need to be put in place to avoid further erosion of the Animal Genetic Resources, including chickens. Due to limited resources, cost-effective strategies are expected and these depend on accurate identification of unique populations. Moreover, it is important to preserve the existing breeds as these derived from the ancient local breeds.

In reaching the aims and objectives to analyse the chicken genetic resources in four countries of Mediterranean area a network of scientists from five Mediterranean countries all having genuine interest in further studying their local chicken breeds was created and biological samples of the available breeds were contributed towards this study.

The local breeds were identified based on data from the various institutions within this network, and in particular the studied breeds were chosen for their geographical origin and on the bases of previous scientific collaborations between different institutions.

This study is the first attempt to create a collaborative network in Mediterranean chicken genetics, and generated results will act as a platform on which other projects can evolve.

RESEARCH TOOLS

(i) Phase I

A questionnaire to generate information on production systems, management and breeding practises was conducted to allow for proper interpretation of molecular data .

(ii) Phase II

1. Twenty-seven microsatellite markers were used to determine within and between population genetic diversity. These markers were recommended by the Food and Agriculture Organization (FAO, 2004) and the International Society of Animal Genetics (ISAG) for assessing chicken diversity. The reference populations had been genotyped in previous projects. There are distributed over 15 chromosomes of the chicken genome. A preliminary DNA genotyping comparison test was performed in order to merge my dataset with the AVIANDIV dataset. Allele size correction was performed based on the distribution of the allele frequencies per each marker.

2. A total 506 bp of the mtDNA D-loop region was also used to infer genetic diversity and phylogeographic structure of the chicken breeds. Haplotypes from GenBank (Liu et al., 2006) were aligned with haplotypes observed in this study as references.

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2nd CHAPTER

A microsatellites-based survey on the genetic structure of two Italian local chicken breeds

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2.1 ABSTRACT

The biodiversity safeguard is an important goal of poultry production in every developed country. Nowadays, the high chicken meat demand from the world market has been leading to a large spread of strongly producing commercial chicken lines. The creation of these standard types is causing a progressive loss of genetic variability. Ancona and Livorno are two Italian autochthonous chicken breeds which represent a great resource in terms of specific genetic richness. Aim of this study is to investigate the genetic diversity of these breeds as potential valuable genetic variability source. In fact, in spite of their endangered status, these chicken breeds are very appreciated for their ability to adapt themselves to extensive organic rearing systems. Blood samples from 131 individuals were collected and genotyped through a thirty microsatellites-based analysis. All the observed descriptive statistical indexes suggested a heterozygosity deficiency and an inbreeding level (mean observed heterozygosity = 0.46, mean expected heterozygosity = 0.53, F_{IS} in Ancona and Livorno = 0.251 and 0.086). The tree from inter-individual DAS distance using Neighbour-Joining algorithm and the FCA analysis showed a higher internal variability in Livorno than in Ancona. STRUCTURE analysis showed the genetic uniqueness of the breeds and the presence of sub-groups in Ancona originating from a possible genetic isolation. This research could be a suitable starting point to set up improved selection schemes and a potential preliminary genotypic test for all the cocks to be used in the selection.

Key words: Chicken, Microsatellites, Ancona, Livorno, Genetic diversity

2.2 INTRODUCTION

The poultry biodiversity safeguard is a strong objective in every developed country (Zanetti *et al.*, 2007). The breed genetic variability gives the chance to select the individuals more able to be adapted to climatic changes, diseases and market variations. Because of the several different environments, up to decades ago Italy showed a considerable biodiversity in livestock breeds and populations. Within the last one-hundred years, the number of the endangered autochthonous breeds is dramatically increased (Zanon and Sabbioni, 2001) leading to an irreversible loss of genetic resources. The reasons of this negative trend are mainly the use of a few breeds selected to maximise the yields and the creation of specialised cross-breeds for the several productions. As a consequence of this loss of genetic diversity, many chicken local breeds reared in Italy until some decades ago are now disappeared (Gandini and Villa, 2003). The autochthonous extant breeds, which have been excluded from intensive rearing systems for a long time, represent an important source of variability. Their disappearance might lead to the loss of a potentially useful genetic patrimony. Ancona and Livorno (Leghorn Italian type), are two of these autochthonous chicken breeds (Domestic Animal Diversity Information System, 2010). The Ancona produces white or sometimes tinted eggs and is also considered an excellent layer because of its good all-year-round egg laying capacity. The Livorno is worldwide spread with different livery colors; this breed is an excellent white egg layer. The mean production can reach two-hundred and eighty eggs per year; the feed-to-egg conversion rate is excellent.

The production systems standardisation takes advantage of commercial strains which have been selected for improved performance and intensive rearing system; such cosmopolitan types are affected by a progressive reduction of genetic variability, which on the other hand is still present in the local traditional breeds (Spalona *et al.*, 2007), particularly suitable for extensive rearing systems.

Microsatellites markers are one of the most common and powerful tool to investigate genetic variability. Such molecular markers have been widely used in several studies regarding genetic diversity of domestic animals such as pig (Vicente et al., 2008), sheep (Lasagna *et al.*, 2011), cattle (Li *et al.*, 2009), goat (Mahmoudi *et al.*, 2009), horse (Giacomoni et al., 2008) and chicken (Hillel *et al.*, 2003).

Aim of this study is to investigate the genetic diversity of the autochthonous Ancona and Livorno breeds. In fact, these local breeds are under threat of extinction, as demonstrated by their drastic decline in number and their low consistency (Mugnai *et al.*, 2009). In spite of their endangered status, these chicken breeds are very appreciated for their ability to adapt themselves to extensive organic rearing systems. Besides that, they were proposed as egg layers models for an en plain air rearing system (Castellini *et al.,* 2006; Mugnai *et al.*, 2009; Dal Bosco *et al.*, 2011). The molecular results on these breeds will be useful to set up improved selection schemes and to conserve strategies to avoid inbreeding.

2.3 MATERIALS AND METHODS

Animal sampling and microsatellite analysis

Blood samples were randomly collected from different animals belonging to Ancona (50), White Livorno (51) and Sasso (30) breeds. Animals from the French breed Sasso were included to have an out-group. These animals were chosen, as far as we were able to manage, in different farms in order to avoid closely related individuals and to have a representative sample of the breeds. Figure 1 shows geographical areas and number of farms which the individuals belonging to different breeds were sampled from. The most important area where Ancona is reared includes the Italian regions Marche and part of Emilia Romagna. Genomic DNA was extracted from blood using the GenElute Blood Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA). Thirty loci microsatellites (Table 1) were chosen on the basis of their position in the chicken genome. Twenty-nine of them had already been used in the AVIANDIV project (Aviandiv, 2011) and the microsatellite marker LEI0192 (Groenen et al., 2000) was also added. The markers were subjected to a standard multiplex PCR amplification using a Biometra TGradient 96 at the following conditions: initial denaturation step of 5 min at 95°C, 35 cycles of 30 sec at 90°C, 45 sec at the annealing T° of each multiplex PCR, 30 sec at 72°C and a final extension of 15 min at 72°C. The multiplex PCR products were pooled in order to analyze many microsatellites in each electrophoresis. Analyses of fragments were performed using an automated DNA sequencer (ABI PRISM 3130xl, Applied Biosystems, Foster City, CA, USA) and a computer software (GeneMapper version

4.0, Applied Biosystems). Allele calling was adjusted to AVIANDIV project nomenclature (Aviandiv, 2011) including nine standard DNA reference samples.

Statistical analysis

The 30 microsatellites PIC values calculated according to Botstein *et al.* (1980) and observed and expected heterozygosity in the analyzed breeds were estimated by the EXCEL MICROSATELLITE TOOLKIT 3.1.1 (Park, 2001). The number of alleles observed in each locus and mean number of alleles per breed were counted using POPGENE 3.2 software package (Yeh *et al.*, 1999). The number of private alleles was calculated through direct count on allelic frequencies calculated by the software CONVERT (Glaubitz, 2004). The Hardy-Weinberg equilibrium was tested by the software GENEPOP 4.0 (Raymond and Rousset, 1995). A Markov Chain Monte Carlo method (20 batches, 5000 iterations per batch, and a dememorisation number of 10,000) was applied to perform exact probability tests, according to the algorithm described by Guo and Thompson (1992). To assess the population genetic structure of the chicken breeds, Wright's F-statistic was estimated. Fixation indices per locus (F_{IS}, F_{IT} and F_{ST}) were calculated according to Weir and Cockerham (1984) using the software GENETIX 4.05 (Belkhir *et al.*, 1996-2004), which was also employed to obtain the F_{IS} per population calculated with 1000 bootstraps. The significance of the fixation indices was tested using the software ARLEQUIN 3.11 (Schneider *et al*., 1997), according to the analysis of molecular variance (AMOVA). The DAS genetic distance (Chakraborty and Jin, 1993) among the individuals was calculated using the software POPULATIONS 1.2.28 (Langella, 2002). The Neighbour-Joining methodology was applied and a tree was built from the inter-individual distances by using the MEGA 4 package (Tamura *et al*., 2007). Factorial correspondence analysis (FCA) (Benzécri, 1982), assessed by the employment of GENETIX 4.05, was used in order to investigate further the differentiation of the individuals within each population. STRUCTURE version 2.2 (Pritchard *et al*., 2000) was employed to confirm the genetic pattern of each individual belonging to the different breeds and to reveal possible clustering substructures. The Bayesian assignment of individuals to populations considered an ancestry model with admixture and correlated allele frequencies. Ten independent runs with 1,000,000 MCMC (Markov Chain Monte Carlo) iterations

and a burn-in of 300,000 were carried out for 2≤K≤6 (K, number of clusters) to estimate the most likely number of clusters present in the data set. This numerical value was then established by calculating ΔK, as in Evanno et al. (2005). The clustering pattern was visualised using the software DISTRUCT 1.1 (Rosenberg, 2004).

2.4 RESULTS AND DISCUSSION

In spite of the presence of some loci microsatellites showing a low level of polymorphism, the used panel turned out to be good and reliable for genetic diversity analysis. Hillel et al. (2003) got comparable results in a study involving more chicken populations. The total number of alleles found in the thirty microsatellite markers was 177. In Spanish chicken breeds, Dávila et al. (2009) detected a lower number of total alleles across all the population. LEI0234 showed the highest number of alleles observed in each locus (14), whereas MCW0248 and MCW0103 the lowest (2). With regard to PIC per locus, about half markers showed slightly high values (>0.50), while the others revealed lower values (<0.50) (Table 1). These results were not much different from those which Tadano et al. (2007) pointed out in a study involving Japanese chicken breeds. However, the informativeness of this microsatellites panel was lower if compared with the results obtained by Qu et al. (2006) and Beigi-Nassiri et al. (2007). The mean number of alleles per breed (Table 2) ranged from 3.50 for Ancona to 4.03 for Sasso. Rosenberg et al. (2001) found higher values in a study which took twenty European chicken breeds into account. The same findings arose from the analysis of the genetic diversity of Chinese indigenous chicken breeds (Qu et al., 2006), which were characterised by a more substantial number of alleles. An explanation of the lower number of alleles found in Ancona and Livorno could be due to the fact that the genetic variability parameters are generally lacking in small autochthonous chicken breeds, compared with larger and more differentiated populations. The Ancona breed showed 17 private alleles whereas Livorno 26 (Table 2). On the other hand Sasso was characterised by the highest number of breed-specific alleles (32) and this is consistent with his cosmopolitan status and with the fact that this breed was genetically influenced by other breeds not included in this work. The mean values of observed heterozygosity (0.49) and

expected heterozygosity (0.52) in the total analysed population (data not showed) are not very high, suggesting a low genetic variability. In more details, Sasso displayed the highest value of observed heterozygosity (0.68) while Ancona the lowest (0.35) (Table 2). The mean expected heterozygosity ranged from a maximum of 0.60 in Sasso to a minimum of 0.47 in Ancona. With regard to Ancona breed, the numerical deviation of the observed heterozygosity compared to the expected heterozygosity is consistent with the values found by Dalvit et al. (2009). In their analyses, Qu et al. (2006) obtained higher values, probably due to the presence of more populations in the study. However, the results found in this work are comparable with those observed by Dalvit et al. (2009) in other two Italian autochthonous chicken breeds (Robusta Maculata and Ermellinata di Rovigo). It might therefore be logic speculating the presence of a general low level of genetic variability within the Italian autochthonous chicken breeds. The F_{IS} calculated in each breed (Table 2) were significantly different from zero ($P<0.05$) in Ancona (0.251) and Livorno (0.086), indicating heterozygosity deficiency in these breeds. The positive and significantly different from zero F_{IS} values might arise from the presence of inbreeding or the presence of sub-populations within the breeds. It is reasonable to speculate that both the hypotheses are possible for the studied breeds, especially for Ancona. Ancona is a small breed and exchange of genetic material among breeders rearing it is not very common. Sasso showed a negative F_{IS} value (-0.142), revealing a heterozygosity excess. This situation is clearly confirmed and actually is the consequence of the observed heterozygosity value which is higher than the expected heterozygosity. Negative F_{IS} values are generally present in populations showing geneflow due to the introduction of individuals belonging to other breeds for the reproduction. Twenty-six loci, out of thirty, deviated ($P<0.05$) from the Hardy-Weinberg equilibrium in the whole population composed by the pooled samples (Table S1, Appendix). This high percentage of deviation from the equilibrium ideal condition is due to a non-random mating which led to a homozygote excess and it is indeed confirmed by the markers F_{IS} values. Deviations from the Hardy-Weinberg equilibrium are expected if individual populations are sub-structured into flocks within populations that are isolated from each other (Granevitze *et al.,* 2007). Dalvit et al. (2009) highlighted a very highly significant deviation from the Hardy-Weinberg equilibrium in two Italian

local chicken breeds before they started out an in situ marker assisted conservation scheme. In Table S1 (Appendix)Wright fixation indices per locus in the whole population are shown. The mean F_{IS} value was significantly different from zero (0.082) ($P<0.05$) confirming again the presence of heterozygosity deficiency and not completely random matings in the studied sample. As expected, the mean F_{IT} index was 0.307 ($P<0.05$), highlighting the presence of some factors which influenced the normal gene flow among the animals resulting in a strong heterozygote deficiency in the total population. The value of the last mean fixation index, F_{ST} (0.245) ($P<0.05$), displayed the existence of a significant segmentation and a very great genetic differentiation among the different breeds. Arcos-Burgos and Muenke (2002) stated that F_{ST} could be significantly greater than zero when a population establishes a pattern of subdivision from other ones because of some kind of genetic isolation, which eventually lead to a condition of homozygote excess.

In this study, Livorno and especially Ancona could reasonably be in this situation. The tree from inter-individual DAS distance using Neighbour-Joining algorithm (Figure 2) displayed a very defined cluster for all the investigated breeds. The spatial representation of the genetic inter-individual distances highlights that Ancona and Livorno are characterised by homogeneous genetic patterns. The animals belonging to the different breeds were placed in three well defined areas; however, very curious is the situation occurring in Livorno.

It is worth noting that this breed differed somewhat from the other two breeds, for his taking place at various nodes, and that is in accordance with a greater within-breed inter-individual distance reflecting more internal variability. It is well known that in chicken, where no pedigree information is available and no breeding plans are usually organised, every animals nucleus is a sub-group of the whole population and it is characterised by more genetic variability than the entirety of the total animals sample (Rosenberg *et al.*, 2001). The differentiation of the individuals within each breed was further assessed with the FCA by the construction of a two-dimensional plot in which the different animals took place (Figure 3). This analysis gives the chance to show the results through a graphic model with a considerable descriptive value (Guinand, 1996). The first axis explained the 10.97% of the total variation and separates the different breeds from

each other, whereas the second axis explained the 8.92%. Other authors, such as Ferreira et al. (2006) and Wheeldon and White (2009) took advantage of this methodological approach for genetic analysis on animal populations obtaining comparable statistical results. In the present study Livorno and Ancona animals formed two separated and well-defined groups. The Livorno showed only some animals which moved themselves away from the ideal grouping area, whereas within the Ancona all the animals took part in a very homogeneous area. This is consistent with the presence of more internal variability in Livorno. Anyway Livorno and Ancona are the closest breeds in the graphical representation. STRUCTURE-based analysis was carried out to estimate the most likely number of clusters present in the data set, to detect the underlying genetic structure among a set of individuals genotyped at multiple markers and to possibly reveal the potential presence of substructures within the breeds. Following Evanno *et al.* (2005), the most likely number of cluster turned to be 3, since the highest ΔK value was obtained for K=3 (Figure S1, Appendix). This result was expected, since the most likely number of clusters was the same as the number of the studied breeds, and this genetic frame reflects what we found with the inter-individual genetic distance tree and FCA-based analyses. Taking advantage of various methodological approaches, all these analyses in different ways confirmed the genetic uniqueness of the studied breeds. Analysis of the percentage of correctly assigned individuals ($q>0.90$) for K= 3 (Table 3) showed the highest values for Ancona and Sasso (100%), with all the animals correctly assigned. With respect to Livorno, fifty animals out of fifty-one were correctly assigned (98%). The proportion of membership in the different clusters is totally comparable among the breeds, even if Ancona exhibited the highest value (0.994) (Table 3). All the breeds displayed a very high percentage of assignment (0.994, 0.993 and 0.985 for Ancona, Sasso and Livorno respectively). These data numerically confirmed the results showed by the FCA analysis and the spatial representation of the genetic inter-individual distances. Figure 4 shows the clustering pattern arising from the STRUCTURE analysis. At K=2 Sasso and Ancona surprisingly clustered together, whereas Livorno clustered separately. This first subdivision was not expected since Sasso is the non Italian breed and was taken as an out-group in this study. An explanation could be that genetic similarities exist more between Ancona and Sasso than

between Ancona and Livorno, even though they come from the same country. At K=3, which is the most likely number of partitions, the three breeds perfectly clustered in three really definite clusters. All the animals were correctly assigned to their clusters, with just extremely small amounts of shared genetic components. As already stated, the studied breeds, particularly Livorno and Ancona, represent specific and unique genetic extents, and therefore they should be considered genetic resources to be preserved. Even though we found the highest ΔK for K=3 following Evanno's method, which perfectly and easily describes the genetic structure of the studied breeds, it is worth showing and discuss the picture we get if we consider the clustering for K=5 (Figure 5). At K=5 Sasso and Livorno did not change their clustering pattern, whereas Ancona resulted sub-structured. Ancona was characterised by a sub-clustering frame: it was therefore possible to distinguish three different genetic contributions for this breed, which could reflect a geographic partition, as confirmed by the highest Fis value (0.251) detected just in Ancona. The animals forming the Ancona cluster resulted segmented according to the different farms where they were sampled from. In fact, the STRUCTURE analysis for K=5, even though with some exceptions, did not show an admixture pattern within the single individuals, but it mainly showed an admixture pattern among the individuals, which generally reflects a farming subdivision. It is worth saying that, even though 3 reasonably was the correct estimation of the most likely number of partitions, the genetic pattern showed at K=5 was very interesting and noteworthy. On one hand, K=3 clearly showed that the three breeds were consistently and perfectly separated from each other and did not share any significant common genetic pattern; on the other hand K=5 showed that the genetic features of Ancona perfectly follow what the local breeders practically do in the reality. The Ancona is a small autochthonous breed, mainly spread across Marche and part of Emilia Romagna. The different breeders have been permanently working at his defence, protection and development in order to safeguard and preserve his existence and his typical peculiarities. Every farm could be considered a conservation temple, where Ancona is maintained at his original genetic standard without any possible contamination from outside. This situation leads to two main consequences. On one hand Ancona keeps his phenotypic and genotypic characteristics unchanged, and that is important for the safeguard of this

breed, on the other hand every farm experiences a kind of genetic isolation because of the lack of Ancona males. Every nucleus includes several hens and a few cocks, resulting in matings always based on the same fertilising males. This eventually leads to inbreeding and to a situation called breeding effect, which is the same as genetic drift. This situation is so marked that it could be possible to speculate the presence of potential sub-populations within the same main breed.

2.5 CONCLUSIONS

To sum up, this study highlights the general lack of genetic variability in the Italian local studied breeds, Ancona and Livorno. After all, the autochthonous breeds are thought to progressively lose their genetic variability because of the wider and wider spreading of commercial breeds; this negative trend was confirmed in Ancona and Livorno through the employment of molecular tools such microsatellites. Microsatellites also resulted a powerful tool to study the genetic diversity and the evolution of domestic animals such the local chicken breeds Ancona and Livorno.

Interestingly, microsatellites gave the chance to demonstrate the genetic uniqueness of the considered breeds and the presence of potential sub-populations within the Ancona breed due to genetic isolation. It would be therefore desirable to set up improved selection schemes in order to save the genetic diversity, to avoid inbreeding and to overcome the presence of population sub-structures. This study confirmed the possibility to discriminate with molecular markers among different breeds by using statistical assignment analysis. These results also might give a suitable starting point to set up a potential preliminary genotypic test for all the cocks to be used in the fertilisation plans, in order to genetically characterise individuals having specific and valuable genetic features and belonging to specific breeds, and to avoid therefore the employment of undefined animals.

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2.6 TABLES AND FIGURES

Fig. 1: sampling geographical areas.

BREED	NUMBER OF FARMS
Ancona	6
Livorno	4
Sasso	1

Table 1: microsatellite markers, chromosomes involved (Chr.), alleles detected (All.), size range and mean PIC (Polymorphism Information Content) per locus.

Locus	Chr.	All.	Size range (bp)	Mean PIC	Locus	Chr.	All.	Size range (bp)	Mean PIC
MCW0248	1	2	205-283	0,16	MCW0078	5	3	135-147	0,42
MCW0111	1	5	102-120	0,42	MCW0081	5	7	112-135	0,62
ADL0268	1	8	102-216	0,59	MCW0014	6	4	164-182	0,26
MCW0020	1	4	179-185	0,48	LEI0192	6	10	244-370	0,57
LEI0234	2	14	216-364	0,66	MCW0183	7	7	296-326	0,36
MCW0206	2	5	221-249	0,31	ADL0278	8	6	114-126	0,46
MCW0034	2	8	212-246	0,61	MCW0067	10	5	176-186	0,53
MCW0222	3	4	220-226	0,44	ADL0112	10	4	120-134	0,37
MCW0103	3	2	266-270	0,14	MCW0216	13	5	139-149	0,39
MCW0016	3	6	162-206	0,47	MCW0104	13	11	190-234	0,54
LEI0166	3	3	354-370	0,48	MCW0123	14	10	76-100	0,47
MCW0037	3	3	154-160	0,41	MCW0080	15	7	264-280	0,53
MCW0295	4	5	88-106	0,50	MCW0330	17	4	256-300	0,47
LEI0094	4	10	247-287	0,59	MCW0165	23	3	114-118	0,48
MCW0098	4	3	261-265	0,45	MCW0069	26	9	158-176	0,51

Table 2: studied breeds, sample size of each breed, mean number of observed alleles, private alleles, mean observed and expected heterozygosity and Fis per breed.

Breed	Sample Size	No. of Alleles		Mean heterozygosity		F_{is}
		Observed (mean)	Private	Observed	Expected	
SA	30	4.03	32	0.68	0.60	-0.142*
AN	50	3.50	17	0.35	0.47	0.251*
LI	51	3.73	26	0.45	0.49	0.086*

SA, Sasso; AN, Ancona; LI, Livorno

*: significantly different from zero (P < 0.05)

Table 3: Percentage of correctly assigned animals with q>0.90 and proportion of membership of the three chicken populations for K = 3.

Breed[a]	% Corr. Assign (q > 0.90)[b]	Clusters[c]		
		1	2	3
AN	100%	0.002	0.003	**0.994**
LI	98%	0.005	**0.985**	0.010
SA	100%	**0.993**	0.003	0.004

a SA, Sasso; AN, Ancona; LI, Livorno.

b Percentage of correctly assigned animals with q ≥ 0.90

c Contributions higher than 0.400 are in bold

Fig. 2: tree from inter-individual DAS distance using Neighbour-Joining algorithm.

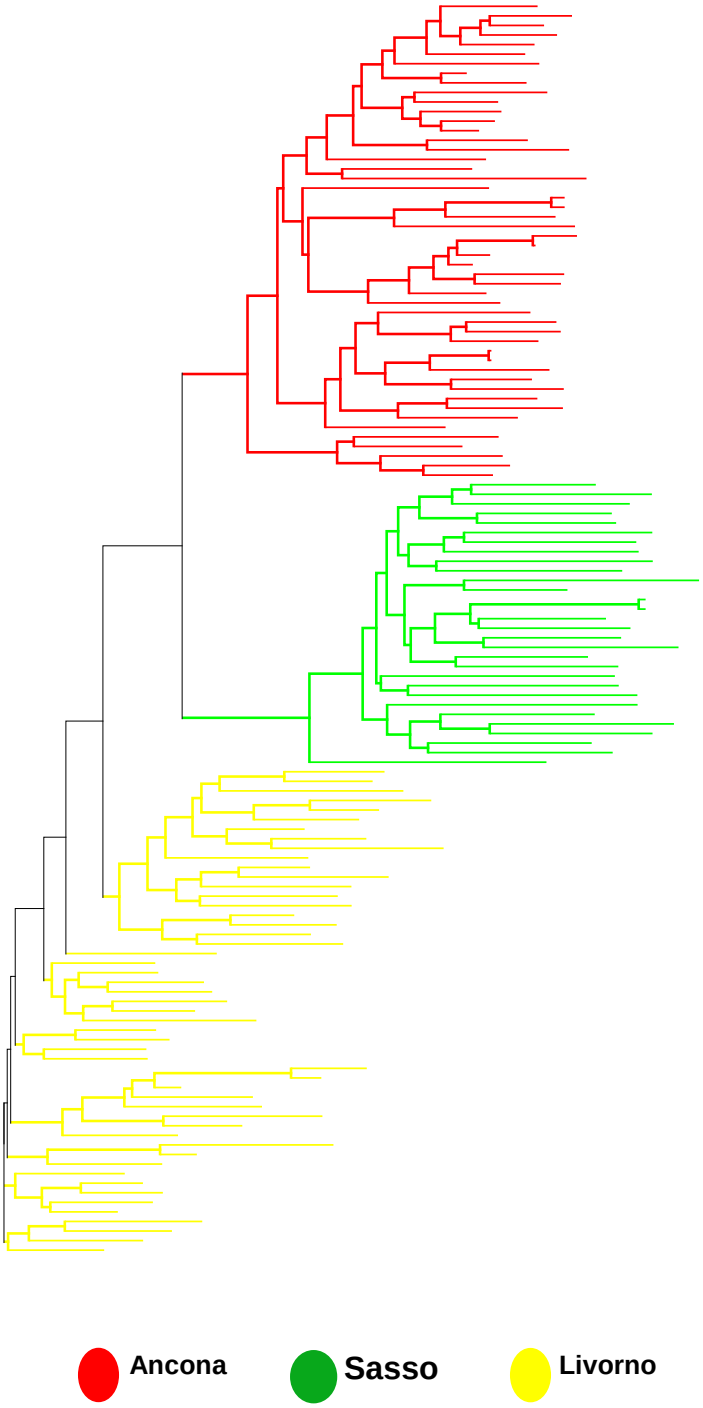


Fig. 3: Factorial Correspondence Analysis (FCA) of the studied chicken individuals.

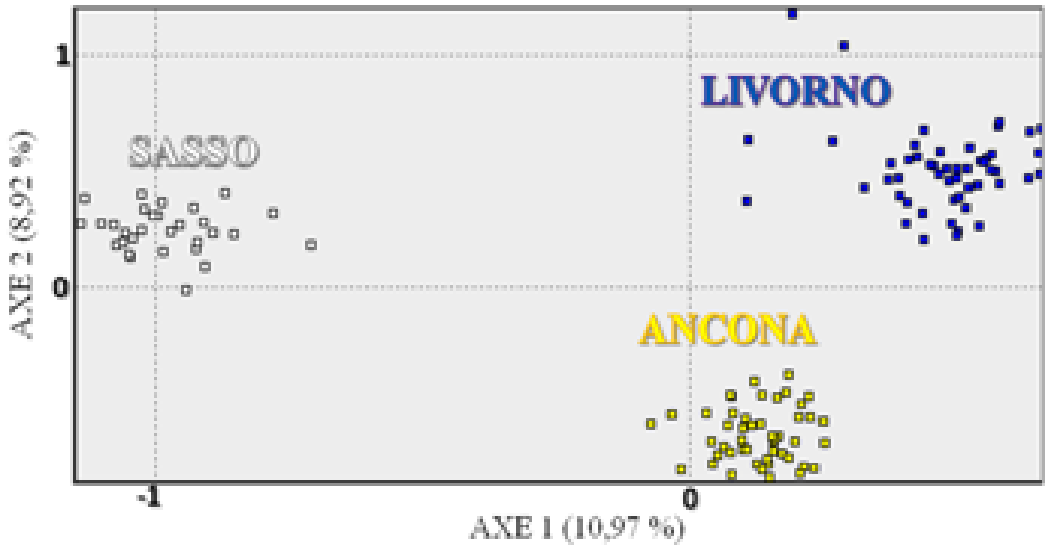


Fig. 4: STRUCTURE cluster analysis of the studied chicken breeds.

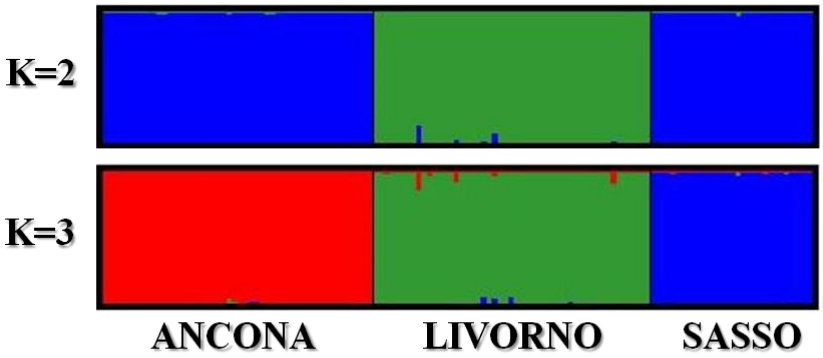
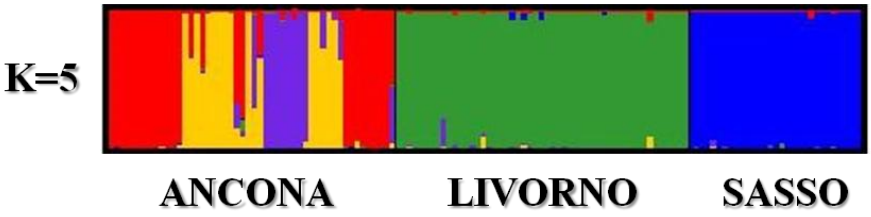


Fig. 5: STRUCTURE cluster analysis of the studied chicken breeds at K=5.



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3rd CHAPTER

Phylogeny, genetic relationships and population structure of five Italian local chicken breeds

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3.1 ABSTRACT

Preserving genetic diversity is an important goal in the poultry industry as is true for all farm animal species. The Italian chicken breeds' situation is critical. The first aim of this study is to investigate the maternal genetic origin of five Italian local chicken breeds (Ancona, Livorno, Modenese, Romagnola and Valdarnese bianca) based on mitochondrial DNA (mtDNA) information. Secondly, the extent of the genetic diversity, population structure and the genetic relationships among these chicken populations, by using 27 microsatellite markers, were assessed. To achieve these targets, a 506 bp fragment of the D-loop region was sequenced in 50 chickens of the five breeds. Eighteen variable sites were observed which defined 12 haplotypes. They were assigned to three clades and probably two maternal lineages. Results indicated that 90% of the haplotypes are related to clade E, which has been described originating from the Indian subcontinent. For the microsatellite analysis, 137 individual blood samples from the five Italian breeds were collected. A total of 147 alleles were detected at 27 microsatellite loci. The five Italian breeds showed a slightly higher inbreeding index (F_{IS} = 0.08) when compared to commercial populations used as reference. Structure analysis showed a separation of the Italian breeds from these reference populations; a further sub-clustering allowed to discriminate the five different Italian breeds. This research provides useful indication for planning preservation schemes of the studied breeds.

Keywords: Chickens**,** Genetic diversity, Mitochondrial DNA, Microsatellites.

3.2 INTRODUCTION

Attention and awareness to genetic conservation has significantly increased in recent years (Allendorf and Luikart, 2007). Preservation of genetic variability plays a crucial role in animal science because its decline may reduce populations' ability to adapt to environmental changes (Lande, 1988). Moreover, autochthonous breeds might be an important resource for research purposes and future breeding programmes.

Poultry is one of the most important livestock species providing high quality nutritious food for human consumption (Blackburn, 2006). In Italy, the number of native chicken breeds has suffered a dramatic decline leading to the current critical situation. Zanon and Sabbioni (2001) reported the historical presence in Italy of 90 poultry breeds (9 ducks, 11 guinea fowls, 53 chickens, 5 gooses and 12 turkeys): 61.0% of these breeds are extinct, 13.3% are endangered, and only 6.7% are involved in conservation programmes. On the other hand, only few specialized chicken lines are used by global breeding companies to provide animals for industrial production.

In this study, five Italian chicken breeds were studied; Ancona from the Marche region, Livornese bianca and Valdarnese bianca, both from Tuscany, Modenese and Romagnola from the Emilia-Romagna region.

Ancona breed is renowned as a good layer of white shelled eggs and has yellow skin (Mugnai *et al.*, 2009), while Livornese bianca (Leghorn Italian type) is supposedly related to the worldwide spreaded commercial White Leghorn layers (FAO, 2010). Valdarnese bianca shows white feathers and dark yellow shank and can be considered as the only traditional Italian meat-type chicken breed (Marelli *et al.*, 2006), even the productive performance is far from being economically sustainable when compared to commercial broiler lines. Modenese and Romagnola breeds are two light breeds of Mediterranean-type known to produce eggs and meat for the rural family. They are not used for commercial purposes (Sabbioni *et al.*, 2006).

In Italy conservation programmes of local chicken breeds are already in place namely: in Veneto region for Ermellinata di Rovigo, Robusta Maculata, Robusta Lionata, Pépoi and Padovana (Baruchello and Cassandro, 2003), in Emilia Romagna region for Modenese and Romagnola (Zanon *et al*. 2006) and in Tuscany

for Valdarnese bianca (Gualtieri *et al.*, 2006). This study can provide information on the genetic structure and origin of these breeds useful for such programmes. In the absence of comprehensive breed characterization data and documentation of the origin of breeding populations, molecular marker information could provide the most reliable estimates of genetic diversity within and between a given set of populations. Nonetheless, molecular data should be integrated with other information (i.e. adaptive, productive and reproductive performance, extinction probability) in the process of decision making.

Molecular markers were developed to investigate genetic relationships between populations within a species. In this context, mitochondrial DNA (mtDNA) and microsatellites are two techniques which have been widely used. Several authors analysed the mtDNA D-Loop region to assess phylogenetic relationships and maternal origin of different chicken populations (Storey *et al.*, 2012; Mwacharo *et al.*, 2011, Muchadeyi *et al.*, 2008; Fu *et al.*, 2001). Microsatellite markers already have been successfully applied in different studies to measure the genetic variability among local chicken breeds (Eltanany *et al.*, 2011; Mtileni *et al.*, 2011; Muchadeyi *et al.*, 2007; Hillel *et al.*, 2003).

This paper provides a complete report on the genetics of the above mentioned Italian chicken breeds, including their remote genetic origins, the differentiation among them and their present level of biodiversity. For this purpose, sequences of the mitochondrial D-Loop region and microsatellite loci have been obtained and analysed with different statistical procedures to obtain the most relevant genetic information.

3.3 MATERIALS AND METHODS

Animal sampling and DNA extraction

A total of 137 blood samples (2 ml from wing vein of each animal collected in vacutainer tubes, containing EDTA as anticoagulant) were randomly collected from five Italian local chicken breeds: 30 Ancona (AN), 30 Livornese bianca (LI), 23 Modenese (MO), 24 Romagnola (RO), 30 Valdarnese bianca (VA) of both sexes. These breeding animals were selected from different farms to avoid sampling of closely related individuals and to collect representative sample of each breed. Fig. 1 shows the geographical areas, the number of farms and individuals included in the sampling. For VA and MO breeds, a preliminary screening of the farms was carried out to avoid the inclusion of animals which did not fit to the morphological standard of the breed. As a result, only one farm was suitable for each of these two breeds. Whole blood was stored at -20ºC until DNA extraction. DNA was isolated using the GenElute Blood Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA) and stored at 4 °C until genotyping.

Reference populations

Six populations (30 samples for each) were selected, as reference populations (Muchadeyi *et al.*, 2007, Mtileni *et al.*, 2011), from the AVIANDIV project (Weigend *et al.*, 1998). These populations consisted of broiler dam (BRD) and sire (BRS) lines, two brown-egg layers (BLA and BLC) and two white-egg layers (LSS and WLA).

The LSS is an experimental White Leghorn conserved at the Institute for Animal Breeding (Germany) as a conservation flock (Hartmann, 1997). The other populations are commercial lines.

Mitochondrial DNA analysis

A subset of 50 DNA samples of the five Italian breeds under study was randomly chosen (10 samples for each breed). In relation to the complete mitochondrial sequence of chickens (accession number NC007236; Nishibori *et al.*, 2005), mtDNA amplification was performed from nucleotide position (np) 16,750 to np 522 including part of the D-loop region. PCR amplification was performed in a 25 µl volume with 3 mM $MgCl_2$, 50 mM of each dNTP, 1 mM of each primer and 1 unit of

Taq® DNA Polymerase (Sigma Aldrich, St. Louis, MO, USA), using a Biometra TGradient 96 Thermocycler at the following conditions: initial denaturation step of 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at the 60°C, 75 s at 72°C, and a final extension of 5 min at 72°C.

The PCR products were sequenced at the Central DNA Sequencing Service (Universidad de Zaragoza, Spain) using a Applied Biosystems 3730xl DNA analyzer.

A fragment of 506 base pairs in size (from np 1 to np 505 of complete chicken mitochondrial sequence) were used for analysis. Sequences were aligned using the software Sequencher™ 4.10 (Gene Codes Inc., Ann Arbor, MI, USA). Indexes such as haplotype diversity (Hd) and nucleotide diversity (π), average number of nucleotide differences (k) and Fu's *Fs* statistic (Fu, 1997) were estimated by DnaSP 5.10 software (Librado and Rozas, 2009).

ARLEQUIN 3.1 software was applied to carry out a hierarchical analysis of molecular variance (AMOVA) in order to analyze the partitioning of genetic diversity within and between the five Italian chicken breeds (Excoffier *et al.*, 2006). The calculations were performed based on 1,000 permutations.

Evolutionary relationships of sequences were evaluated through a median-joining network constructed using the software Network 4.6 (www.fluxus-engineering.com). The network also included nine haplotypes representing the main clades (clades A to I) in the Chinese and Eurasian region (Liu *et al.*, 2006) as references. Haplotypes from GenBank were aligned with haplotypes observed in this study.

Microsatellite analysis

From a total of 30 microsatellite markers recommended for biodiversity studies of chicken by ISAG/FAO (FAO, 2004), 27 markers (Table S1) were used in this study. The markers were genotyped in standard multiplex PCR amplification using a Biometra TGradient 96. Annealing temperatures were set to values reported at the AVIANDIV website (2012). Allele calling was adjusted using nine standard DNA samples. Analyses of fragments were performed using an automated DNA sequencer (ABI PRISM 3130xl, Applied Biosystems, Foster City, CA, USA) and the

software package GeneMapper version 4.0 (Applied Biosystems, Foster City, CA, USA).

Analysis of microsatellite genotypes

The observed and expected heterozygosity within breeds was estimated using EXCEL MICROSATELLITE TOOLKIT 3.1.1 (Park, 2001). The POPGENE software (version 3.2, Yeh *et al.*, 1999) was used to estimate the number of alleles observed at each locus and the mean number of alleles per breed. GENEPOP 4.0 software (Raymond and Rousset, 1995) was used to carry out a test for Hardy-Weinberg equilibrium. A Markov Chain Monte Carlo method (20 batches, 5,000 iterations per batch, and a dememorisation number of 10,000) was applied to estimate unbiased exact P-values according to the algorithm described by Guo and Thompson (1992). Weir and Cockerham (1984) estimates of Wright's fixation indices (F_{is}, F_{it} and F_{st}) within and across populations were calculated using FSTAT software (Goudet, 2002). Standard errors were generated by jack-knifing over loci and populations. Fixation index per population (F_{is}) was estimated, with 1000 bootstraps, using software GENETIX 4.05 (Belkhir *et al.*, 1996-2004). Reynolds weighted genetic distance (Reynolds *et al.*, 1983) among the populations was calculated using PHYLIP software 3.6 (Felsenstein, 2005).

The algorithm implemented in STRUCTURE software version 2.2 (Pritchard *et al.*, 2000) was used to assess genetic clustering of each individual to the various breeds and to reveal possible admixture. The analysis involved an admixture model and correlated allele frequencies.

One hundred independent runs were carried out with 20,000 interactions as burn–in phase followed by 50,000 interactions for sampling from $2 \leq K \leq 16$ (K= number of assumed clusters) to estimate the most likely number of clusters present in the data set. Further analysis was performed by analyzing the five Italian chicken breeds separately from the population references. The most likely K value was identified as described by Evanno *et al.* (2005). The clustering pattern was visualised using the software DISTRUCT 1.1 (Rosenberg, 2004).

3.4 RESULTS AND DISCUSSION

Mitochondrial DNA phylogeny

The present paper represents the first approach to the phylogeny of Italian chicken breeds inferred by mtDNA analysis.

The sequences of the first 506 bp fragments of the chicken mitochondrial D-loop were used for analysis. The number of polymorphic sites, mtDNA haplotypes and haplotype diversity are shown in Table 1. In this study 12 haplotypes were defined and a total of 18 nucleotide substitutions (only transitions) were observed.

All the populations, except AN, were polymorphic with a number of haplotypes per population ranging from three (LI, MO and RO) to five (VA). The highest haplotype diversity (Hd), was found in VA chicken (0.8440±0.0800), whereas the lowest value (excluding the monomorphic AN) was observed in RO (0.3780±0.1810). These values are similar to what observed in Hungarian breeds by Revay *et al.* (2010). Mitochondrial D-loop monomorphism in the AN breed may be related to higher degree of inbreeding of this breed confirmed later by the microsatellite analysis.

The nucleotide diversity (π) is a more suitable parameter than haplotype diversity to estimate the genetic diversity in population. In fact, the ð value addresses both the frequency of haplotypes and nucleotide differences between haplotype. The average nucleotide diversity was 0.0045±0.0013 across all the Italian chicken breeds (excluding the monomorphic AN), and ranged from 0.0097±0.0018 in LI to 0.0007±0.0004 in RO. Thereby, a higher nucleotide diversity was observed in LI than in the other breeds. These values are similar to that estimated by Liu *et al.* (2006) for chicken sampled in Europe, Middle East, South East and East Asia.

In the light of the mtDNA AMOVA results, the genetic variation among chickens within breed is 67.83% while genetic variation among breeds is 32.17% (Table S2).

A high genetic differentiation was observed for the mtDNA data (F_{ST} = 0.322, P<0.001), supporting the hypothesis of a definite separation among the five Italian chicken breeds.

Median-joining network analysis of the mtDNA D-loop haplotypes using mtDNA sequence polymorphism in the Italian chicken breeds together with reference haplotypes (Liu *et al.,* 2006) revealed that Italian breeds clustered in one major

and two minor haplogroups, derived from three different lineages (A, B and E) resulting from different ancient domestication events (Fig. 2).

Ninety % of the birds of the five Italian breeds grouped with E-lineage derived haplotype LIUE1, while other animals clustered with reference sequences LIUA1 (4%) and LIUB1 (6%), derived from lineage A and B, respectively.

Interestingly, seven of the eight haplotypes that clustered within haplogroup E were separated from major haplotype E1 by only one mutation. It should be noted that two different sequences (from MO and LI) were included in haplogroup A (Liu *et al.*, 2006). Finally three identical sequences (from LI) made part of the haplogroup B, with shared haplotype together with LIUB1.

Haplogroup E has been reported to be widespread in Europe, Middle East and India, while haplogroups A and B are widely distributed in South China and Japan (Liu *et al.*, 2006). Other authors (Revay *et al.*, 2010; Grimal *et al.,* 2011) observed these last two haplogroups in Hungarian and Spanish chicken breeds. In particular, Revay *et al.* (2010) found two sequences in haplogroup B that were identical to those existing in commercial lines of white egg layer. Therefore, it cannot be excluded that the presence of this haplogroup is a result of introgression from commercial layer lines. No scientific references about mtDNA were found on possible genetic influences of South Eastern Asia chickens in Italian breeds. However, the arrival of these haplotypes to Europe as a result of commercial activity are well documented at least for the last eight centuries and can not be disregarded.

Finally, the presence of two sequences in MO and LI, differing only in one single nucleotide mutation in the same lineage A, could support genetic proximity among these two breeds.

Microsatellites

After the spread of a domestic species in a particular area as a result of one or several domestication or immigration events several phenomena, resulting in changes in the autosomal loci alleles frequencies, usually occur. Among them, population isolation, selection for a particular phenotype and especially genetic drift due to population size reduction have important effects on allele frequencies of the populations and may cause dramatic reductions in the genetic variability

and high level of inbreeding (Henson, 1992). It is therefore necessary to evaluate the current genetic structure of the autochthonous populations prior to start any conservation or selection programme.

In our trial we found 147 alleles in the five Italian breeds across all 27 loci investigated (Table S1). Zanetti et al. (2010) detected a lower number of alleles (112) in other six Italian breeds (Ermellinata di Rovigo, Robusta Maculata, Robusta Lionata, Pépoi, Padovana and Polverara) using twenty microsatellite markers whereas Bianchi et al. (2011), using thirty microsatellite loci, observed a higher number of alleles (177) in a preliminary work on the Ancona and Livorno breeds. This first study, carried out sampling randomly animals in different farms, highlighted the general lack of genetic variability in these two Italian local breeds.

The number of alleles at each locus (Table S1) ranged from 2 (MCW0248 and MCW0103) to 11 (MCW0034 and LEI0094) whereas the number of alleles per breed (Table 2) ranged from 2.63 (MO) to 3.67 (VA). These values are similar to that obtained by Zanetti *et al.* (2010) on a study involving chicken breeds from North Italy.

It should be noted that all these local breeds are generally reared in small rural flocks (Dalvit *et al.*, 2005). This similarity could indicate that the local Italian chicken breeds are in the same demographic conditions, therefore all the breeds show a low genetic variability. Nevertheless, three of the studied breeds (MO, RO and VA) are currently involved in conservation schemes; because of the hard shortness of breeding animals available to these activities, a founder effect could determine a loss of genetic variations (Wilson *et al*., 2005).

VA displayed the highest value of the observed and expected heterozygosity (0.53) while AN and MO the lowest (0.39). The observed and expected values of heterozygosity in each breed showed similar values to that found by Dalvit *et al.* (2009), Bodzsar *et al.* (2009) and Granevitze *et al.* (2007), in different Italian and European poultry breeds, respectively (Table 2).

A deficiency of heterozygosity (F_{IS}) was observed in both AN (0.19244, $P<0.05$) and LI (0.10920, $P<0.05$) breeds suggesting the possible presence of inbreeding probably due to the mating between related and infrequent exchange of breeding animals among different rural farms (Table 2).

In contrast, observed frequencies of heterozygotes were similar to those expected in MO, RO and VA, and F_{IS} estimates were not significantly different from zero, suggesting that these populations are close to Hardy-Weinberg equilibrium state.

The mean F_{IT}, F_{ST} and F_{IS} estimates of the five Italian chicken breeds and of the six commercial lines (previously estimated by Muchadeyi *et al.*, 2007) are reported in the Table 3. The average inbreeding value at the total sample level (F_{IT}) was 0.349 ± 0.017 ($P<0.01$) and higher in commercial lines than in Italian breeds. The genetic differentiation (F_{ST}) of Italian chicken breeds was lower (0.225 ± 0.019) than the corresponding value of the commercial lines (0.354 ± 0.025), indicating a lower than between commercial lines but still substantial sub-structuring of the Italian breeds. Coefficient of inbreeding within population (F_{IS}) in Italian chicken breeds was higher than that of commercial lines, confirming the inbreeding presence in these populations reared in closed small flocks on rural farms.

Phylogenetic relationships based on Reynolds genetic distance among the populations were visualised through a Neighbour-joining tree (Fig. 3). The tree showed that the two White Leghorn strains (WLA and LSS) clustered with MO and LI breeds. LI is closely related to the founder population of White Leghorn used as commercial egg layers and the results confirm the common historic origin of White Leghorn strains. As expected, MO and LI appeared closed in the tree because of the ancient crossbreeding practices among these two breeds as reported by Mazzon (1932). As stated before, the genetic proximity between MO and LI was also detected in the mitochondrial analysis.

Two more clusters were observed: VA clustered with brown egg layers and broilers were in a cluster between brown egg layers and white egg layers. Genetic introgression of heavier dual purpose chickens could explain the clustering of VA with brown egg layers (Gualtieri *et al.*, 2006, Sacchi, 1960). AN and RO appear in a separate branch and this could be due to geographical proximity favoring the exchange of AN and RO animals in the past.

Results of STRUCTURE analysis are given in Figure 4. The analysis was carried out to detect the potential presence of substructures within the breeds.

Most likely clustering was tested using the ΔK statistic introduced by Evanno *et al.* (2005). The highest ÄK values were obtained for K=4. At the lower K values (K=2 and 3) four (BLA, BLC and WLA, LSS) of the six reference populations are

separated from the Italian breeds. At K=4, the six commercial lines were divided into three different clusters while the Italian breeds clustered together, even if VA, MO and AN show slightly relation to broilers, and LI and MO to White egg layers. These results may indicate that the five Italian breeds make up a gene pool different from commercial chicken lines.

The five Italian breeds were further sub-clustered, according to the approach used by Rosenberg *et al.* (2002) and Jakobsson *et al.* (2008). Figure 4 shows the results of this second step of sub-clustering. Clustering was carried out from K=2 to K=5. In this approach, the highest ÄK value was obtained for K = 5. At this K-value, the five Italian breeds were discriminated into separate clusters, even if LI and MO are more related to each other than other breeds.

This finding is in agreement with the results of mitochondrial data, the Neighbour-joining tree and F_{ST} value and confirms the genetic differences of the five studied breeds.

3.5 CONCLUSIONS

The mtDNA data suggest that the Italian chicken breeds mainly origin from the Indian subcontinent, at least from the maternal lineage standpoint, since most individuals are included in the E lineage. However, a possible origin in South China and Japan could be possible for the small proportion of birds belonging to the A and B lineages.

The results obtained indicate a low genetic variability in the five Italian chicken breeds as shown in microsatellite analysis. The loss of variability is probably due to the big social and demographic transformations occurred in Italy in the last fifty years, that determined a dramatically reduction of the number and size of local poultry breeds.

Surely farmers are not able to manage such small flock and mated birds too much relative or/and introduced individuals of other breed like the case of VA, which suggest a potential genetic introgression from the heavy type chickens. Possible crossbreeding between breeds located in neighbouring geographical locations was also detected in AN and RO.

Nevertheless such not suitable breeding practice, all the five Italian chicken breeds studied showed distinct genetic differences with evidence of a sub-populations structure.

The conservation programs in these breeds must take into account these results (especially as it refers to low variability, genetic substructures and genetic distances) in order to minimize any undesired negative effects such as inbreeding increase. It is therefore urgent to preserve these Italian breeds by applying adequate strategies controlled by the public authorities.

Acknowledgements

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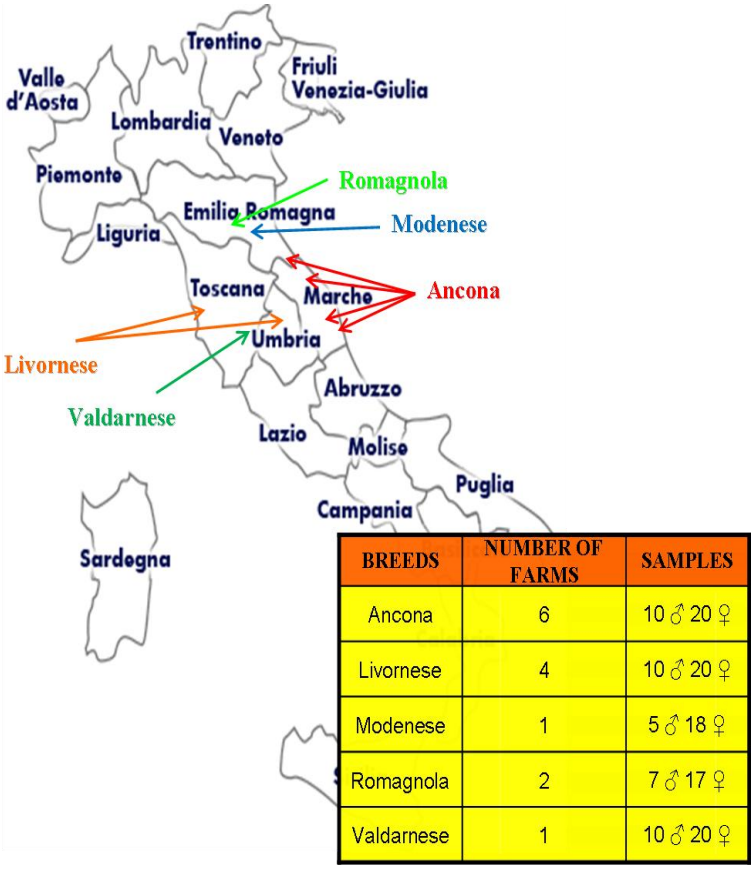
3.6 TABLES AND FIGURES

Fig 1: Geographical sampling areas



BREEDS	NUMBER OF FARMS	SAMPLES
Ancona	6	10 ♂ 20 ♀
Livornese	4	10 ♂ 20 ♀
Modenese	1	5 ♂ 18 ♀
Romagnola	2	7 ♂ 17 ♀
Valdarnese	1	10 ♂ 20 ♀

Table 1: mtDNA diversity indices of the five Italian chicken breeds.

Breed	ID name	N	π	nh	Hd	S
Ancona	AN	10	0.0000±0.0000	1	0.0000±0.0000	0
Livornese	LI	10	0.0097±0.0018	3	0.6390±0.1260	11
Modenese	MO	10	0.0045±0.0027	3	0.6000±0.1310	10
Romagnola	RO	10	0.0007±0.0004	3	0.3780±0.1810	2
Valdarnese	VA	10	0.0029±0.0003	5	0.8440±0.0800	4
Overall		**50**	**0.0045±0.0013**	**12**	**0.7250±0.0650**	**18**

N: number of used sequences, ð: nucleotide diversity, nh: number of haplotypes, Hd: haplotype diversity, S: number of segregation sites.

Fig 2 Median-Joining network tree for the twelve haplotypes of Italian chicken breeds and the nine reference sequences by Liu et al. (2006) based on the polymorphic sites of the mitochondrial D-loop region. Circled areas are proportional to the haplotype frequencies.

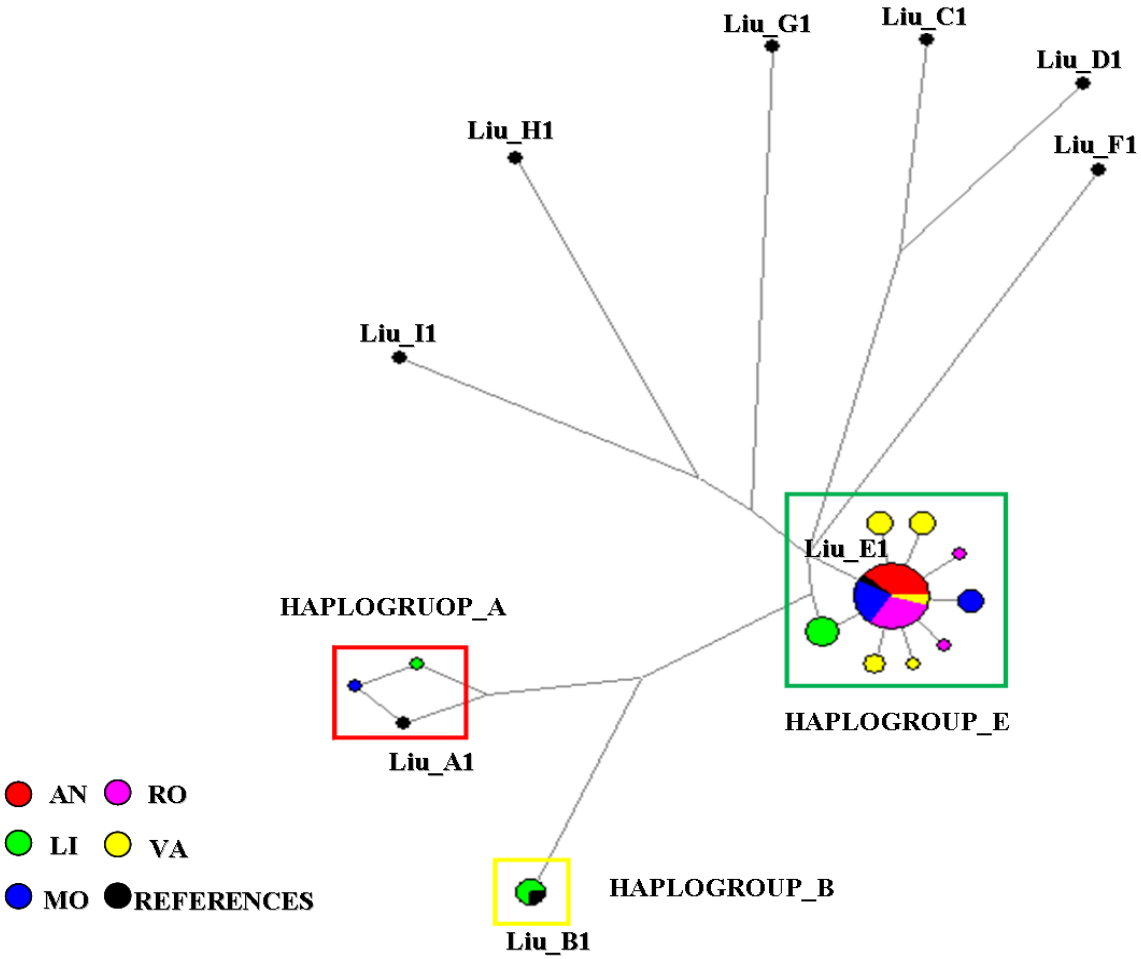


Table 2 Chicken breeds studied, sample size of each breed, mean number of observed alleles (MNA), mean observed (HO) and expected heterozygosity (HE), and inbreeding coefficient (F_{IS}) per breed.

Breed	Sample Size	MNA ± SD	HO ± SD	HE ± SD	F_{IS}
AN	30	3.26 ± 1.10	0.39 ± 0.017	0.48 ± 0.041	0.19244[a]
LI	30	3.11 ± 0.97	0.40 ± 0.019	0.45 ± 0.036	0.10920[a]
MO	23	2.63 ± 0.93	0.39 ± 0.020	0.39 ± 0.040	-0.00902
RO	24	3.59 ± 1.45	0.47 ± 0.020	0.50 ± 0.040	0.07704
VA	30	3.67 ± 1.11	0.53 ± 0.018	0.53 ± 0.039	0.00006
Mean value		3.25 ± 0.42	0.43 ± 0.06	0.47 ± 0.05	

[a]: significantly different from zero ($P < 0.05$)

Table 3: Overall population (F_{IT}), between-population (F_{ST}) and within-population (F_{IS}) inbreeding coefficients and their standard errors (SE) of the Italian and Commercial population.

Population	F_{IT} ± SE	F_{ST} ± SE	F_{IS} ± SE
Italian	0.285 ± 0.026**	0.225 ± 0.019**	0.077 ± 0.027**
Commercial	0.374± 0.025**	0.354 ± 0.025**	0.030 ± 0.014*
Overall	0.349 ± 0.017**	0.314 ± 0.015**	0.051 ± 0.015**

Significantly different from zero at *P<0.05, **P<0.01

Fig 3: Neighbour-joining tree obtained from the Reynolds weighted genetic distance among the five Italian chicken breeds (1,000 bootstraps). Bootstrap values above 50% are shown at each node. AN = Ancona, LI = Livornese bianca, MO = Modenese, RO = Romagnola, VA = Valdarnese bianca, WLA = white egg layer line A, LSS = white egg layer experimental line, BLA = brown egg layer line A, BLC = brown egg layer line C, BRDA = broiler dam line A, BRSA = broiler sire line A.

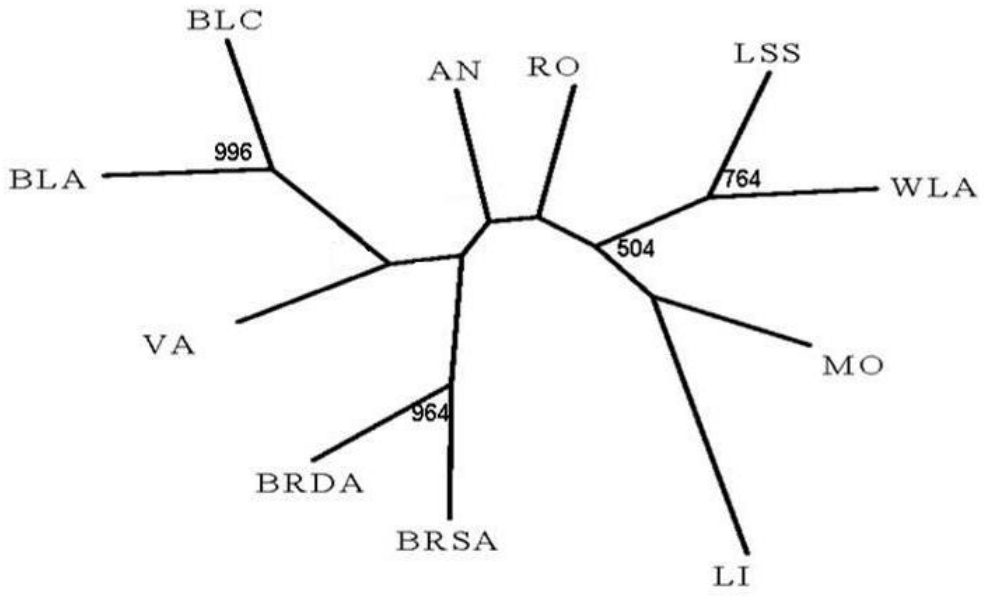


Fig 4 STRUCTURE cluster analysis of the sample: AN = Ancona, LI = Livornese bianca, MO = Modenese, RO = Romagnola, VA = Valdarnese bianca, WLA = white egg layer line A, LSS = white egg layer experimental line, BLA = brown egg layer line A, BLC = brown egg layer line C, BRDA = broiler dam line A, BRSA = broiler sire line A.

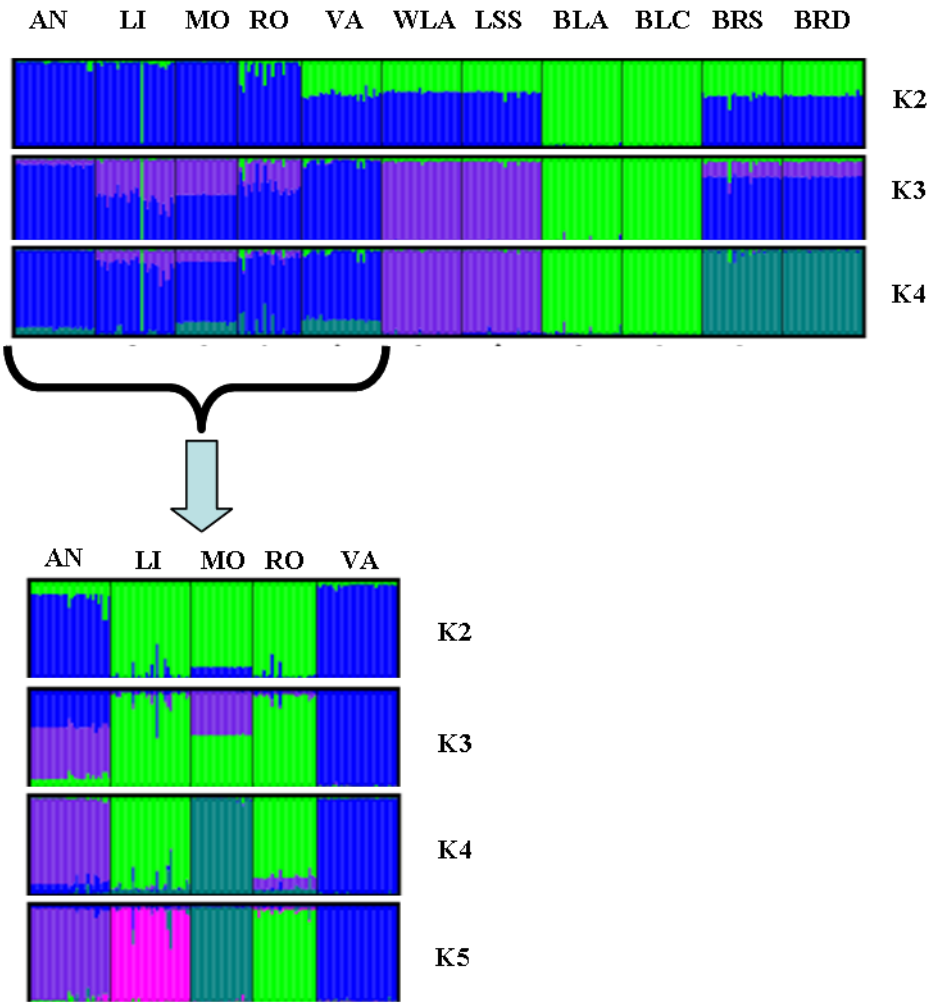


Table S1: Microsatellite loci, chromosomal position (Chr), size range and number of alleles observed (Na) at each locus.

Locus	Chr	Size range (bp)	Na	Locus	Chr	Size range (bp)	Na
MCW0248	1	215-223	2	MCW0078	5	135-145	4
MCW0111	1	98-114	7	MCW0081	5	112-135	8
ADL0268	1	104-116	4	MCW0014	6	164-182	7
MCW0020	1	179-185	4	MCW0183	7	296-326	8
MCW0206	2	223-249	6	ADL0278	8	114-124	4
MCW0034	2	220-242	11	MCW0067	10	174-184	5
MCW0222	3	220-226	4	ADL0112	10	122-132	4
MCW0103	3	266-270	2	MCW0216	13	141-147	4
MCW0016	3	144-184	7	MCW0104	13	178-226	9
LEI0166	3	356-366	3	MCW0123	14	80-94	7
MCW0037	3	154-158	3	MCW0330	17	258-290	4
MCW0295	4	88-106	6	MCW0165	23	114-118	3
LEI0094	4	247-285	11	MCW0069	26	158-170	7
MCW0098	4	261-265	3				

Table S2: Results from the hierarchical AMOVA in the five Italian chicken breeds*.

Source of variation	df	Sum of square	Variance components	Percentage of variation	F_{ST}	P
Between breeds	4	21.40	0.449	32.17	0.322	0.001
Whitin breeds	44	41.70	0.948	67.83		

* obtained from mtDNA data

df = degrees of freedom

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4th CHAPTER

Genetic diversity and phylogeographic structure in some Mediterranean chickens using mitochondrial DNA analysis and microsatellite assay

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Manuscript to be submitted

4.1 SUMMARY

Genetic diversity and relationship among sexteen Mediterranean chicken populations were assessed by sequencing mitochondrial DNA and using a panel of 27 microsatellite markers. To achieve these targets, a 506 bp fragment of the mtDNA D-loop region was sequenced in 160 DNA samples from sexteen populations. Twenty-five variable sites that defined 21 haplotypes were observed and assigned to three clades and probably three maternal lineages. The major haplotype (E1) was present in the Mediterranean populations, originates from the Indian subcontinent as previously described in other studies. Different sequences were included in haplogroup A and B that are distributed in South China and Japan. For the microsatellite analysis, 465 individual blood samples from of the sixteen Mediterranean chicken populations were collected. A total of 242 alleles were found across 27 microsatellite loci with a mean number of 8.96 alleles per locus. Some breeds show to be inbreed, suggesting the need of appropriate measures taken to avoid its negative effects. The *theta* values indicated that about 22% of the total variation originated from variation between the Mediterranean populations as previously reported in other European chicken breeds. Structure analysis exhibited extensive genetic admixture in many studied populations.

These results indicate that Mediterranean chicken populations retain moderate levels of genetic diversity and that originated from three maternal lineages. Suitable conservation measures should be implemented for these breeds in order to minimize inbreeding and uncontrolled crossbreeding. A special care is required for the conservation and preservation of these potentially vulnerable breeds.

Keywords: Genetic variability, population structure, mtDNA, microsatellite.

4.2 INTRODUCTION

The loss of livestock biodiversity in the face of increasing pressures from modern farming is a cause for global concern (FAO, 2007). Domestic chickens have long been important livestock species to use for food, religion activities, entertainment and decorative uses (Blackburn, 2006; Liu et al., 2006). Chicken are not a migratory species, have a small home range, do not fly well over long distances, and are not equipped for swimming. As results, their current global distribution ca be largely attributed to human mediated dispersal (Storey et al. 2012). In the history of livestock, the Mediterranean sea had a key role in postneolithic times, when populations like Phoenicians, Romans, Greeks and Berbers introduced a variety of domesticated plants and animals, including chickens, into southwest Europe (Serjeantson, 2009).

The Mediterranean type chicken are most associated to the Red Jungle Fowl (*Gallus gallus*), which were the first chickens brought into Europe (Moiseyeva *et al.*, 2003). Much later, the local breeds were subjected to intensive selection and crossbreeding with Asian breeds, and this fact contributed at the modern biodiversity of chicken populations (Hillel et al., 2003).

In a more recent time, the family poultry farms were largely responsible for the local production of eggs and meat but now this role has steadily dwindled in the Mediterranean countries; in fact local production is entirely replaced by intensively reared poultry (Mallia, 1999). The current breeding strategies are involved in intensive selection of only few chicken strains specialized, to increase the industrial production both of eggs and meat (Weigend *et al.*, 1999).

The cosmopolitan domestic breeds are not at risk of extinction, therefore the main attentions is focused on the local and less popular chicken breeds (Zanetti *et al*. 2011). The autochthonous breeds are an important resource of gene for future breeding and research purposes.

The local breeds may contain much of the genetic variation because of their adaptation to special environments. The genetic diversity within and between populations is a crucial tool in decision making process for biodiversity conservation strategies (Wilkinson *et al.,* 2011), moreover it is important to minimize the loss of genetic variation as a consequence of inbreeding (Arif and Khan, 2009).

Efficient molecular techniques have been developed to investigate the genetic relationship between populations and the phylogenesis of the chicken breeds. In these fields mitochondrial DNA (mtDNA) and microsatellites are largely used.

Several authors analysed the mtDNA D-Loop region to clarify the phylogenetic relationships, to investigate the maternal origin of different chicken populations and to evaluate diversity among them and their phylogeographic structure (Storey *et al.*, 2012; Mwacharo *et al.*, 2011, Muchadeyi *et al.*, 2008; Fu *et al.*, 2001). The microsatellite markers have been applied in several studies to measure the genetic variability among local chicken breeds (Eltanany *et al.*, 2011; Mtileni *et al.*, 2011; Muchadeyi *et al.*, 2007; Hillel *et al.*, 2003). MtDNA research provides a preliminary description of the breed structure and history, but nuclear markers (as microsatellites) would supply important information to complete the analysis (Kvist *et al.* 2011).

A first objective of this study was to investigate the maternal lineages of chicken populations from several Mediterranean countries (Spain, Italy, Albanian, Serbia and Malta) and their evolutionary relationship in order to enhance current knowledge of breeds history by sequencing mitochondrial DNA D-loop region.

A further approach was to evaluate the levels of genetic variability, the genetic structure and the level of admixture of these chicken populations, using a panel of microsatellite markers.

4.3 MATERIALS AND METHODS

Samples collection

Sixteen local chicken breeds, of both sexes, from five countries of the Mediterranean area were included in this study. A total of 465 blood samples (2ml for each animal, collected with Vacutainer® system, in tube added with EDTA as anticoagulant) were randomly collected from: five Italian chicken breeds (30 Ancona - AN, 30 Livornese bianca - LI, 25 Modenese - MO, 25 Romagnola - RO, 30 Valdarnese bianca - VA), six Spanish chicken breeds (30 Pita Pinta Asturiana - PI, 30 Gallina de Sobrarbe - SO, 30 Gallina Valenciana de Chulilla - CH, 30 Sureña - SU, 30 Combatiente Español - CO, 30 Extremeña azul - EX), three Serbian chicken breeds (30 Somborska Crested - SK, 30 Banat Nacked Neck - BG, 30 Svrljig Hen - SV), one Albanian chicken breed (30 Albanian population - AB) and one from Malta Island (25 Black Maltese - MA). These animals were chosen from different farms to avoid the sampling of closely related individuals and to collect a representative sample of each breed. Figure 1 shows the geographical areas, the number of sampled farms and the individuals per breed. Blood samples were stored at -20 °C until the DNA extraction. Genomic DNA and mtDNA was extracted from whole blood using the GenElute Blood Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA). DNA was stored at 4 °C until genotyping and sequencing.

Reference populations

Six populations (30 samples for each) were selected from the AVIANDIV project (Aviandiv project home page, 2012) as reference populations. Other authors used these populations as reference (Muchadeyi *et al.*, 2007, Mitleni *et al.*, 2011). These populations consisted of Broiler dam (BRDA) and sire (BRSA) lines, two brown-egg layers (BLA and BLC) and two white-egg layers (LSS and WLA). The white-egg layer (LSS) was an experimental White Leghorn maintained at the Institute for Animal Breeding (Germany) as a conservation flock (Hartmann, 1997). The other populations were commercial lines.

Mithocondrial DNA

A subset of 160 DNA samples of the sixteen breeds under study was randomly chosen (10 samples for each breed) as showed in Table 1.

In relation to the complete mitochondrial sequence (accession number NC007236; Nishibori *et al*., 2005), mtDNA amplification was performed from nucleotide position (np) 16,750 to np 522 including part of the hypervariable region of the chicken mitochondrial genome (D-Loop or control region, running from np 1 to np 1232). PCR amplification was performed in a 25 µl volume with 3 mM $MgCl_2$, 50 mM of each dNTP, 1 mM of each primer and 1 unit of Taq® DNA Polymerase (Sigma Aldrich, St. Louis, MO, USA), using a Biometra TGradient 96 Thermocycler at the following conditions: initial denaturation step of 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at the 60°C, 75 s at 72°C, and a final extension of 5 min at 72°C.

The PCR products were sequenced at the Central DNA sequencing service (Universidad de Zaragoza, Spain) by means of a Applied Biosystems 3730xl DNA analyzer.

A fragment of 506 base pairs in size (from np 1 to np 505 of complete chicken mitochondrial sequence) was used in the analysis. Sequences were aligned using the software Sequencher™ 4.10 (Gene Codes Inc., Ann Arbor, MI, USA).

Statistical analysis of mtDNA information

Indexes such as haplotype diversity (Hd) and nucleotide diversity (π), average number of nucleotide differences (k) and Fu's *Fs* statistic (Fu, 1997) were estimated by DnaSP 5.10 software (Librado and Rozas, 2009).

Evolutionary relationships of sequences were evaluated through a median-joining network constructed using the software Network 4.6 (www.fluxus-engineering.com). The network also included nine haplotypes representing the main clades (clades A to I) in the Chinese and Eurasian region (Liu *et al.*, 2006) as references. Haplotypes from GenBank were aligned with haplotypes observed in this study.

Microsatellite analysis

From a total of 30 microsatellite markers recommended for biodiversity studies of chicken by ISAG/FAO (FAO, 2004), 27 markers (Table S1) were used in this study. The markers were genotyped in standard multiplex PCR amplification using a Biometra TGradient 96. Annealing temperatures were set to values reported at the AVIANDIV website (2013). Allele calling was adjusted using nine standard DNA

samples. Analyses of fragments were performed using an automated DNA sequencer (ABI PRISM 3130xl, Applied Biosystems, Foster City, CA, USA) and the software package GeneMapper version 4.0 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis of microsatellite genotypes

Allele frequencies for each locus, total number of alleles per locus (MNA) and estimated observed (H_o) and unbiased expected (H_e) heterozygosities were calculated by the EXCEL MICROSATELLITE TOOLKIT 3.1.1 (Park, 2001). Test for deviation from Hardy-Weinberg equilibrium across all loci for each population were performed in GENEPOP 4.0 software (Raymond and Rousset, 1995), applying the "exact test" and using the Markov chain algorithm with default setting to calculate P-values (Guo and Thompson, 1992) and corrected for multiple tests using Bonferroni methods (Rice, 1989). The amount of inbreeding within population (f), and the amount of differentiation among populations (Theta) per locus were estimated according to Weir and Cockerham (1984) and using FSTAT 2.9.3 software (Goudet, 2002), with corresponding P-values obtained based on 1000 randomizations. The same software was also employed in calculations of allelic richness (Rt), an estimation of the mean number of alleles per locus, corrected by sample size. The within-breed inbreeding coefficient (F_{IS}) was calculated, with a 95% confidence interval, determined by 1000 permutations and 10000 bootstraps, using the software GENETIX 4.05 (Belkhir *et al*., 1996-2004). Reynolds genetic distances between pairs of breeds were estimated following Reynolds *et al.* (1983) and were plotted as a neighbor network using SplitsTree4 (Huson and Bryant, 2006).

The algorithm implemented in STRUCTURE software version 2.2 (Pritchard *et al.*, 2000) was used to confirm the genetic pattern of each individual belonging to the different breeds and to reveal possible breed substructures. The analysis involved an admixture model and correlated allele frequencies. One hundred independent runs were carried out with 20,000 interactions burn–in phase and 50,000 interactions from $2 \leq K \leq 22$ (K= number of clusters) to estimate the most likely number of clusters present in the dataset. The most probably K value was then

established by calculating ÄK, as in Evanno *et al*. (2005). The clustering pattern was visualised using the software DISTRUCT 1.1 (Rosenberg, 2004).

4.4 RESULTS

Mitochondrial DNA phylogeny

The sequences including the first 506 bp fragments of the chicken mitochondrial D-Loop, were used for analysis. The number of polymorphic sites, number of mtDNA haplotypes and haplotype diversity of the studied breeds are shown in Table 1. In this study 21 haplotypes were defined and a total of 25 nucleotide substitutions (only transitions) were observed.

All the Mediterranean chicken breeds, except AN, CO and SK, were found to be polymorphic with the number of haplotypes ranging from two (EX and AB) to five (VA, SO, BG and MA).

The highest haplotype diversity (Hd), 0.861 ± 0.087, was observed in MA chicken, whereas the lowest value (excluding the monomorphic breeds) was founded in AB, 0.200 ± 0.154.

The nucleotide diversity average value was 0.0040±0.0011 and ranged from 0.0097±0.0018 (LI) to 0.0004±0.0003 (AB), excluding monomorphic breeds.

The Fu's Fs statistic was negative, although not significant, and it could indicate a departure from neutrality, therefore a population expansion (-7.71, P>0.10, data not shown).

The distribution of clades is shown in Figure 2. The Median-Joining network tree of mtDNA D-loop haplotypes of the Mediterranean chicken breeds and of the reference haplotypes (Liu *et al.,* 2006), revealed that all the breeds clustered in one major haplogroup but also two isolate haplogroups were shown by the analysis. Most of the sequences grouped with haplotype LIUE1 (91%), while other few sequences clustered with reference sequences LIUA1 (7%) and LIUB1 (2%). It should be noted that several sequences (MO, LI, CH, EX and MA) were included in haplogroup A (Liu *et al.*, 2006). Three identical sequences (from LI) made part of the haplogroup B, with shared haplotype together with LIUB1.

Haplogroup E is reported as widespread in Europe, Middle East and India, while haplogroups A and B are widely distributed in South China and Japan (Liu *et al.*, 2006).

Microsatellite polymorphism

A total 242 alleles were detected across the 27 investigated loci (data not shown). MCW0104 exhibited the highest number of alleles observed (21), whereas MCW0098 and MCW0165 had the lowest number (3) (Table S1).

The expected frequencies for each locus ranged from 0.146 (MCW0103) to 0.713 (MCW0037) and the observed heterozygosity frequencies ranged from 0.107 (MCW013) to 0.624 (MCW0037). The estimated amount of inbreeding within populations (*f*) had an overall mean of 0.093 ± 0.061 ($P<0.05$), moreover the amount of differentiation among populations (*theta*) per locus was also significant ($P<0.05$), with an overall mean of 0.213 ± 0.052 (Table S1).

The mean number of alleles per population (MNA), expected (H_E) and observed (H_O), H-W equilibrium observed, private alleles, and inbreeding coefficient (F_{IS}) per breed are showed in Table 2. The MNA per population ranged from 2.63 (MO) to 4.96 (SU, AB), with an average across all the breeds of 3.67 ± 0.71 alleles per locus. The average expected and observed heterozygote frequencies within populations across loci were 0.50 (ranging from 0.36 to 0.64) and 0.46 (ranging from 0.35 to 0.61) respectively. After Bonferroni correction, 1.9 of the 27 loci deviated from HWE. LI, CH, CO, EX and SK were at HWE for all loci. In total, 59 private alleles in 13 breeds were detected. Fifty of the unique alleles had a frequencies <0.1%; while the remaining nine were found in AN (1), LI (1), VA (2), PI (1), CO (1), BG (1) and MA (2) (data not shown). R_t values (mean value of 2.37 ± 0.26) were similar in all populations, varying within a short range between 1.96 (MO) and 2.86 (BG), assuming a minimum sample size of three individuals.

The inbreeding coefficients calculated in each breeds were significantly different from zero in six breeds (AN, LI, PI, SO, AB and MA), which showed heterozygote deficit. This index reached a maximum value in SO (0.27254) and a minimum value in CH (-0.00445).

The genetic differentiation (*Theta*) between pairs of breeds (Table 3) ranged from 0.07 (AB *vs* BG) to 0.40 (CH *vs* CO and MO *vs* CO); overall breeds average *theta* value was 0.22. Reynolds' pairwise genetic distance ranged from 0.09 (BG *vs* AB) to 0.50 (SK *vs* CO).

The neighborNet dendrogram is presented in Figure 3. The tree showed that Serbian SK formed a cluster with the brown layers. Serbian SV and BG appeared in

other cluster with broiler lines. Spanish SO is closely related to the White Leghorn (LSS and WLA) used as commercial layer, while the other Spanish populations are separated on the base of a geographic distribution. The North Spain populations (CH and PI) are included in a separate branch, as the South (SU, CO and EX). About the Italian populations (AN, VA and RO) clustered in the same branch, while LI and MO appeared close in the tree. AB is in another cluster with MA. Reference populations showed longer branches than the sixteen Mediterranean populations studied.

Structure analysis using a Bayesian approach was performed with increasing numbers of inferred populations. The results indicate that, for the 22 breeds analysed, the most likely number of populations is 19 (Fig. S1), suggesting that the most significant subdivision was by breeds or by groups of closely related breeds.

The STRUCTURE clustering solutions (Figure 4) indicate that, for K=2, one cluster includes the Serbian breeds (SK,BG and SV), Spanish Gallina Valenciana de Chulilla (CH), Pita Pinta Asturiana (PI) and broiler lines (BRSA and BRDA), with q>0.900. A second cluster includes the two white-egg layer. All the remaining populations showed different levels of admixture. Populations that split to form a separate clusters at lower K values can be interpreted as being relatively genetically distinct (Rosemberg et al., 2001). For K=5, the reference populations were clearly differentiated. The South Spanish breeds and the Serbian breeds grouped in two different clusters. A further cluster includes Italian and North Spanish breeds. At K=8, three Italian breeds (LI, MO and RO) and MA made one cluster. Another Italian breed (VA) split to form a distinct genetic cluster. For K=14, MO, RO, SO and the three Serbian breeds (SK, BG and SW) formed their own cluster. All breeds clustered independently when 19 groups were considered, with the exception of AN, PI, EX, SV, AB and MA.

The results of Bayesian cluster analysis are summarized in Table S2, where the average q values in each clusters are shown for the different breed. The membership fraction among the breeds ranged between 0.442 in AB and 0.947 in MO.

4.5 DISCUSSION

The majority of the studied populations showed mtDNA haplotypes that clustered in clade E and only few animals were included in clades A (EX, LI, MO and MA) and B (LI). Based on skeleton of supposed regions of domestication, this finding suggests the existence of three maternal lineages for the Mediterranean chicken breeds which presumably originated from Indian subcontinent (Aplogroup E), Yunnan and surrounding regions in China (Aplogroups A and B). This hypothesis would be in agreement with historical records of chicken introduction in the Mediterranean area. In fact, chickens reached Europe along two main trading routes: a northern route through China and Russia and a southern route through Persia and Greece (Crawford, 1990). The clades A and B, as reported by Liu et al. (2006), have a similar geographical distribution and a close phylogenetic relationship; therefore can be presumed that both lineages originated from the same ancestral population. Other authors (Revay *et al.*, 2010; Grimal *et al.,* 2011) observed these two haplogroups in Hungarian and Spanish chicken breeds. In particular, Revay *et al.* (2010) found two sequences in haplogroup B that were identical to those existing in commercial chicken lines (white egg layer). Therefore, it cannot be excluded that the presence of this haplogroup is a result of introgression from commercial layer lines. About the sequences in clade A it is important to highlight that seven of these are from Spanish breeds (EX and CH), two from Italian breeds (LI and MO) and one from Maltese breed (MA). In a previous study on Spanish breeds, Grimal *et al.* (2011) already found two sequences of CH and one of PI in the clade A. The presence of one Maltese black's sequence in the clade A is due to the possible genetic introgression of Spanish and Italian breeds (Andalusian and Leghorn breeds) as reported by Shepard (1920) and Patrick (1975).

Although the majority of the Mediterranean chicken populations were assigned to clades E and A, thirteen of these were polymorphic for the mtDNA D-Loop region, with the number of haplotypes ranging from 0.20 to 0.86. These values are comparable to the results obtained from Muchadeyi *et al.* (2008) and Cuc *et al.* (2006) in Zimbabwean village chickens and Vietnamese chickens respectively. The star topology, which was more pronounced in clade E, is associated with ancestral haplotypes undergoing population expansion (Lopes et al., 2005).

Results of microsatellite markers revealed that 22% of the genetic variation across individuals could be ascribed to between-breed differences, as evidenced by the estimated average number of alleles (MNA=3.67), expected heterozigosity within breeds (H_E =0.50) and *theta* values with levels comparable to those reported in other European chicken breeds (Granevitze et al., 2007; *Bodzsar et al.*, 2009). Observed and expected frequencies of heterozygotes were similar in some breeds, consequently, F_{IS} estimates were not significantly different from zero, suggesting that these breeds are close to Hardy-Weinberg equilibrium state.

A deficiency of heterozygosity was observed in six populations (AN, LI, PI, SO, AB, MA). Consequently, the estimates of within-population inbreeding coefficients (Table 2) were considerably higher than that in the studied populations. It was not possible to assess the influence of mating close relatives because of the absence of pedigrees. The relatives mating although could be a contributing factor in many breeds, the most important source of heterozygote deficit is likely to be genetic substructuring (Figure 4). The moderate genetic variability among the Mediterranean chicken populations could be due to their common origins, as confirmed by the analysis of mtDNA sequences.

A part of population AB, the genetic differentiation among the studied breeds was high (Table 3 and Figure 3), with levels comparable to those among other European local chicken breeds (Bodzsar et al., 2009; Wilkinson et al., 2011). In general, chicken breeds appear to be genetically distinct populations with limited gene flow. The small population size, the short generation interval and the random genetic drift has likely contributed to the elevated levels of observed genetic differentiation among many of the Mediterranean chicken breeds.

In the NeighborNet representation of the Reynolds genetic distance, seven different clusters can be recognized and each one of them may be considered a different path of chicken dispersion into the Mediterranean area or, in some cases, a recent germoplasm introgression from other breeds. One cluster included SK and two brown eggs layer (BLA and BLC). SK was crossed with dual purpose breeds like New Hampshire and White Rock; less it is crossed with line hybrids for egg production (Isa Brown and Hisex hybrids) (Miloševiæ et al., 1997). A second cluster included SV, BG and two broiler lines (BRSA and BRDA). SV is the most

popular autochthonous broiler breed in Serbia and was developed in the mid-20th century by crossing the native hen breed with other breeds, mostly Australorp and Langhan (Mašiæ et al., 1996, 1997; Mitroviæ et al., 2011). Besides BG arised from random crossbreeding of serbian domestic chicken with Transylvanian naked neck (Grujiæ, 1928) that it is a good meat producer (Mitroviæ et al., 2011).

AN, VA and RO are three Italian populations that appear in a separate branch, because of their geographical proximity that in the past allowed the exchange of breeding animals.

The tree showed that the two White Leghorn (WLA and LSS) clustered with MO, LI, CH and SO breeds. LI is closely related to the founder population of White Leghorn used as commercial egg layers and the results confirm the common historic origin of White Leghorn strains. MO and LI appeared close in the tree, as it was expected, because of the ancient crossbreeding practices among these two breeds as reported by Mazzon (1932). It is not possible to explain the genetic relationships amongst SO, PI and the two White eggs layers.

PI formed one separated cluster. PI is a breed of the Atlantic type and it was recreated by the biologist A. Equino Marcos (1985), starting with the most typical characteristics of the old breed from Asturia (North of Spain).

The three Spanish Andalusian breeds are closed in the same cluster and this fact reflects their geographical distribution. The last cluster includes breeds (MA and AB) that not have historical influence between them. In Albania, it is very difficult to find a uniform and distinct chicken breed; as a matter of fact during the last 60 years in Albania were imported several improved breeds.

STRUCTURE analysis confirmed the general features observed in the NeighborNet dendrogram. In general, European chicken breeds have been observed to be distinct homogenous genetic populations with little evidence of substructure within breeds (Bodzsar et al., 2009; Zanetti et al., 2011). On the contrary, many of the Mediterranean chicken populations exhibited extensive genetic admixture (Figure 3 and 4), thus explaining the differences between these two graphic representations. For example, it was not possible to evidenziate the genetic relationships between the brown and broiler lines with the Serbian populations, because these strains formed a separate clusters in STRUCTURE. In contrast the Andalusian Spanish breeds appears together already at low levels of K. Interesting

is the situation of MA population; this breed showed an admixture by LI and EX and the results confirm the possible genetic introgression of Spanish and Italian breeds (Andalusian and Leghorn breeds) as reported from Shepard (1920) and Patrick (1975).

These results indicate that appropriate management programs must be implemented to ensure that the genetic pool represented by these breeds is not lost due to further genetic erosion or uncontrolled crossbreeding.

In conclusion, the Mediterranean chicken populations retain great levels of genetic diversity as shown in mtDNA analysis and microsatellite. This study also indicate that Mediterranean breeds originated from three maternal lineages and the Indian subcontinent is the main origin of these chicken populations, at least from the maternal lineage standpoint, since most individuals are included in the E lineage.

Inbreeding was detected in some breeds, suggesting the need of appropriate measures to be taken to avoid its negative effects. The results presented herein can be used to support breeds recognition and promotion, and to assist all stakeholders in the conservation measures and breeding programs.

Acknowledgements:

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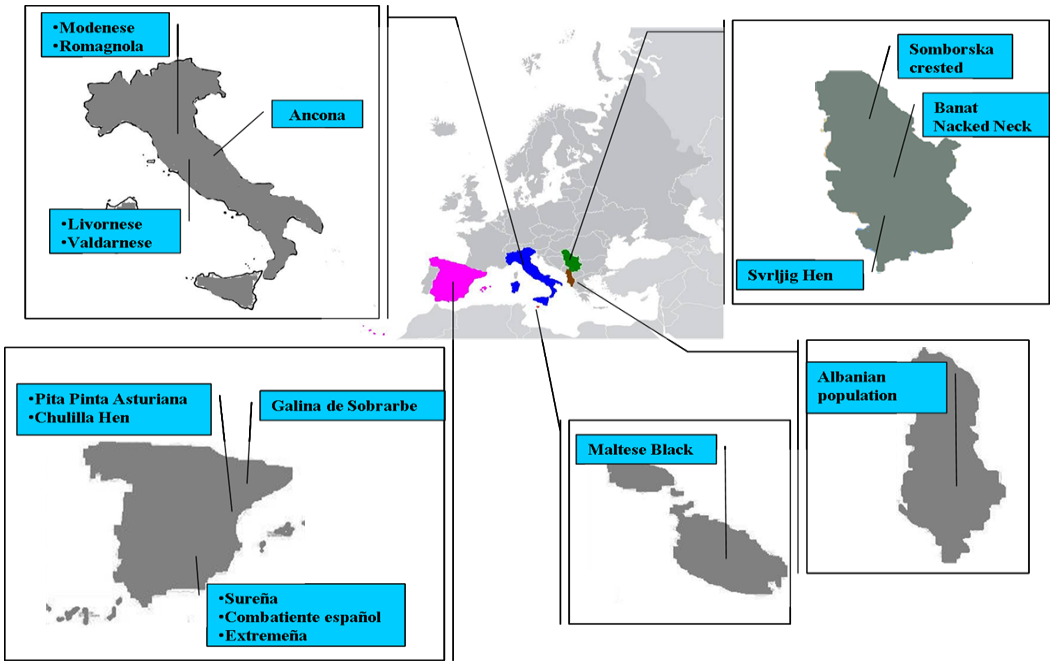
4.6 TABLES AND FIGURES

Figure 1 Geographical sampling areas



BREED	SAMPLES
Ancona	10♂ 20♀
Livornese bianca	10♂ 20♀
Modenese	8♂ 17♀
Romagnola	7♂ 18♀
Valdarnese bianca	10♂ 20♀
Pita Pinta Asturiana	5♂ 25♀
Chulilla Hen	5♂ 25♀
Galina de Sobrarbe	10♂ 20♀
Sureña	10♂ 20♀
Combatiente Español	10♂ 20♀
Extremeña	10 ♂ 20♀
Albanian chicken	10♂ 20♀
Somborska Crested	10♂ 20♀
Banat Nacked Neck	10♂ 20♀
Svrljig Hen	10♂ 20♀
Black Maltese	5♂ 20♀

Table 1 mtDNA diversity indices of the sixteen chicken breeds.

Breed	ID name	N	π	nh	Hd	S
Ancona	AN	10	0.0000±0.0000	1	0.0000±0.0000	0
Livorno	LI	10	0.0097±0.0018	3	0.6390±0.1260	11
Modenese	MO	10	0.0045±0.0027	3	0.6000±0.1310	10
Romagnola	RO	10	0.0007±0.0004	3	0.3780±0.1810	2
Valdarnese	VA	10	0.0029±0.0003	5	0.8440±0.0800	4
Pita Pinta Asturiana	PI	10	0.0014±0.0005	3	0.5560±0.1650	2
Gallina Valenciana de Chulilla	CH	10	0.0056±0.0028	3	0.6070±0.1640	10
Gallina de Sobrarbe	SO	10	0.0033±0.0006	5	0.6670±0.1410	5
Sureña	SU	10	0.0030±0.0005	3	0.7220±0.0970	3
Combatiente Español	CO	10	0.0000±0.0000	1	0.0000±0.0000	0
Extremeña azul	EX	10	0.0084±0.0015	2	0.5330±0.0950	8
Albanian population	AB	10	0.0004±0.0003	2	0.2000±0.1540	1
Somborska Crested	SK	10	0.0000±0.0000	1	0.0000±0.0000	0
Banat Nacked Neck	BG	10	0.0027±0.0005	5	0.8440±0.0800	4
Svrljig Hen	SV	10	0.0030±0.0006	4	0.8220±0.0051	4
Black Maltese	MA	10	0.0067±0.0025	5	0.8610±0.0870	12
Overall		**160**	**0.0040±0.0011**	**21**	**0.6364±0.1158**	**25**

N: number of sequence used, π: diversity of nucleotide, nh: number of haplotype, Hd: diversity of haplotype, S: number of segregation site.

Figure 2 Median-Joining network tree for the twelve haplotypes of Mediterranean chicken populations, nine reference sequences by Liu et al. (2006) based on the polymorphic sites of the mitochondrial D-loop region. Circled areas are proportional to the haplotype frequencies.

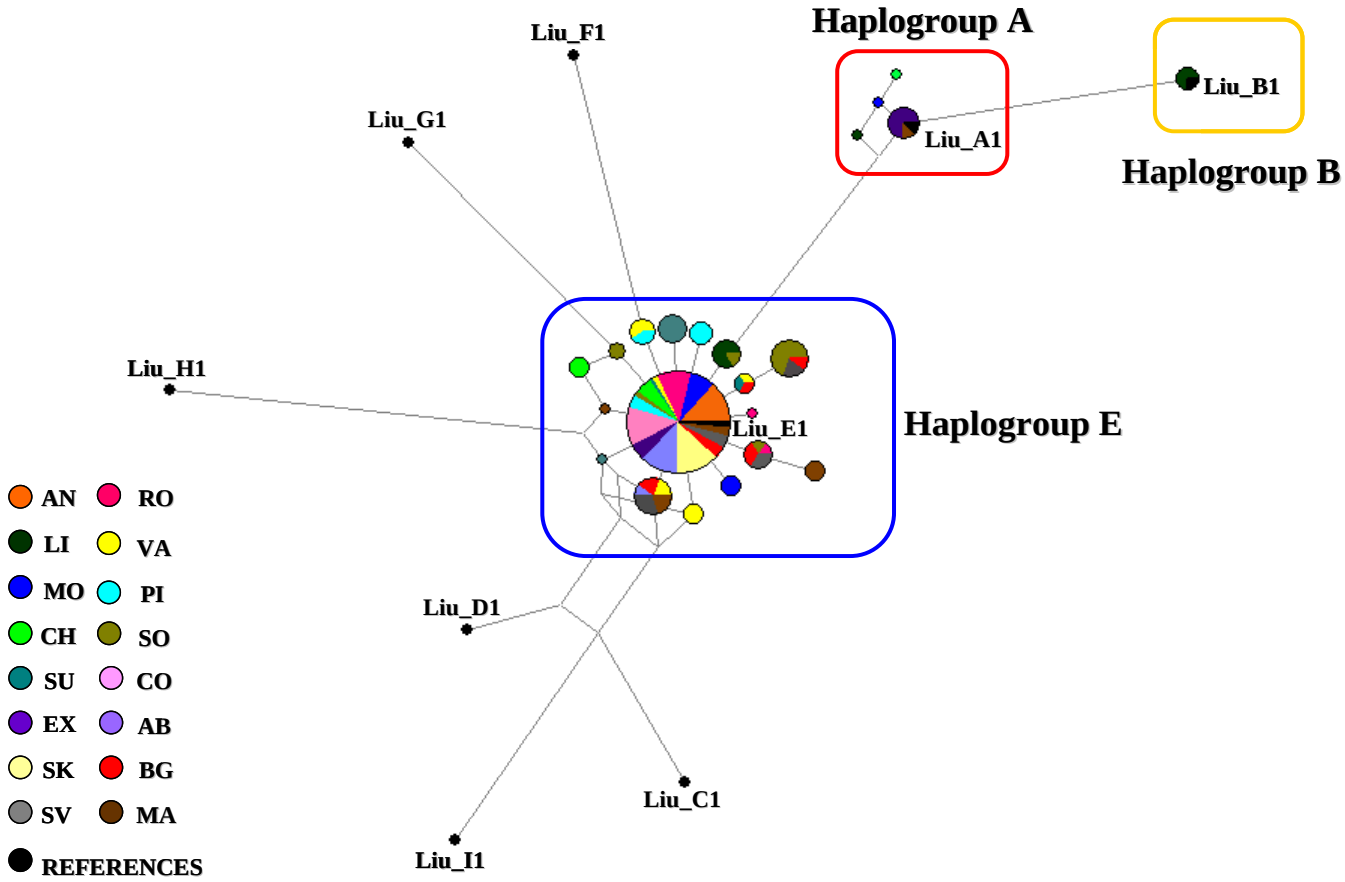


Table 2 Chicken breeds studied, sample size of each breed, mean number of observed alleles (MNA), mean observed and expected heterozygosity, mean allelic richness per locus corrected for sample size and breed (R_t), number of locus deviated from Hardy-Weinberg equilibrium per breed (dHWE) and inbreeding coefficient (F_{IS}) per breed.

Breed	Country	ID	Sample Size	MNA	H_E	H_O	A_p[1]	Rt	dHWE[2]	F_{IS}
Ancona	Italy	AN	30	3.26 (1.10)	0.48 (0.017)	0.39 (0.041)	2	2.30	3	0.19244 [a]
Livornese bianca		LI	30	3.11 (0.97)	0.45 (0.019)	0.40 (0.036)	1	2.14	0	0.10920 [a]
Modenese		MO	25	2.63 (0.93)	0.39 (0.020)	0.39 (0.040)	1	1.96	1	-0.00902
Romagnola		RO	25	3.59 (1.45)	0.50 (0.020)	0.47 (0.040)	2	2.36	1	0.07704
Valdarnese bianca		VA	30	3.67 (1.11)	0.53 (0.018)	0.53 (0.039)	3	2.47	1	0.00006
Pita Pinta Asturiana	Spain	PI	25	3.89 (1.50)	0.54 (0.020)	0.42 (0.035)	3	2.48	4	0.22792 [a]
Gallina Valenciana de Chulilla		CH	25	2.96 (1.00)	0.43 (0.019)	0.44 (0.042)	0	2.11	0	-0.00445
Gallina de Sobrarbe		SO	30	3.63 (1.21)	0.50 (0.018)	0.37 (0.038)	1	2.36	2	0.27254 [a]
Sureña		SU	30	4.96 (2.16)	0.57 (0.018)	0.52 (0.029)	13	2.64	2	0.07903
Combatiente Español		CO	30	3.22 (1.89)	0.36 (0.017)	0.35 (0.050)	7	1.98	0	0.03146
Extremeña azul		EX	30	3.70 (1.32)	0.51 (0.018)	0.49 (0.039)	5	2.37	0	0.02611
Albanian population	Albania	AB	30	4.96 (2.30)	0.62 (0.018)	0.52 (0.026)	9	2.80	1	0.16273 [a]
Somborska Crested	Serbia	SK	30	3.58 (1.24)	0.53 (0.018)	0.53 (0.032)	0	2.45	0	0.00804
Banat Nacked Neck		BG	30	4.85 (2.30)	0.64 (0.018)	0.61 (0.024)	10	2.86	1	0.04563
Svrljig Hen		SV	30	3.74 (1.26)	0.54 (0.018)	0.52 (0.032)	0	2.49	3	0.05458
Black Maltese	Malta	MA	25	3.04 (0.98)	0.42 (0.023)	0.35 (0.042)	2	2.16	2	0.16724 [a]
Mean				3.67 ± 0.71	0.50 ± 0.08	0.46 ± 0.08		2.37 ± 0..26	1.31 ± 1.25	0.089 ± 0.090

[1]Number of breed-specific private alleles.

[2]Number of locus that deviate from Hardy-Weinberg equilibrium [after Bonferroni correction, P<0.00012, Rice (1989)].

[a] Significantly different from zero (P < 0.05)

Table 3 Genetic differentiation among the analysed populations[1]

Breed	AN	LI	MO	RO	VA	PI	CH	SO	SU	CO	EX	AB	SK	BG	SV	MA	Reynolds	*Theta*
AN		0.22	0.33	0.25	0.25	0.20	0.30	0.21	0.32	0.44	0.37	0.18	0.42	0.25	0.28	0.24	0.28	0.25
LI	0.21		0.27	0.25	0.27	0.22	0.18	0.19	0.29	0.44	0.31	0.08	0.31	0.21	0.28	0.21	0.25	0.22
MO	0.32	0.22		0.29	0.35	0.28	0.27	0.28	0.29	0.47	0.37	0.23	0.38	0.28	0.25	0.29	0.31	0.28
RO	0.18	0.17	0.25		0.22	0.25	0.31	0.22	0.22	0.30	0.28	0.17	0.35	0.21	0.29	0.23	0.26	0.20
VA	0.19	0.23	0.30	0.18		0.22	0.31	0.24	0.23	0.39	0.31	0.16	0.29	0.18	0.22	0.21	0.26	0.22
PI	0.20	0.21	0.27	0.19	0.17		0.22	0.17	0.27	0.43	0.30	0.14	0.27	0.13	0.22	0.19	0.23	0.20
CH	0.30	0.22	0.27	0.25	0.27	0.19		0.22	0.30	0.49	0.36	0.15	0.31	0.22	0.22	0.28	0.28	0.25
SO	0.22	0.17	0.25	0.15	0.19	0.15	0.24		0.25	0.41	0.32	0.12	0.33	0.16	0.22	0.18	0.23	0.20
SU	0.23	0.21	0.24	0.17	0.18	0.18	0.24	0.18		0.18	0.13	0.20	0.30	0.19	0.21	0.28	0.24	0.18
CO	0.34	0.34	0.40	0.24	0.30	0.33	0.40	0.28	0.14		0.27	0.36	0.50	0.36	0.44	0.42	0.39	0.31
EX	0.29	0.28	0.33	0.23	0.24	0.25	0.30	0.24	0.14	0.25		0.23	0.29	0.22	0.29	0.35	0.29	0.24
AB	0.18	0.11	0.20	0.12	0.15	0.13	0.15	0.10	0.13	0.24	0.15		0.20	0.09	0.18	0.14	0.18	0.14
SK	0.32	0.28	0.32	0.26	0.24	0.21	0.23	0.24	0.21	0.36	0.21	0.14		0.16	0.25	0.35	0.31	0.24
BG	0.21	0.19	0.24	0.16	0.14	0.12	0.19	0.14	0.13	0.27	0.16	0.07	0.13		0.18	0.18	0.20	0.17
SV	0.25	0.26	0.27	0.24	0.20	0.19	0.23	0.21	0.15	0.33	0.24	0.15	0.19	0.13		0.27	0.25	0.22
MA	0.25	0.23	0.31	0.22	0.25	0.25	0.31	0.22	0.24	0.36	0.32	0.14	0.30	0.20	0.27		0.25	0.26

[1]Pairwise genetic distance between breeds by Reynold's genetic distance (above the diagonal). Pairwise genetic differentiation between breeds (below the diagonal) estimated according to Weir and Cockerham (Weir and Cockerham, 1984). The two columns right of the table represent average breed Reynolds' genetioc distance and average breed *Theta*.

Figure 3 NeighbourNet dendrogram constructed from Reynolds genetic distances among 22 studied populations.

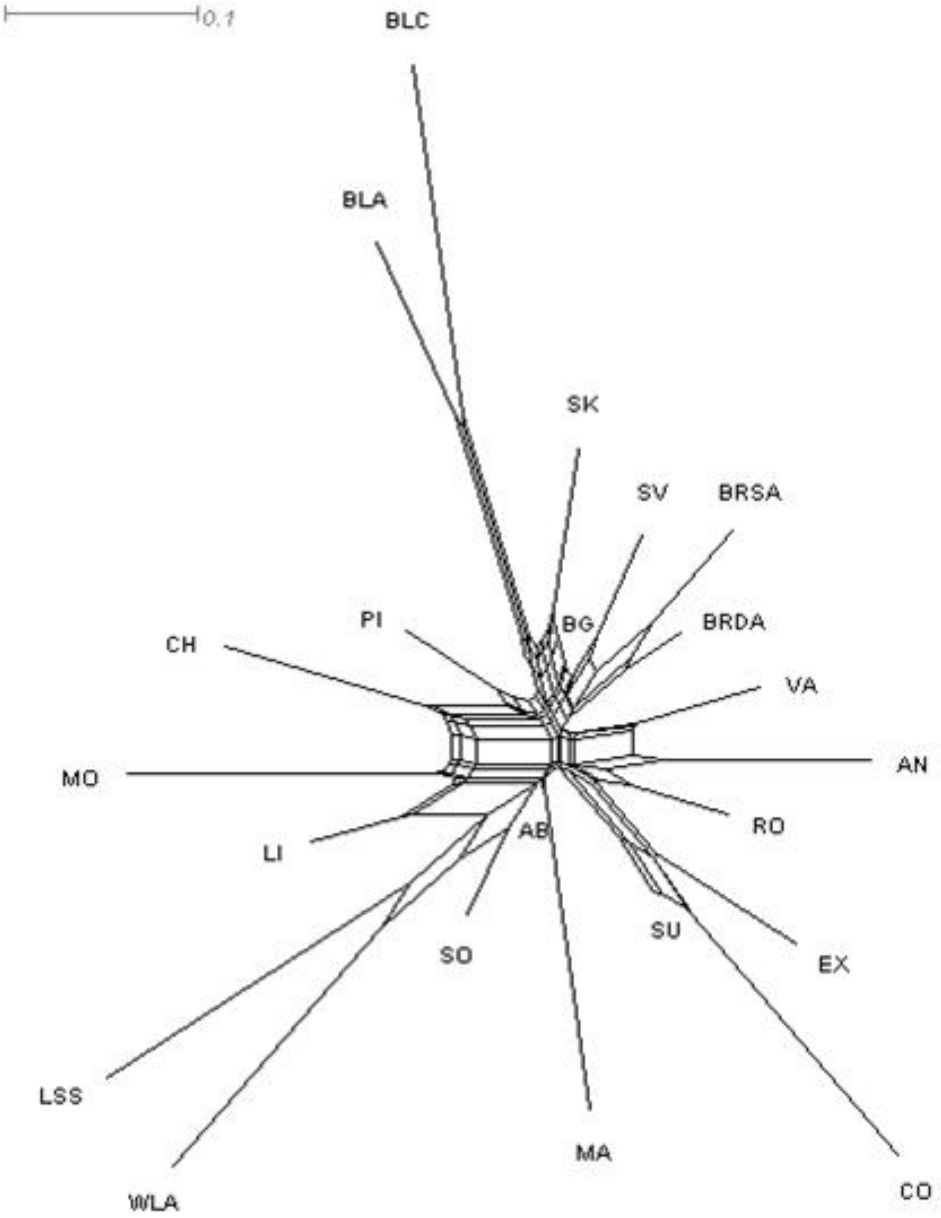


Figure 4 STRUCTURE cluster analysis of the studied populations

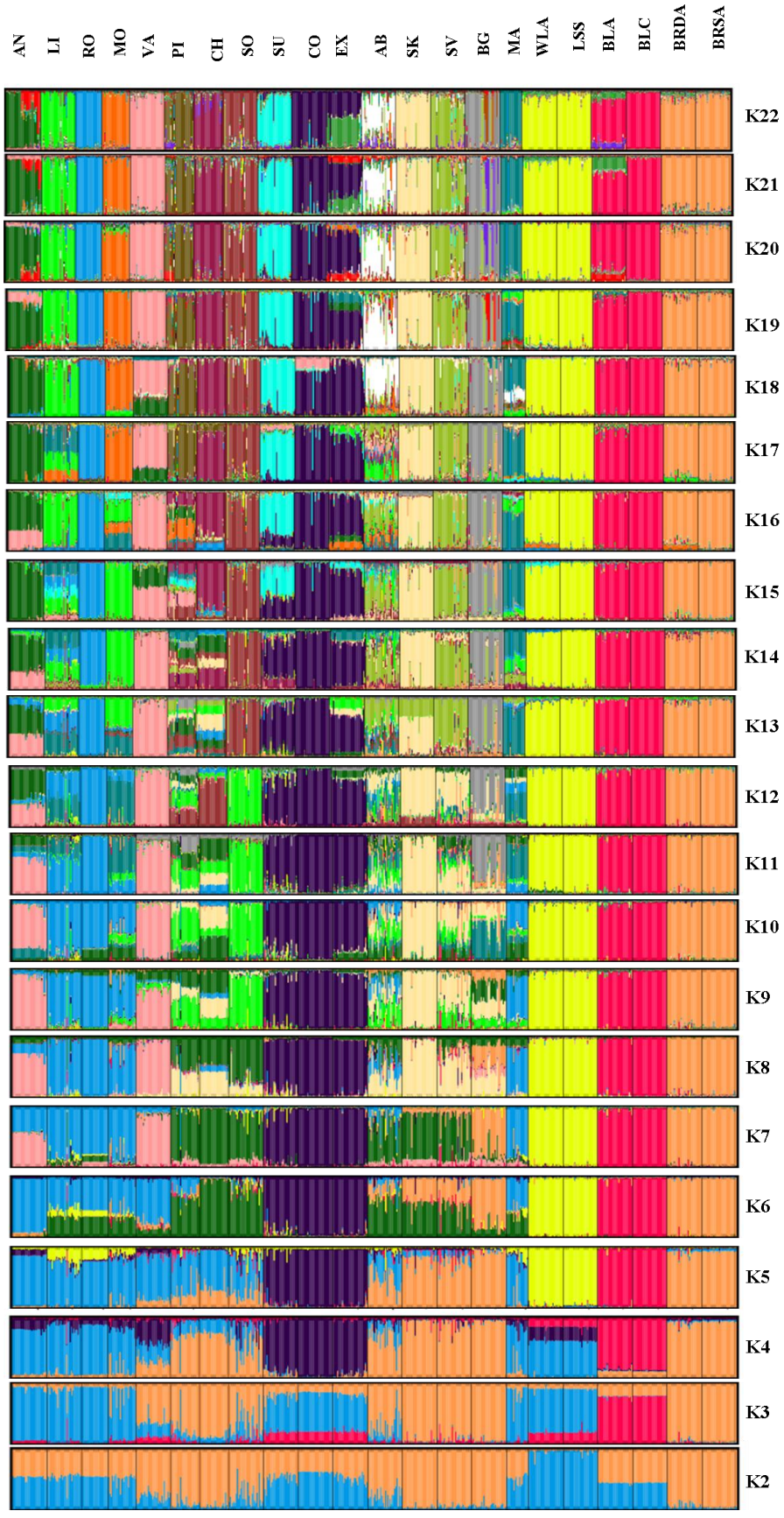


Table S1. Microsatellite loci, chromosomal position (Chr.), size range (S.R.), number of alleles observed (Na) at each locus, expected (He) and observed (Ho) heterozygosities, F-statistics *f* (the amount of inbreeding within populations), *theta* (the amount of diffentiation among populations) and their standard errors (SE) estimated across 16 Mediterranean studied populations.

Locus	Chr.	S.R. (bp)	Na	He	Ho	*f* ± SE*	*theta*± SE*
MCW0248	1	215-223	9	0.607	0.587	0.273 ± 0.219	0.323 ± 0.099
MCW0111	1	98-114	7	0.486	0.479	0.089 ± 0.054	0.233 ± 0.061
ADL0268	1	104-116	8	0.669	0.609	0.036 ± 0.041	0.193 ± 0.030
MCW0020	1	179-185	4	0.594	0.545	0.075 ± 0.031	0.182 ± 0.041
MCW0206	2	223-249	10	0.542	0.481	0.049 ± 0.045	0.157 ± 0.039
MCW0034	2	220-242	13	0.524	0.323	0.115 ± 0.051	0.147 ± 0.027
MCW0222	3	220-226	5	0.475	0.453	0.256 ± 0.049	0.159 ± 0.049
MCW0103	3	266-270	7	0.146	0.107	0.097 ± 0.054	0.327 ± 0.050
MCW0016	3	144-184	10	0.485	0.429	0.063 ± 0.082	0.513 ± 0.094
LEI0166	3	356-366	7	0.340	0.314	-0.031 ± 0.048	0.147 ± 0.031
MCW0037	3	154-158	4	0.713	0.624	0.182 ± 0.041	0.207 ± 0.070
MCW0295	4	88-106	9	0.552	0.530	0.156 ± 0.077	0.274 ± 0.074
LEI0094	4	247-285	19	0.598	0.620	0.070 ± 0.034	0.184 ± 0.045
MCW0098	4	261-265	3	0.308	0.273	0.049 ± 0.074	0.209 ± 0.053
MCW0078	5	135-145	6	0.540	0.491	-0.021 ± 0.073	0.220 ± 0.050
MCW0081	5	112-135	13	0.570	0.527	-0.040 ± 0.044	0.180 ± 0.049
MCW0014	6	164-182	9	0.461	0.343	0.098 ± 0.084	0.216 ± 0.054
MCW0183	7	296-326	13	0.406	0.341	0.098 ± 0.049	0.016 ± 0.040
ADL0278	8	114-124	6	0.562	0.507	0.005 ± 0.047	0.363 ± 0.068
MCW0067	10	174-184	8	0.449	0.463	0.035 ± 0.039	0.118 ± 0.023
ADL0112	10	122-132	9	0.360	0.344	0.101 ± 0.052	0.228 ± 0.067
MCW0216	13	141-147	9	0.526	0.483	0.053 ± 0.040	0.165 ± 0.039
MCW0104	13	178-226	21	0.516	0.444	0.119 ± 0.073	0.148 ± 0.044
MCW0123	14	80-94	9	0.511	0.424	-0.084 ± 0.061	0.186 ± 0.029
MCW0330	17	258-290	9	0.552	0.564	0.169 ± 0.075	0.224 ± 0.061
MCW0165	23	114-118	3	0.565	0.509	0.392 ± 0.069	0.198 ± 0.060
MCW0069	26	158-170	12	0.453	0.485	0.106 ± 0.043	0.237 ± 0.050
Means (SD)			8.96±4.27	0.500±0.116	0.456±0.119	0.093 ± 0.061	0.215 ± 0.052

*All the F-statistics indices are significant (P<0.05)

Figure S1. Description of ΔK values computed by STRUCTURE software (Prichard et al., 2000) following Evanno et al. 2005 procedure at K=2 to K=24 in 22 chicken breeds

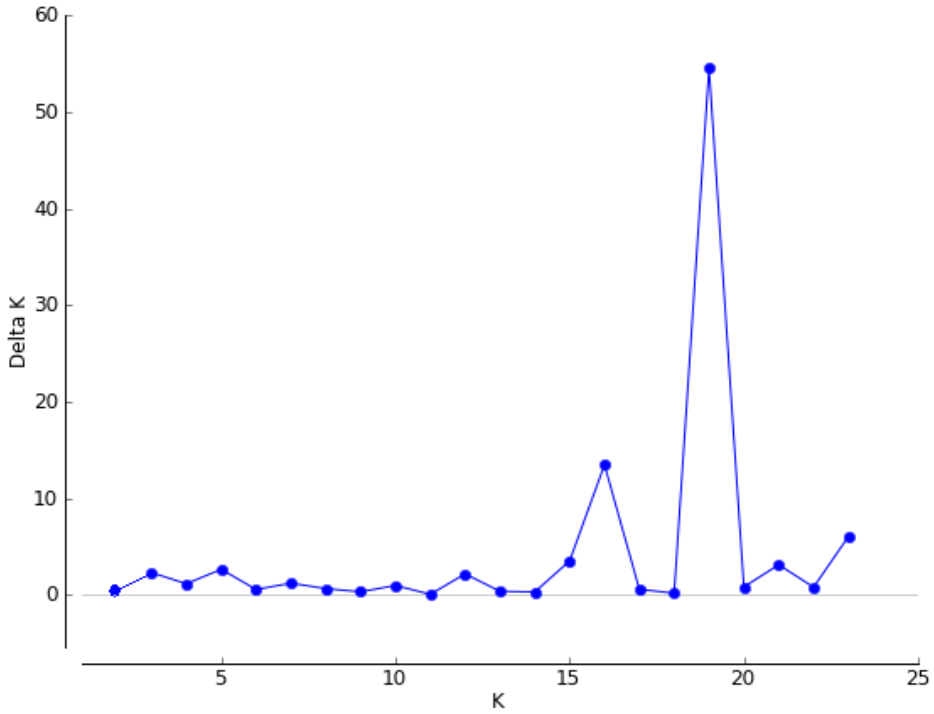


Table S2. Estimated membership fractions in each cluster (q), as inferred by STRUCTURE for K=19.

	Inferred Cluster																		
BREED	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
AN	0,031	0,003	0,003	0,005	0,007	0,004	0,022	0,004	0,007	***0,622***	0,004	0,007	0,004	0,005	0,090	0,152	0,011	0,003	0,017
LI	0,011	0,006	0,008	0,006	0,006	0,009	0,006	0,006	0,008	0,010	0,010	0,006	0,006	0,024	0,010	0,010	***0,820***	0,010	0,029
MO	0,003	0,002	0,002	0,003	0,002	0,003	0,002	0,003	0,003	0,003	0,004	0,003	0,002	0,003	0,004	0,003	0,006	0,002	***0,947***
RO	0,006	0,011	0,011	0,004	0,005	0,008	0,004	0,005	0,005	0,006	0,042	0,005	0,005	0,005	***0,814***	0,006	0,042	0,003	0,014
VA	0,007	0,006	0,005	0,014	0,006	0,005	0,005	0,004	0,008	0,040	0,005	0,006	0,004	0,009	0,004	***0,860***	0,005	0,003	0,005
PI	0,034	0,007	0,014	0,011	0,035	***0,662***	0,031	0,007	0,085	0,009	0,015	0,008	0,018	0,018	0,007	0,006	0,006	0,009	0,017
CH	0,007	0,010	0,004	0,003	0,010	0,007	0,007	0,003	***0,870***	0,004	0,004	0,003	0,004	0,023	0,005	0,004	0,005	0,003	0,026
SO	0,022	0,005	0,010	0,007	0,032	0,021	0,008	0,005	0,010	0,008	0,008	0,006	0,015	***0,805***	0,004	0,007	0,007	0,014	0,005
SU	0,010	0,005	0,008	0,009	0,006	0,005	0,013	0,052	0,008	0,007	0,006	***0,833***	0,005	0,006	0,007	0,005	0,005	0,007	0,005
CO	0,004	0,002	0,002	0,003	0,003	0,003	0,003	***0,903***	0,003	0,003	0,004	0,037	0,005	0,005	0,008	0,003	0,004	0,003	0,004
EX	0,147	0,008	0,008	0,006	0,013	0,005	0,009	***0,598***	0,006	0,136	0,005	0,019	0,016	0,005	0,005	0,004	0,004	0,003	0,004
AB	***0,442***	0,017	0,012	0,012	0,051	0,086	0,046	0,006	0,033	0,058	0,075	0,007	0,027	0,028	0,041	0,008	0,029	0,013	0,011
SK	0,009	0,017	0,005	0,010	0,022	0,005	0,006	0,003	0,006	0,004	0,005	0,004	***0,876***	0,009	0,004	0,004	0,005	0,003	0,004
BG	0,031	0,038	0,017	0,013	***0,730***	0,019	0,020	0,008	0,009	0,010	0,010	0,020	0,024	0,019	0,007	0,012	0,005	0,004	0,005
SV	0,006	0,023	0,008	***0,662***	0,082	0,005	***0,153***	0,003	0,015	0,004	0,004	0,004	0,009	0,005	0,003	0,005	0,003	0,003	0,004
MA	0,007	0,007	0,004	0,016	0,005	0,007	0,004	0,006	0,009	0,006	***0,767***	0,006	0,010	0,007	0,006	0,008	0,072	0,005	0,047
WLA	0,022	0,003	0,022	0,010	0,003	0,005	0,005	0,004	0,004	0,003	0,003	0,005	0,005	0,004	0,003	0,002	0,014	***0,879***	0,006
LSS	0,004	0,004	0,004	0,005	0,003	0,002	0,006	0,003	0,003	0,003	0,007	0,003	0,002	0,003	0,004	0,004	0,002	***0,937***	0,002
BLA	0,007	0,007	***0,866***	0,006	0,005	0,006	0,006	0,003	0,020	0,004	0,004	0,003	0,006	0,012	0,005	0,021	0,008	0,006	0,005
BLC	0,021	0,002	***0,906***	0,002	0,002	0,003	0,003	0,001	0,001	0,002	0,003	0,002	0,002	0,002	0,002	0,040	0,002	0,002	0,001
BRDA	0,006	***0,890***	0,011	0,005	0,005	0,003	0,005	0,008	0,005	0,008	0,005	0,006	0,006	0,004	0,007	0,008	0,006	0,005	0,006
BRSA	0,005	***0,921***	0,003	0,006	0,005	0,005	0,008	0,003	0,004	0,003	0,008	0,003	0,003	0,004	0,003	0,004	0,004	0,002	0,007

Contribution of the more important cluster per breed is represented in bold

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5th CHAPTER

GENERAL CONCLUSION

InEurope and in particular in the Mediterranean region, radical changes in the structure if human population caused an early awareness of the possible erosion of the animal genetic resources; since the beginning, this was linked to the conservation of the breeds in danger. This lead an increase of attention worldwide to the question of animal genetic resources and thei conservation.

In the Mediterranean area during the post Neolithic time, a large variety of domesticated plants and animals, including chicken, were introduced.

The contributes presented shared the objective to study and characterise some local Mediterranean chicken breeds. Different approaches have been developed to understand the different aspects that contribute to breed differentiation. In this way, a modern molecular technologies have opened up more reliable ways of investigating genetic diversity and population structures.

The mitochondrial DNA D-loop sequence on the other hand is a highly mutable marker that is clonally transmitted by female lines. MtDNA variation has been particularly useful in establishing relationships between different chicken breeds and their wild relatives, to identify domestication sites and to trace an ancient maternal origin of populations. Besides, the mtDNA sequences are capable of providing information on genetic structure in particular when combined with other nuclear markers such as microsatellites. Microsatellites are common in all eukaryotic genomes and highly polymorphic codominant markers, making them very useful for the study of genetic variation. For chickens, standardized panels of about 30 microsatellites distributed across the genome have been recommended by the FAO. The autosomal nuclear microsatellite loci used in this study are bi-parental markers whose inheritance is affected by recombination.

The first contribute, dealing with the genetic molecular characterisation performed by means of microsatellites analysis, highlighted the moderate level of genetic diversity among two Italian local breeds (Ancona and Livornese bianca). The microsatellites used in this study are assumed to be neutral markers and give an indication of overall population differentiation. Whatever, using the method for analyse genetic differentiation (i.e. genetic distance, structure clustering), Ancona breed highlighted a potential sub-populations due to genetic isolation.

This study confirmed the possibility to discriminate with molecular markers among different breeds by using statistical assignment analysis.

The second contribute was focused on five Italian chicken breeds (Ancona, Livornese bianca, Modenese, Romagnola and Valdarnese bianca). The mtDNA data suggest that the Indian subcontinent is the origin of the Italian chicken breeds, at least from the maternal lineage standpoint.

The microsatellite results indicate a low genetic variability in the five Italian chicken breeds. The loss of variability is probably due to the fact that these breeds are reared in small farms with a low number of animals. Moreover, it is possible to speculate in Valdarnese breed a potential genetic introgression from heavy type chickens. Possible crossbreeding between breeds spread in close geography positions was also detected in Livornese bianca e Modenese. The five Italian chicken breeds studied showed genetic differences and a sub-populations structure. Furthermore, the results from this study also indicated that the studied breeds are genetically distinct from commercial chicken lines. This genetic distinction could be explained by current genetic isolation and restricted gene flow between the populations.

The third contribute was carried out on the all sixteen studied populations, reared in the Mediterranean basin. The analysis of mtDNA sequences showed that the Mediterranean populations could be assigned to three distinct maternal lineages (E, A and B) based on a skeleton reflected and suggested regions of domestication in chickens. This skeleton plays an important role because it is constructed from clades, which indicate apparent geographic affiliation for domestication events. The skeleton was based on the most frequent haplotypes of the nine clades of Liu's network (Liu et al., 2006). Clade E is presumably originated from Indian subcontinent, while Clade A and B from Yunnan, South and Southwest China and surrounding areas.

The studied breeds showed a great level of genetic variability, comparable to those reported in other European chicken breeds.

In general, several Mediterranean breeds showed genetic substructure, if compared with other European breeds. These ones showed distinct homogenous genetic populations with evidence of substructure in some populations.

On the whole the contributes evidenced, in different ways, the great diversity existing among the studied populations. The results presented in this study can be used to support breeds recognition and promotion, and to assist all stakeholders in the conservation measures and breeding programs.

CONCLUSION

From this study several conclusion can be drawn:

(i) The Italian local chicken breeds originated from the Indian subcontinent.

(ii) At the autosomal level, the Italian chicken breeds represent genetically distinc populations.

(iii) The Italian studied breeds are genetically separated from the six purebred lines.

(iv) The Mediterranean breeds show different levels of genetic variability and some populations are genetically closed (same geographical distribution).

(v) Probably, the Mediterranean studied breeds originated from three maternal lineages, that can be largely attributed to historical human dispersal in the Mediterranean basin.

6th APPENDIX

APPENDIX A

Questionnaire to record information about the breeds sampling

Strain

1. Description of strain
2. Local breed name .
3. Number of adult birds *

Male		Female	

(* specify the sex for each samples)

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4. Type of population (inbred, selected,, standardized....)
5. Status of population (open or closed)
6. Conservation program exists

Origin/source of breed

	No.	Location
1. Within flock		
2. Communal area farm		
3. Commercial Farm		
4. Other (specify)		

***SHORT DESCRIPTION OF THE CHICKENS SAMPLED FOR DNA ANALYSIS**

*(history of the breed if know, phenotypic traits, etc...)

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APPENDIX B

- **ALBANIAN POPULATIONS**
  - **Albanian Black chicken**

Phenotypic traits	
Average live weight -hens	1.2 -1.4 kg
-cocks	1.5 -1.8 kg
Laying capacity per year	140 – 155 eggs
Average weight of eggs	35-45 g
Starts of egg laying	135-140 days
Size of population1 -hens	825
-cocks	182
Trend of population decreasing	
Economic use for producing of eggs and meat for family farm	

[1]Statistical evaluation

- **SERBIAN BREEDS**
  - **Sombor crested**

Phenotypic traits	
Average live weight -hens	~2.5 kg
-cocks	~3.5 kg
Laying capacity per year	200 – 220 eggs
Average weight of eggs	-
Starts of egg laying	-
Size of population[1]	100
Trend of population decreasing	
Economic use for producing of eggs and meat for family farm	

[1]Statistical evaluation

- **Banat Nacked Neck**

Phenotypic traits	
Average live weight -hens -cocks	 ~2.0 kg ~2.5 kg
Laying capacity per year	120-160 eggs
Average weight of eggs	-
Starts of egg laying	-
Size of population[1]	100-150
Trend of population decreasing	
Economic use for producing of eggs and meat for family farm	

[1]Statistical evaluation

- **Svrljig Hen**

Phenotypic traits	
Average live weight -hens -cocks	 ~2.5 kg ~3.0 kg
Laying capacity per year	-
Average weight of eggs	-
Starts of egg laying	-
Size of population[1]	250
Trend of population decreasing	
Economic use for producing of eggs and meat for family farm	

- **SPANISH BREEDS**
  - **Galina de Sobrarbe**

http://www.gallinadelsobrarbe.es ©

Phenotypic traits	
Average live weight -hens -cocks	 1.7-2.0 kg 2.5-3.0 kg
Laying capacity per year	170 eggs
Average weight of eggs	55 g
Starts of egg laying	-
Size of population	-

  - **Chulilla Hen**

http://www.chulival.com ©

Phenotypic traits	
Average live weight -hens -cocks	 ~2.1 kg ~3.0 kg
Laying capacity per year	150 eggs
Average weight of eggs	56 g
Starts of egg laying	-
Size of population	-

- **Pita Pinta Asturiana**

http://www.mundoavicola.com ©

Phenotypic traits	
Average live weight -hens -cocks	 2.5-3.0 kg 4.0-5.4 kg
Laying capacity per year	245 eggs
Average weight of eggs	60-65 g
Starts of egg laying	-
Size of population[1]	400

[1]Statistical evaluation

- **Sureña**

http://www.todogallinas.com ©

Phenotypic traits	
Average live weight -hens -cocks	 0.6-0.8 kg 0.7-09 kg
Laying capacity per year	165 eggs
Average weight of eggs	38 g
Starts of egg laying	-
Size of population[1]	400

[1]Statistical evaluation

- **Combatiente Español**


http://www.todogallinas.com ©

Phenotypic traits	
Average live weight -hens -cocks	 1.0-1.5 kg 1.5-2.0 kg
Laying capacity per year	-
Average weight of eggs	55 g
Starts of egg laying	-
Size of population	-

- **Extremeña**

http://www.lagallinaazul.es/Extremenas.html ©

Phenotypic traits	
Average live weight -hens -cocks	 ~2.5 kg ~3.6 kg
Laying capacity per year	120 eggs
Average weight of eggs	60 g
Starts of egg laying	-
Size of population	-

- **ITALIAN BREEDS**
  - **Valdarnese Bianca**

Ceccobelli ©

Phenotypic traits	
Average live weight -hens	2.5-3 kg
-cocks	3.1-3.5 kg
Laying capacity per year	135 eggs
Average weight of eggs	60 g
Starts of egg laying	-
Size of population	-

  - **Livornese Bianca**

Phenotypic traits	
Average live weight -hens	2-2.3 kg
-cocks	2.4-2.7 kg
Laying capacity per year	280 eggs
Average weight of eggs	55 g
Starts of egg laying	-
Size of population	-

- **Modenese**

Phenotypic traits	
Average live weight -hens	1.9-2.6 kg
-cocks	2.5-3.2 kg
Laying capacity per year	-
Average weight of eggs	55 g
Starts of egg laying	-
Size of population	300

- **Romagnola**

Phenotypic traits	
Average live weight -hens	2.0 kg
-cocks	2-2.5 kg
Laying capacity per year	150 eggs
Average weight of eggs	60 g
Starts of egg laying	-
Size of population	500

- **Ancona**

Ceccobelli ©

Phenotypic traits	
Average live weight -hens	1.8-2.1 kg
-cocks	2.5-2.8 kg
Laying capacity per year	250-300 eggs
Average weight of eggs	50 g
Starts of egg laying	-
Size of population	-

- **MALTA BREED**
  - **Maltese black**

Phenotypic traits	
Average live weight -hens	2.0-2.7 kg
-cocks	2.5-3.0 kg
Laying capacity per year	120-170 eggs
Average weight of eggs	-
Starts of egg laying	-
	-

LIST OF PUBLICATIONS

During the PhD thesis I also worked on other different project related to molecular genetics and diversity:

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“We still do not know
one thousandth of one
percent of what nature
has revealed to us.”

Albert Einstein