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Endemism in Sardinia: Evolution, ecology, and conservation in the butterfly Maniola nurag
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I.
Introduction

Several ways of looking at a butterfly

When two things are different, they can not be equal; but when two things are equal they can still be different. Were I to announce to you the discovery of an animal which for the first two thirds of its life is a serpent, then weaving a veil of pure silk around its body, contracts itself into a motionless amphora without external mouth or limbs, and, after remaining in this state for a while without food or drink, bursts its silken box and flies into the sky like a winged bird, you would be amazed and bewildered to say the least. And you would not believe me; not if you were living at the turn of the 18th century, when the general public still considered butterflies to be the soul ("psyche") of the dead, and no relation was laid between the leaf eating 'worms' creeping along grasses in spring and the colourful 'summerbirds' floating above flowery fields later in the year.

It was then, in 1698, when from the port of Amsterdam a ship set sail for the Dutch colony of Surinam in South America. On board was the artist and pioneer entomologist Maria Sibylla Merian (1647-1717). After two-months of cramped, dirty sea voyage, she landed in tropical Paramaribo, Surinam. When she returned to the Netherlands two years later, she brought back a work which was both a significant scientific discovery and an aesthetic masterpiece. Her amply illustrated *Metamorphosis Insectorum Surinamensium*, published in 1705, was one of the finest examples of bookmaking of that time (Merian, reprinted 1980). She was one of the first of her era to demonstrate the metamorphosis from egg, through larva and pupa, to adult insect:

"This caterpillar has a particular smell like fruit when many different kinds are stored together. When it has reached its full size it sheds its coat or skin entirely and this it leaves hanging above it as I have illustrated. It fastens itself to a wall with its head downwards and attaches the hind part of its body as firmly as if it were glued on. In the middle of its body it spins a white thread round itself in order to stay firmly suspended. Then within half a day it turns into a date stone, in shape like a baby wrapped in swaddling clothes so that one can almost detect a human face in it...this 'date stones' are grey, in part also green in colour. In this form they hang until April or May."

Two millenniums before Merian had published her book (Merian, 1717), the metamorphosis of insects had already been described in considerable detail by
Aristotle (384-322 B.C.) He, who is often referred to as 'the first naturalist' or 'the first biologist' (Hoyt & Schultz, 1999), created an extensive body of work that proved to be the dominant view for more than two thousand years. To my knowledge, not many other natural scientists have achieved this. Aristotle reports in considerable detail on metamorphosis, and on the discovery of a fabric that could be made from the cocoons of certain types of caterpillars. This was more than eight hundred years before the true silkworm came to Greece (D'Arcy Wentworth, 1910):

"The so-called psyche or butterfly is generated from caterpillars which grow on green leaves, chiefly on leaves of the raphanus, which some call crambe or cabbage. At first it is less than a grain of millet; it then grows into a small grub; and in three days it is a tiny caterpillar. After this it grows on and on, and becomes quiescent and changes its shape, and is now called a chrysalis. The outer shell is hard, and the chrysalis moves if you touch it. It attaches itself by cow-web like filaments, and is unfurnished with mouth or any other apparent organ. After a little while the outer covering bursts asunder, and out flies the winged creature that we call psyche or butterfly. At first, when it is a caterpillar, it feeds and ejects excrements; but when it turns into the chrysalis it neither feeds nor ejects excrement."

In summary, three things that look different can still be the same. Metamorphosis means change in shape and structure. The body of an insect is extensively remodelled during the three stages from larva via chrysalis to adult; to such an extent that for a long time these three stages were considered to be three entirely different individuals.

Since the times of Aristotle we know that maggots change into flies, but until today, we are only beginning to understand why they do so. Similar to metamorphosis, 'evolution' means 'change'. But the change evolutionary biologists refer to is not only the change in external characters. In its narrowest and at the same time broadest definition, the one population geneticists use, evolution means 'change in the gene frequencies of a population in time'. Evolutionary biologists aim at answering the question 'why'. Why do larvae change into butterflies, and ultimately why is DNA the basis of the genetic code?

In this thesis, I try to combine the evolutionary question 'why' with the taxonomic question 'which' and the ecological question 'how'. A 'how' question seeks for proximate causes, e.g., how does a male individual manage to locate a female? A 'why' question seeks for ultimate causes, e.g., why has a species evolved the capacity to distinguish females of its own type? The aims of the study at hand are (1) to describe the diversity of endemics on the Mediterranean island of Sardinia,
with a specific focus on butterflies, (2) to investigate how an endemic species survives, and, from the patterns observed today, (3) to reconstruct why this species originally might have evolved, and finally (4) to describe ways to allow these species to coexist next to us while we use the land to build our economy. For this purpose, I start with a general overview of the variety of endemic species occurring in Sardinia, then zoom in to butterflies, and finally focus on a single species, which I will take as a model to explore the factors determining the differentiation of an endemic.

**Why a butterfly?**

About two millennia after Aristotle, Alfred Russel Wallace (1823-1913), who along with Charles Darwin was architect of the theory of evolution by natural selection, spent many years travelling in South America and the Malay Archipelago, supporting himself by selling specimens. His travel accounts testimony his enthusiasm for nature, in particular butterflies (Wallace, 1869).

"I have rarely enjoyed myself more than during my residence here. As I sat taking my coffee at six in the morning, rare birds would often be seen on some tree close by, when I would hastily sally out in my slippers....The next day I went again to the same shrub and succeeded in catching a female, and the day after a fine male. I found it to be as I had expected, a perfectly new and most magnificent species of Ornithoptera, and one of the most gorgeously coloured butterflies in the world. The beauty and brilliancy of this insect are indescribable, and none but a naturalist can understand the intense excitement I experienced when I at length captured it. On taking it out of my net and opening the glorious wings, my heart began to beat violently, the blood rushed to my head, and I felt much more like fainting than I have done when in apprehension of immediate death. I had a headache the rest of the day, so great was the excitement."

These few words of one of the fathers of evolutionary biology are, in a way, the explanation for why an extensive and growing body of evolutionary literature is based on research done with Lepidoptera. The ‘flying flowers’, as their Greek name πεταλοθέτες calls them, have always fascinated naturalists, they are beautiful to be with, rather easy to sample, and turned out to be good model organisms for investigating evolutionary questions. Obviously my own choice to focus on a butterfly, instead of a bat or a bumblebee, was based on these ample reasons.
The beginnings of nature conservation

In one of his gloomier moments, Diogenes the Cynic (4th century B.C.) said that, locked within their walls, men who first crowded into cities to escape the fury of those outside, committed every outrage against each other as if this were the sole object of their coming together (Lovejoy & Boas, 1965). This discontent of the civilised with civilisation arose from an emotional impulse to seek a life in harmony with 'nature' rather than a rational reflection. Civilisation, which means nothing else than 'living in cities', had appeared in Southern Mesopotamia in the late fourth millennium B.C. (Botteró, 1992). The transformation to civilization depended on the invention of agriculture, irrigation, technical development, and supervision by a literate bureaucracy (Chatwin, 1996). A 'civilised' is someone who lives within a literate urban civilisation. The opposite of it is a nomad following his animals from pasture to pasture. Dwelling within permanent domiciles allowed direct control over the immediate environment. Settlements allowed mankind to fix certain aspects of the continuously changing natural world and thereby provide a predictable environment. These urban human civilisations of the Old World spread outwards over all places and landscapes, until they became a serious problem for all other species sharing the same geographic space.

In our age, when the term 'nature conservation' has become part of everyday life, and humanity is struggling with species’ extinctions going faster than we can catalogue them, one can only look back with astonishment at the fact, that it has taken man more than two thousand years to realize, what early philosophers already seemed to intrinsically understand, that one is part of nature too, and, what is more, depends on it. By means of science and technology we now try to correct the damages industrialization and urban growth reeked. Practical technology has become the major link between humans and their environment. While today we usually divide the world into the manageable categories of the various disciplines of science and art, classical philosophy and its rebirth in the Renaissance, emphasized the interconnectedness of disciplines.

Leonardo da Vinci’s ‘universal man’ or ‘Vitruvian man’ (Figure 1) originally was not drawn to symbolize humanism in general, as it is usually viewed...
today, but represents the harmony between man and nature (Crown, 1995). The drawing is based on the assumption that the world (or cosmos) is in harmony throughout and the human body is itself an echo of that harmony. This idea of the interconnectedness of all things, whether man-made or natural, is yet another variant of present days' theories on morphogenetic fields (Sheldrake, 1995), and the superstring theory where the vibrations of a string in a 10-dimensional space are the basis of all material and life (Boyer et al., 2003). In the ancient and Renaissance perception, the concept of beauty was inseparable from theoretical science and practical engineering, as well as from the basic laws governing the universe. Human notion and the human sense of what is beautiful and what is not were integral to that whole system.

Ancient ideas of Pythagorian parallels between musical harmony, geometry, and the functioning of the entire universe have continued to influence scientists and architects over the centuries. Pythagoras found from simple observation that if two strings are set to vibrate under identical conditions, the pitch of a string will be one octave above the other if its length is half the length of the other. If their lengths are in a ratio of two to three, the tonal difference will be a fifth and so forth. The Pythagoreans were confident that they had the key to nature in their hands, and that all regularities in nature were musical. 'The music of the spheres', which according to Pythagoras accounted for the orbits of the planets, induced Johannes Kepler to develop his first heliocentric scheme in the early 17th century. Eventually, he found that the shape of the orbits was elliptic rather than perfectly circular. Nevertheless, his impulse to search for geometric principles still led him to striking discoveries about fundamental structures of the universe.

The search for order and structure and, ultimately, beauty is not only driving scientific discovery, but an eminent characteristic of humanity. Immanuel Kant dismissed the view that our minds are tabulae rasae getting filled with impressions, sense, and cultural information, but picked up the Aristotelian view that we have an innate drive to order the impressions of the world around us. Kant's theory of a continuous search for order in our environment is in rough agreement with Darwinian ideas: the mindset ensures survival in a variable and unpredictable environment.

In medieval and classical times, building was a conscious attempt to harmonize architecture with what man saw as being nature's own structural symmetries. Nowadays, most other species would surely benefit if we were more aware of our intrinsic association with the natural environment, and applied the renaissance
ideas of harmony in combination with modern technology when shaping our environment. This does not imply that our streets have to look like game trails, but suggests we should introspect and reveal the ‘universal man’ inside ourselves, and accept that we are also seated on a branch of the tree of life.

Speciation

When two things are equal, they can still be different, meaning that when two individuals look similar, they can still be genetically very different. But what are the forces that make individuals different or similar, and ultimately induce the split of one population into two or more separate entities that can in the end become separate species? (And now, I have to warn the less scientifically oriented reader, that from here onwards, things will become more and more subject specific, and you might want to consult Box I. for a first orientation in definitions of frequently used biological terms.)

Until the beginning of the 19th century, when Lamarck and Darwin developed their revolutionary ideas on the evolutionary potential of species, species were considered as fixed, non-evolving entities. Despite the great impact Darwin had on biological science, he only began to solve a few of the many questions in evolution (Darwin, 1879). In the Darwinian view, species are pictured as ‘varieties’, i.e., groups of individuals defined by differences in morphology. Darwin and his contemporaries focused mainly on explaining the evolution of phenotypic characters, while they hardly addressed the question of how barriers to gene exchange evolve. It is the latter however, that we recognise today as the essence of speciation (Futuyma, 1998). Why are species necessary? Is it, like Dobzhansky (1937) suggested, that organisms form species because the environment presents discrete ecological niches for them to fill? Different species do usually occupy different niches and have different co-adapted gene pools. However, this does not necessarily imply that speciation is only an adaptive process. Mayr (1957, 1963) suggested that reproductive isolation can evolve after genetic changes that occur for other reasons, like geographic isolation, so that speciation is an incidental, non-adaptive consequence of the divergence of populations in allopatry.

According to the biological species concept, a species is defined as an entity that is reproductively isolated from other species. Isolating mechanisms between species can be divided into three main types: (1) premating mechanisms (e.g., spatial, temporal, or ecological isolation), (2) prezygotic isolation (e.g., incompatibility of
gametes), (3) postzygotic isolation (e.g., hybrid inviability or sterility).

Genetic drift, natural selection, and geographic separation are commonly accepted as the main diversifying forces (Futuyma, 1998), that can eventually lead to the fixation of alternative advantageous alleles, i.e., differentiation and, if followed by reproductive isolation, speciation. Classic examples of speciation theories include Dobzhansky’s premise that allopatry can lead to postzygotic isolation through the accumulation of genetic incompatibilities between loci in different geographically isolated populations (Dobzhansky, 1937), and Mayr’s suggestion that bottlenecks (founder events) can produce rapid differentiation (Mayr, 1954). The founder effect hypothesis has been postulated as particularly relevant for islands: speciation could take place as a result of colonisation of a small number of, or even a single gravid female(s). An oft-cited example for such an event are the endemic Hawaiian Drosophila flies, whose enormous variety of 800 species were largely explained by a founder-induced model (Carson & Templeton, 1984).

Studying the degree of variability levels and genotype distribution within a population and compare these with other populations of the same, or a closely related species, can provide insights in the evolutionary history of that population, in that it may allow us to detect historical bottlenecks, or founder effects, and quantify the amount of gene-flow between the studied populations. These genetic data are ideally combined with demographic and ecologic information (Bossart et al., 1998). Population size, mating system, sex ratio, and distribution of individuals in a certain habitat all influence the population genetic structure (Raijmann, 1996). Therefore, in this thesis, I combine population genetic techniques with ecological field data.

**Sympatric speciation**

In contrast to the allopatric model of speciation, where complete geographical isolation is the barrier to gene-flow between two populations, sympatric speciation occurs when two segments of an originally panmictic population differentiate despite continuing gene-flow. Besides these two contrasting modes of speciation, there is another model buttressed between: parapatric speciation takes place if two populations diverge along an ecological gradient, as a result of adaptation to different ecological niches although there is a contact zone between both populations. Allopatric speciation is the null hypothesis to explain species diversity (Mayr, 1942; 1982). It is intuitively more plausible, and there are many examples for speciation under allopatric conditions (especially from islands, see for example...
Grant, 1998), but only few empirical examples of sympatric speciation. One of the most appealing ones is the case of Geospiza conirostris, the large cactus finch (Grant & Grant, 1989). Sympatric speciation is primarily driven by disruptive, frequency- or density dependent natural selection on resource use. It does not imply selection for pre-mating reproductive isolating mechanisms; such prezygotic isolation may evolve implicitly, as a result of selection for genes involved in assortative mating. Sympatric speciation initiates when in a group of individuals sharing the same resources, some of the group shift in resource preference (e.g., host plant or habitat). In such a situation, there is increasing competition among those individuals that are best adapted to the particular ecological niche the population is using because they are the most frequent ones (frequency-dependent-competition). Individuals most unlike the others experience the least competition and will therefore be favoured by natural selection (Pfennig & Murphy, 2002 and references therein). The result is that speciation is speeded up and the populations increasingly differentiate. Differentiation thus seems to be particularly incited, if the differences of the ecological niche the speciating groups of individuals occupy are small (Kondrashov & Kondrashov, 1999; Doebeli & Dieckmann, 2003; Tautz, 2003a).

After an initial subdivision of a population under sympatric conditions the following main phases of differentiation have been proposed (slightly modified from Tautz, 2003b):

I. Early stage of differentiation. Disruptive selection on traits that allow the use of alternative niches, coupled with an increasing degree of assortative mating. In this phase one would expect to find differently adapted types that mate assortatively. Most alleles are still shared between the populations, and gene-flow between different subpopulations still occurs, at least at genetic regions that are not involved in differential adaptation. This phase supposedly lasts less than a 100 generations.

II. Within a 1000 generations, morphotypes and assortative mating should become more pronounced. There is a strong reduction in gene-flow, and neutral alleles in the two subgroups increasingly become subject to independent drift, resulting in different frequencies of the alleles.

III. Within 10 000 generations, significant genetic differences build up. Alternative fixation and lineage sorting of neutral alleles take place. One will find new mutations that are a single mutational step away
from pre-existing alleles and can be used as diagnostic markers.

IV. Millions of generations after the initial split, a prediction of further evolution of adaptive characters is difficult. Additional adaptations are equally possible as relative stasis with respect to the initial adaptations. There is now a clear molecular distinction in allelic types and frequencies. Many population-specific alleles have evolved, that differ by multiple steps from previously existing alleles. The accumulation of many mutations has led to postzygotic isolation.

Schluter (1999) argues that ecology must influence speciation as the rate of speciation varies greatly with ecological circumstances. He summarizes evidence that young species with a sufficiently different ecology persist despite gene-flow, even if they have originated in sympathy. Further, he distinguished two hypotheses of how ecology drives speciation. The first suggests that speciation results from divergent selection stemming from the use of alternative environments and resource competition. Reproductive isolation evolves as a by-product of phenotypic differentiation and may involve reinforcement of prezygotic isolation later on. If divergent ecological selection induces mating preferences leading to reproductive isolation, sexual selection is a variant of ecological speciation. As a consequence, speciation rates are high in groups with adaptive radiation because reproductive isolation evolves most quickly when divergent selection is strongest. The second hypothesis proposes, partly overlapping with the first, that ecological processes mainly act on the viability of diverging populations. In novel environments species accumulate rapidly because more populations are able to avoid extinction for long enough to develop reproductive isolation. The absence of predators, parasites and competitors may for example lead to high population densities with reduced chance of extinction. This second idea is based on Mayr (1963) who underlined the importance of niche shift: "We see again and again that an incipient species can complete the process of speciation only if it can find a previously unoccupied niche (p. 574)."

Measuring the likeness of organisms

Allozyme markers
Allozyme markers still are one of the most efficient tools for detecting intra-specific genetic variation within populations, as well as inter-specific differentiation in closely related species. Due to its relatively low cost, the large number of loci that can be obtained, and its straightforwardness, this method is still being widely
Box I. Glossary of frequently used biological terminology (slightly changed after Futuyma, 1998).

Species = in the sense of biological species, the members of a group of populations that interbreed or potentially interbreed with each other under natural conditions. It is also a fundamental taxonomic unit, to which individual specimens are assigned, which often, but not always corresponds to the biological species.

Gene = the functional unit of heredity.

Gene-flow = The incorporation of genes into the gene pool of one population from one or more other population.

Genetic drift = Random changes in the frequencies of alleles or genotypes within a population.

Bottleneck = Severe temporary reduction in population size.

Mutation = An error in the replication of a nucleotide sequence, or any other alteration of the genome that is not manifested as reciprocal recombination.

Adaptive radiation = Evolutionary divergence of members of a single phylogenetic line into a variety of different adaptive forms; usually the taxa differ in the use of resources or habitats, and have diverged over relatively short interval of geologic time.

Allele = One of the several forms of the same gene, presumably differing by mutation of the DNA sequence, and capable of segregating as a unit Mendelian factor.

Allele frequency = The proportion of gene copies in a population which are a given allele; i.e., the probability of finding this allele when a gene is taken randomly from the population.

Natural selection = The differential survival and/or reproductive success of classes of entities that differ in one or more characteristics.

Founder effect = The principle that the founders of a new colony carry only a fraction of the total genetic variation in the source population.

Locus = A site on a chromosome occupied by a specific gene; more loosely, the gene itself in all its allelic states.

Character displacement = Refers to a pattern of geographic variation in which a character differs more between sympatric than between allopatric populations of two species; sometimes used to describe the evolutionary process of accentuation of differences between sympatric populations of two species, owing to interactions between them.

Dispersal = Movement of individuals or organisms to different localities; in biogeography, extension of the geographic range of a species by movement of individuals.

Disruptive selection = Selection in favour of two or more modal phenotypes and against those intermediate between them.

Divergence = The evolution of increasing difference between lineages in one or more characters.

Ecological niche = The combinations of all relevant environmental variables under which a species or population can persist; often also used to describe the resources a species utilizes.

Population = A group of conspecific organisms that occupy a more or less well defined geographic region and exhibit reproductive continuity from generation to generation; ecological and reproductive interactions among these individuals are more frequent than with other members of other populations of the same species.

Genetic variability = Variation in a trait within populations, measured by the variance that is due to genetic differences among individuals.

Homozygous = The same allele at each of the copies of a genetic locus.

Heterozygous = Different alleles at each of the copies of a genetic locus.

Reinforcement = Evolution of enhanced reproductive isolation between populations, due to natural selection for greater isolation.

Overdominance = The expression of two alleles in heterozygous condition of a phenotypic value for some characteristic that lies outside the range of the two corresponding homozygotes.

Viacariance = Separation of a continuously distributed ancestral population into separate populations, due to development of a topographic or ecological barrier.
used in Lepidoptera, so that staying with it facilitates comparisons among taxa enormously. Allozymes are electrophoretically distinguishable forms of an enzyme that are encoded by different alleles, which can be visualized with enzyme-specific staining reactions. In electrophoresis, a tissue extract or homogenate of the whole animal, as in many small insect species, is placed on a starch or cellulose acetate gel, or another medium through which proteins can move. When an electrical current is applied to the gel, the proteins move through it at a speed depending on molecule size and net electric charge. Some aminoacid substitutions can alter the net electric charge, so that variants of the same protein, encoded by different alleles, can be distinguished by their mobility. When a locus is monomorphic, all individuals exhibit the same electrophoretic mobility, when it is polymorphic, different homozygotes and heterozygotes have varying mobility, by which they can be distinguished. The banding pattern obtained on the gel can thus be interpreted to identify homozygous and heterozygous individuals, and draw conclusions about genetic polymorphism, the breeding system of individuals, population structuring, and the existence of morphologically indistinguishable species (Menken & Ulenberg, 1987; Murphy et al., 1996).

There are many statistical models to interpret genetic population structure. For allozyme data, the most important of these are the Hardy-Weinberg principle (Murphy et al., 1996 and references therein), and F-statistics (Wright, 1951; Weir & Cockerham, 1984). The Hardy-Weinberg principle states that "in the absence of selection, drift, and migration, the frequencies of alleles in a randomly mating population will maintain a stable equilibrium with genotype frequencies of \( AA = p^2 \), \( Aa = 2pq \), and \( aa = q^2 \), where \( p \) is the frequency of the allele \( A \), and \( q \) is the frequency of the alternative allele \( a \)." Deviations from Hardy-Weinberg equilibrium indicate that one or more of the assumptions are not met by the population.

\( F \)-statistics are the most commonly used indirect method to estimate gene-flow. Wright's (1951) one locus model with two neutral alleles shows that in an island model under equilibrium conditions, the among population variance in allele frequencies is \( F_{ST} = 1/(1+4N_m) \). To use allele frequency data to infer population structure requires a significant assumption, namely, that alternative alleles at a locus are selectively neutral (Kimura, 1983). Allozyme studies begin thus with neutrality as a working assumption, which thus makes the implicit assumption that evolution is mainly determined by genetic drift. This assumption holds until there is evidence for selection at a particular locus. Assuming that substitution rates in allozymes are constant over time, rough estimates of time scales for population subdivision are possible, which I will discuss below.
Measuring species' differentiation by clustering methods

Inferring the evolutionary history of a population (or species) from molecular data is based on the acceptance of a tree-like model of evolution, where ancestral characters are inherited, and the assumption that the population's evolutionary history is defined by changes in these characters (Vijverberg, 2001 and references therein). There are several methods to select the most probable evolutionary scenario among the nearly infinite set of possible phylogenies, which are either based on an algorithm that leads to the determination of a tree, or on an 'optimal criterion' that evaluates alternative phylogenies and decides which one is the 'better'. The optimal methods include the principle of parsimony, which seeks solutions that minimize the amount of evolutionary change, and likelihood methods, that estimate the actual amount of change (Swofford et al., 1996; Vijverberg, 2001). The algorithmic methods include all forms of pair-group cluster analyses, e.g. the unweighted pair-group method (UPGMA), which is based on arithmetic averaging (Sneath & Sokal, 1973), and other distance methods, e.g. neighbour-joining (Saitou & Nei, 1987). The algorithmic methods are computationally fast, but do not evaluate different trees. Consequently, the answer obtained by these methods may not be the most likely phylogeny. Algorithmic methods are therefore best used to explore the data, and find a starting tree for more thorough searches with the criterion method (Swofford et al., 1996).

In the frame of this thesis, I will rely on cluster analyses, as cluster analyses can be applied as a means of representing genetic similarity or distance data (Sneath & Sokal, 1973), this is a convenient method of exploring the allozyme data. The UPGMA method is the most commonly used clustering method and uses averages of distances within groups to determine the minimal distance between groups in building the phenogram (=tree). The tree is constructed by linking the most similar pairs of taxa, followed by successively linking more distant groups (Swofford et al., 1996). Only if the data are ultrametric, i.e., all lineages evolve at equal rates, the representation provided by the tree will be exact. It is therefore essential to keep in mind that UPGMA clusters only account for the extent of genetic similarity between groups, while the historical branching order is neglected (Swofford et al., 1996). The neighbour-joining method is conceptually related to traditional cluster analysis (Saitou & Nei, 1987), but removes the assumption that the data is ultrametric. From the original distance matrix provided, neighbour-joining constructs a modified distance matrix in which the separation between each pair of nodes is adjusted on the basis of their average divergence from all other nodes. By linking the most similar pair of nodes, an additive but unrooted tree is constructed (Swofford et al., 1996).
Estimating divergence time

In the absence of a good fossil record, as it is the case of butterflies, the only way to estimate time scales of species divergence is to infer the timescales from the amount of genetic differentiation between the species in question and its supposed ancestor. This requires the acceptance of a molecular clock hypothesis, which proposes that genes and gene products evolve at rates that are roughly constant over time and across evolutionary lineages (Arbogast et al., 2002). Although there are many controversies around this method's application (Swofford et al., 1996), molecular clocks provide at least rough, comparative estimates for evolutionary events, when there is no fossil evidence. This method has therefore substantially influenced our views on the timing of many important events in evolutionary history, in particular those related to human evolution and migration, Pleistocene speciation, and historical radiations of major groups of plants and animals (for a review see Arbogast et al., 2002). 'Local' molecular clocks, that are applied exclusively within closely related taxa or for particular genes, have been proposed to be more reliable than 'universal' clocks (Yoder & Yang, 2000). Closely related species are often expected to be similar in population size, metabolic rate, generation time, and DNA repair efficiency, which are considered the most likely sources of rate heterogeneity (Martin & Palumbi, 1993), and can consequently be expected to experience similar rates of molecular evolution. Zuckerkandl and Pauling (1965) were the first to suggest that genes and their protein products might evolve at rates constant enough to use the rates as measures of molecular divergence. Subsequent supporters of this hypothesis view molecular divergence time not as a metronome, but as a Poisson process, with regularity of the same order of magnitude as radioactive decay (Hillis et al., 1996).

It is difficult for one to find relevant data to calculate confidence limits for an allozyme (Nei's genetic, D) clock. Avise and Aquadro (1982) summarized the problem "...the major obstacle to critical tests of the electrophoretic protein clock is the almost total lack of reliable independent information about times of speciation." Nonetheless, there has been an enormous range of estimated divergence rates for Nei's D; ranging mostly between 5 and 18 million years (Hillis et al., 1996). Obviously, with such a wide range of estimates rates, any genetic distance estimate is likely to be compatible with some geological data (Avise & Aquadro, 1982). But there have been successful attempts to calculate confidence intervals on an allozyme clock (Beerli et al., 1996), which show that although the confidence levels on the clock are fairly broad, it is possible to use the predictions to exclude some biogeographic scenarios within closely related groups of organisms.
The genus *Maniola*

Butterflies of the genus *Maniola* (Lepidoptera: Nymphalidae) are highly polymorphic. Variation within species includes variability in wing-patterns within single populations as well as in different geographic areas, and ecological variability in terms of phenology, and life history. This ecological flexibility culminates in the fact that females of *Maniola jurtina* conduct a summer-diapause in the southern areas of the Palearctic, whereas in temperate climates they do not. Notably, three of the seven species in this group are island endemics: *Maniola chiachia* THOMSON 1987, from the Greek island of Chios, *Maniola cypricola* (GRAVES 1928), from Cyprus, and *Maniola nurag* (GHILIANI 1852) from Sardinia. The distribution areas of endemic and widespread species are usually mutually exclusive, except in Sardinia, where the ranges of the endemic *Maniola nurag* and the widespread *Maniola jurtina* overlap.

In Sardinia, *M. jurtina* is most abundant at sea level, but can occasionally be found up to 1000 m a.s.l. *M. nurag* flies exclusively above 500 m, and has its distributional centres around the three main mountain areas of the island (Gennargentu, Monte Limbara, and Foresta dei Sette Fratelli). At intermediate altitudes (500 - 900 m), both species are often found sympatrically (Figure 2; chapter 6).

![Figure 2](image_url)  

**Figure 2.** Distribution of *Maniola jurtina* and *Maniola nurag* on Sardinia according to elevation (m a.s.l.). The white bar indicates habitats where exclusively occupied by *M. jurtina*, the grey bar shows areas where both species occur sympatrically, the black bar indicates areas where only *M. nurag* occurs.
The most widespread species in this group is *Maniola jurtina* (Linnaeus 1758). *Maniola jurtina* is distributed throughout Europe, western North Africa, parts of the Middle East, the Irish Aran Islands to the Caspian Sea, and from central Scandinavia to the Canary Islands (see chapter 8). It occurs on all Mediterranean islands (Thomson, 1987), except the Aegean Dodecanes, the island of Chios in Northern Greece where it is replaced by *M. chia* (Thomson 1987) and Cyprus, where it is replaced by the *M. cypriocola* (Graves 1928). *Maniola telmessia* (Zeller 1847) largely replaces *M. jurtina* in southern and western Turkey, although occasionally both species’ distribution areas overlap (Van Oorschot & Van den Brink, 1992; Hesselbarth et al., 1995), and flies commonly in parts of Iran, Iraq and Syria. *Maniola halicarnassus* Thomson, 1990 is recorded from the Bodrum peninsula (Turkey) and the Aegean island of Nissiros. *Maniola megalta* (Oberthur 1909) flies in southern and western Turkey. All *Maniola* species are usually found below 1000 m a.s.l. (Thomson, 1973), with the exception of, *M. nurag*, which only flies in areas above 500 m and often occurs above 1000 m. In the southern Alps, *M. jurtina* has occasionally been observed at higher altitudes than 1000 m as well (Higgins & Riley, 1970).

**Figure 3.** A nuraghe.

**The generalist: Maniola jurtina**

*Maniola jurtina*, the meadow brown, is very common in all parts of Europe, even in highly industrialized regions like Great Britain and the Netherlands. It is most
typically found on mesophile grassland, but also in light woodland and shrubland. The meadow brown is an oligophagous phytophage with larvae feeding on various kinds of grasses (Higgins & Riley, 1970; Schmitt, 1999), particularly *Poa* spp., and *Festuca* spp. Emergence time varies according to altitude and latitude, and ranges from March (Canary Islands) to July (Scandinavia). The species is protandric. Protandry is very common in butterflies, and generally in insects with high densities, sexual size dimorphism, sex ratios biased towards males, and monandry (del Castillo & Núñez-Farfán, 2002). All these characteristics, except monandry, are displayed by *M. jurtina* (García-Barrios, 1987).

As mentioned above, in Southern Europe, females perform an imaginal diapause during the hottest part of the summer with a concomitant delayed ovarian maturation (Verity, 1953; Dowdeswell, 1961, 1962; Scali, 1971, Masetti & Scali, 1972; Scali & Masetti, 1973). During the summer diapause, females hide in the shade of bushes or trees and remain inactive until early September when temperatures drop down below the required threshold. Such aestivation behaviour has also been described for *M. telmessia* (van Oorschot & van den Brink, 1992).

**The summersleeper: biology and life cycle of *Maniola nurag***

*Maniola nurag* is a rare species *sensu* Rabinowitz (1981) in that it has a small geographic range and occupies a very specific habitat. Local populations can be large (500 - 1000 individuals), compared to other island endemics (Casula, 1999), but are still generally smaller than those of *M. jurtina* (see chapters 4 and 5). *Maniola nurag* is most probably oligophagous like *M. jurtina*, but with a narrower spectrum of potential host plant genera. It has been raised from *Festuca morisiana* (Jutze et al., 1997), but cannot be restricted to that single plant, as we did not find *F. morisiana* on any of the sites where we observed the butterfly in the field. It must at least be able to feed on several plant species in the genus *Festuca*, maybe even other grass genera. Nevertheless, it is likely, that the endemic species is more restricted in the choice of larval host plants than its widespread relative.

The field-data presented in more detail in this thesis (chapters 5 and 6) confirm earlier anecdotal evidence in the literature, that *Maniola nurag* has similar life history traits as the southern populations of *M. jurtina*, and also exhibits the above described aestivation behaviour (Simmons, 1930). Adults are on the wing from mid May to the end of September, but most easily observed during male emergence peak at the end of May, beginning of June. *Maniola nurag* is clearly protandric with males arriving 1-2 weeks earlier than females. Males emerge in a shorter
period of time and in higher numbers than females, which emerge gradually and mostly only a few days before aestivation (Scali 1971, Scali and Masetti 1973). As males die before aestivation, females have to be mated in the short pre-aestivation period. Consequently, males are patrolling in search for females. This is also the reason why males are more easily detected and caught in mark-release-recapture studies (Scali 1971, Scali and Masetti 1973). Fertilization usually occurs within a few days after emergence, mostly even within the first 24 hours after eclosion of the female. Females of *M. nurag* do not start oviposition before late summer/begin autumn. The delayed ovarian maturation could be as long as 2 months in some individuals, with a mean of over one month after copulation (Garcia-Barros, 1987).

Although obviously related to climatic factors, the exact mechanism that controls the maturation of the ovaries is still unknown. In Northern European populations, for example, the pre-oviposition period is only one week at most (e.g., Brakefield, 1982). After oviposition, the larvae of *M. nurag* start to feed only 2-3 weeks after they hatched, when the first autumn rains have fallen.

In *Maniola nurag* populations, we observed a slightly bimodal emergence pattern, which has earlier been observed in British *M. jurtina* populations (Dowdeswell, 1961; Thomson, 1971; Brakefield, 1982). Masetti & Scali (1972) observed the same in Tuscan populations. Thomson (1971) explained this phenomenon as a 'temporal sub-speciation' consisting in a bimodal pattern of appearance in both sexes. He tried to explain it with a diapause in the pupation phase of the butterfly. This explanation has received substantial and justified criticism by Garcia-Barros (1987). Following Dennis (1971) a bimodal pattern could be the result of disruptive selection against individuals emerging in the middle of the emergence period, and eventually produce total reproductive isolation of such modes. A bimodal emergence pattern would favour early emerging individuals of *M. nurag* would in years when spring is early, while late emergence would be advantageous in years with a late spring. In *M. jurtina*, Goulson (1993) did not observe any reduction of gene-flow between early and late emerging morphs of, and thus no evidence for beginning reproductive isolation. This complicated phenology behaviour is not unknown in European Satyrids (Verity, 1919), but seems especially complex in *Maniola*.

**Conservation of Maniola nurag**
Rare species are intrinsically more vulnerable to extinction than more widespread ones. A loss of fitness and increased extinction risk due to limited genetic variability has been shown for many island species (Frankenham 1997). Isolated
populations often show a reduced genetic diversity (Cassel & Tammaru 2003). Genetic diversity always decreases over time, as a natural consequence of genetic drift. Large population size counteracts drift, while small population size and inbreeding increases the level of homozygosity in the entire genome basis, which, in turn, might depress fitness as a result of the expression of partly recessive deleterious alleles (inbreeding depression) and the loss of heterozygote advantage, together with the possibility to adapt to environmental changes. Particularly island endemics are therefore often found to be limited in their evolutionary potential (Frankenham, 1997). There is no evidence in our data, that the Sardinian populations of *M. nurag* suffer from inbreeding or have historically experienced severe bottlenecks. Presently, the genetic variation in the Sardinian endemic is similarly high, as in the widespread *Maniola* species, and much greater than in other endemic Sardinian butterflies (Marchi, *et al.* 1996), so that its evolutionary potential seems intact.

Female survival over the summer diapause is a crucial parameter for the viability of *M. nurag*-populations. This ecological characteristic makes them very susceptible to human-induced change of their natural habitat (as was shown for *M. jurtina*, see Scali, 1971) and might be a special nuisance in a global warming scenario. Then imagos would possibly emerge earlier in spring but resume activity later in autumn. The consequence would be a prolonged aestivation phase that might increase the risk of female death before reproduction. It also might be that with increasing temperature individuals from intermediate altitudes move up the mountain slopes. But due to the island situation this would save them only to a limited extent, as at a certain point, they cannot go any further. Despite of showing the same life history traits in their southern populations, *M. jurtina* is, on a global scale, obviously much less prone to extinction than *M. nurag*. First, it is much more widespread and abundant and secondly does not aestivate in the Northern part of its range.

**Etymology of the epithet ‘nurag’**

The Sardinian meadow brown, *M. nurag*, is named after the prehistoric buildings we find scattered across the island, the ‘nuraghi’ (Figure 3). These megalithic towers derive from a Bronze Age culture that was present in Sardinia about 3500 ago. The name ‘nuraghe’ derives from the nuragic word ‘nur’ which means ‘hollow heap’. A nuraghe (Figure 3) is made of roughly worked stones, and might stand as a single tower with a circular base, or in a complex of many towers joined together with connecting walls. The earliest form of nuraghi is dated from 1500
B.C., these were corridor nuraghi which from the outside resembled a pile of rock. More elaborate tower nuraghi have several floors with a staircase running around the interior, each floor covered with a corbeled dome, made by stacking rocks in circular courses. Over 7000 nuraghi have been preserved up to our era. Due to their well-equilibrated structure and architecture, these buildings have remained standing for more than 3000 years, although they are not cemented but rely only on well bonded and coursed stonework. Almost all of them are situated at the top of a hill or the edge of a plateau, in a position where they overview the surrounding land. This fact together with their fortress-like character indicates that the nuraghis were built for passive defence.

Synthesis and outline

Islands have one essential similarity to nature reserves: both are surrounded by an ‘ocean’ of unsuitable habitat. In this vein, island biogeography can be applied in nature conservation (Soulé, 1986). An area that was formerly part of a continuum and is then turned into an isolated reserve will show some of the patterns we find on islands, e.g., the number of species it contains will usually depend on its size (Begon et al., 1990), the number of colonizing taxa will depend on the degree of isolation etc. As centres of endemcity, islands are a priori in the focus of nature conservation. The mountainous areas of Sardinia have been recently designated as Prime-Butterfly-Areas (Van Swaay & Warren, 2003), in a volume compiled by joint efforts from the two most important European butterfly conservation organizations, Dutch and British Butterfly Conservation Recognition of the Prime-Butterfly-Areas by legal authorities and implementation in management policies, would not only benefit butterflies, but a number of other endemic species, simply because they occur in the same areas (see chapter 2). Although my analysis does not identify butterflies as such straightforward indicators for overall species diversity (chapter 3) as they are often proposed to be (Cleary, 2003), they can serve as attractive flagship species to raise awareness of the very particular Mediterranean community they belong to, which will ultimately benefit the entire region. Butterflies have attracted people’s sympathy through the centuries, which makes them an excellent target group to stimulate attention for nature conservation. If butterflies make Sardinians and their visitors realize that their island does not only have a wonderful sea-shore, but also, landscapes with plant and animal communities, that are unique in the world, this thesis would also have a practical impact. How to incorporate Sardinian endemics in the international legal framework, and some suggestions on the establishment of nature reserves are given in chapters 4 and 5.
If we want to understand why species go extinct, we first have to understand what are the factors that initiate species differentiation and maintain their genetic diversity. Accordingly, chapters 6 and 7 give a detailed description of the ecology and genetic differentiation of the endemic satyrid butterfly *Maniola nurag*. The fact that *M. nurag* is the only endemic *Maniola* species flying in sympatry with a more widespread congeneric, *M. jurtina*, and the differential resource utilization I observed on Sardinia, gives rise to the hypothesis that the two coexisting *Maniola* species are an example of sympatric or parapatric speciation along an environmental gradient (part 3 of this thesis). Subtle niche and microhabitat differentiation could have initiated evolutionary branching, which then led to phenotypic divergence. Evolutionary branching occurs when frequency dependent selection splits a phenotypically monomorphic population into two distinct phenotypic clusters (Doebeli & Dieckmann, 2000). The genetic data suggests that the species are at a rather early stage of differentiation, comparable to the beginning of phase II of the 4 differentiation-stages proposed by Tautz (2003b) (see I. 5 of this chapter). The fact that the two species differ most conspicuously in secondary sexual characteristics is indicative of the early action of evolutionary forces on traits involved in mate recognition (Darwin, 1879), which again is evidence for a recent splitting event.

Chapter 8 illustrates morphological differences between several species in the genus *Maniola*, where I concentrate on the description of diagnostic characters in the genitalia. Here, I provide morphological evidence for the hypothesis, developed in part 3, that there are hybrids among the Sardinian individuals of *Maniola*. Finally, in chapter 9, I describe a curiosity of the genitalia structure found in a female *M. jurtina* from Amsterdam.