Hybridization and genetics of the Swiss *Emys orbicularis sp*. populations

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Abstract

Hybridization between subspecies has recently become an important concern for Conservation Genetics, as hybridization may occur between rare and common related species and between two genetic groups from different origins, resulting in hybrid offspring that may be not adapted to the local conditions (outbreeding depression). As the European Pond turtle (*Emys orbicularis sp*., Linnaeus 1758) is planned to be reintroduced in Switzerland, it is therefore crucial to estimate if hybridization occurs between subspecies to avoid introgression of foreign introduced individuals into the genetic pools of Swiss relict populations. Potential sites in cantons Thurgau, Aargau and Bern were investigated with traps in order to determine if Swiss relict populations still exist in Switzerland and samples from individuals found during the last decades were analysed to define their putative location of oprigin. Hybridization between three subspecies (*E. o. orbicularis, E. o. hellenica* and *E. o. galloitalica)* in a natural contact zone (Southern France) was compared to hybridization in an artificial contact zone (Moulin-de-Vert, Switzerland) and estimated with the use of the maternally inherited cytochrome *b* gene and of bi-parentally inherited microsatellites. No Swiss relict population was reported and all the found individuals harboured an allochthonous origin, thus indicating that all the Swiss *Emys orbicularis* populations harbour allochthonous individuals and that no relict individual is reported. Genetic analyses on the populations from Southern France and Moulin-de-Vert showed strong introgression between subspecies, indicating therefore successfull hybridization between subspecies *E. o. orbicularis, E. o. hellenica* and *E. o. galloitalica*.

1. Introduction

1.1. Conservation genetics

Humans have an increased impact on biodiversity since the last few centuries by destroying and fragmenting landscapes and habitats, as well as by direct destruction of populations mainly through hunting, fishery and consumption (Frankham *et al*., 2002). Populations may then become fragmented, isolated and may suffer from bottlenecks (temporary reduced demography with reduced genetic variability) and reduced gene flow within and between populations with a decrease in genetic variability and fitness (Bushar *et al*., 1998). A decreasing genetic diversity may then worsen because of genetic drift and inbreeding, contributing to further loss in genetic diversity, to inbreeding depression and to the extinction of the local population (Lande, 1988; Jimenez *et al*., 1994; Saccheri *et al*., 1998; Crnokrak and Roff, 1999).

Populations with low genetic variability will be therefore less able to respond to changes in their environment, such as the rise of new pathogens and parasites (Hebard, 1994), the loss of parts of their ecological niches and the needs to shift on new resources and to adapt to climate changes, etc.

As a consequence, conservation genetics aims at the preservation, management and restoration of genetic diversity, an important factor for evolution. Genetic studies have become important to preserve and manage genetic diversity in endangered and rare species (Franckham *et al*. 2002. An important attention has recently been given to preservate and restorate genetic diversity since the discovery of a positive correlation between genetic diversity and evolution (Dlugosch and Parker, 2007), individual fitness (McAlpine, 1993; Reed and Frankham, 2003; Da Silva *et al*., 2006; Pitcher and Neff, 2006) and population's viability (Elridge *et al*., 1999).

Conservation genetics involves various research disciplines including population genetics, molecular ecology, systematics and evolutionary biology. Combination of these disciplines allows conservation geneticists to identify small and isolated populations susceptible to genetic drift and extinctions, to minimise inbreeding and loss of genetic diversity, to resolve population structure and taxonomic uncertainties, to define management units within species, to identify suitable individuals and populations for reintroductions, to understand species' biology and to detect hybridization between rare and common related species (Frankham, 2002).

1.2. Hybridization in conservation genetics

Hybridization has recently become an important concern for Conservation Genetics, particularly since the discovery of introgressed genomes of rare species by common related species, the improvement of molecular techniques and efficient genetic markers (microsatellites, allozymes, DNA fingerprints and RAPDs) and the development of statistical resources (Wendel and Percy, 1990; Gottelli *et al*., 1994; Allendorf and Waples, 1996; Haig and Avise, 1996; Young and Murray, 2000; Randi, 2008).

By modifying the environment, Human alters physiogeographic and climatic barriers. As a consequence, previously isolated taxa may meet together in contact zones (or hybrid zones) and may reproduce, resulting in hybrid offspring. Introgression happens when hybridization occurs between 2 genetic groups, resulting in hybrid offspring. Repeated introgression into one gene pool may lead to the loss of genetically rare species, subspecies or populations.

As hybrids harbour new combinations of traits from 2 genetic groups, they may be differently affected in their individual fitness depending on the degree of taxonomic differentiation between both parents (species vs. subspecies vs. population). Moreover, complex interactions involving additive and dominant effects within genomes may provide advantages or disadvantages to the offspring depending on the manner of how parts of the genome are transmitted (Frankham *et al*., 2002).

New combinations of genes may be advantageous in an environment in which both pure parents are non-adapted: new resistance to parasites and new behaviours for the exploitation of new resources or habitats allow hybrid individuals to become more adapted to a novel environment than both pure parents. However, new combination of traits may also have negative impacts, especially when both parents are specialized on 2 different environments or resources. Hybrids are thus not able to exploit neither one nor the other environment or resources on which pure groups are specialised (Frankham *et al*., 2002). Furthermore, repeated introgression of locally adapted individuals by foreign genes may lead to the loss of ecotypes and of local adaptations. Such a case may finally result in outbreeding and outbreeding depression. Moreover, sterile offspring may result when highly differentiated taxa (such as different species) hybridize.

1.3. Ecology of *Emys orbicularis sp*. (Linnaeus, 1758)

Emys orbicularis sp. (Linnaeus, 1758) is the unique Palearctic member of the Neoarctic family *Emydidae* (Fritz, 2003) and was considered as a monotypic species for decades (Boulenger, 1889; Wermuth and Mertens, 1961, 1977; Ernst and Barbour, 1989). However, this species appears now to be one of the most genetically fragmented vertebrates of the Western Palearctic (Lenk *et al*., 1999; Fritz *et al*., 2005a, 2009).

The European Pond Turtle lives in a mosaic of various habitats with permanent waters and wetlands, with shoreline vegetation mainly consisting of *Phragmites sp*., *Nymphaea alba* and *Nuphar lutea* conferring shelter, food and easy access to terrestrial land (Bodies *et al*., 2000; Ficetola *et al*., 2004).

Terrestrial habitats have long been neglected for freshwater turtles, but are of a primary importance as biological corridors between various wetlands and for providing shelter during periodic drought and during seasonal migrations from residential ponds towards overwintering and nesting sites (Ficetola *et al*., 2004; Gibbons, 2003; Utzeri and Serra, 2001). Optimal nesting sites should be exposed southwards, with open sandy dry areas and meadows with shallower ponds close to nesting sites. Distances from residential ponds to nesting sites reach usually 1-2 km (Andreas, 2000; Fritz and Gunther, 1996; Lebboroni and Chelazzi, 1998; Meeske, 2000; Naulleau, 1992; Roe and Georges, 2007; Rovero and Chelazzi, 1996; Schneeweiss and Steinhauer, 1998) but may extend to 3-4 km (Jablonski and Jablonska, 1998). Such distances are considerable in comparison with other freshwater turtles (Semlitsch and Bodie, 2003).

The presence of basking sites constituted of partially immersed dead wood, or located on the rivers' and ponds' banks, as well as the vicinity of nesting sites are considered as the limiting resources for *Emys orbicularis sp*. (Fritz, 2001). Pollution of its habitat does not seem to be problematic for the short-term survival of this species (Balàzs and Györffy, 2006; Kovàcs *et al*., 2004, Cheylan, pers. comm.). However, shell and plastron anomalies are more often observed in polluted than in non-polluted areas (Balàzs and Györffy, 2006). Pollution may thus have an impact on embryonic development and maybe on the reproductive success of *E. orbicularis*.

Sexual maturity is reached at the age of 8-12 years (depending on the locations), or when carapaces' length is 110-145mm for males and 118-170mm for females (Fritz, 2001). *E. orbicularis* has long been considered as a strictly carnivorous species (Lanza, 1983; Ernst and Barbour, 1989), but recent studies have since demonstrated a shift from a more carnivorous diet before the breeding season and at juvenile stadium, towards a more herbivorous diet after the breeding season and with increasing ageing (Lebboroni and Chelazzi, 1991, 1998; Ficetola and de Bernardi, 2006; Ottonello *et al*., 2005), as do other *Emydidae* species (Hart, 1983).

1.4. Genetic differentiation in glacial refugia and Holocene expansion

During Pliocene climatic changes (-7My – 1.8My), disparition of Parathethys Sea and apparition of marked seasons are thought to have played an important role in the adaptive radiation of *E. orbicularis* into different haploclades (Fritz, 1995b; 1998; 2003; Lenk *et al*., 1999). Molecular clock of 0.4% divergence/Myr. in *Testudinoïdea*, based on 40-90 restriction sites in mtDNA (Avise *et al*., 1992), estimates differentiation into haploclades I and III to X to have occured for 3.0 – 4.1 Mya., while haploclade I gave the offshoot haploclade II during a more recent Pleistocene vicariant event (Fritz *et al*., 2005b, 2009).

During Pleistocene (-1.8 My. – 10'000y. BC), climatic oscillations have consisted of glacial periods forcing species to retreat into southern refugia and of warmer interglacial periods allowing them to expand northwards. *E. orbicularis* was thus restricted to favourable refugia along the Mediterranean sea coasts, in Southern Balkans, Southern of the Caspian Sea, in Anatolia and in Caucasus. Because of geographic barriers, such as seas and mountains, populations were fragmented and isolated from each other. Isolation and absence of gene flow between populations thus led to genetic differentiation of these populations. As a consequence, retreated and isolated populations of *E. orbicularis* evolved separately into various haploclades (Fritz *et al*., 2003, 2009), as described in Table 1.

Table 1: Haploclades and *Emys orbicularis sp*. following Lenk *et al*., 1999; Fritz *et al*., 2005a, 2009.

Haploclade	Subspecies
I	*E. o. orbicularis* (+ *E.o. luteofusca*) and *E.o colchica*
II	*E. o. orbicularis*
III	*E. trinacris*
IV	*E. o. hellenica*
V	*E. o. galloitalica* (*E.o. galloitalica sensu stricto*, *E.o. lanzai*, *E.o. capolongoi*)
VI	*E. o. occidentalis* (*E.o. occidentalis sensu stricto*, *E.o. hispanica*, *E.o. fritzjuergenobsti*)
VII	E. *o. iberica* and *E. o. persica*
VIII	undescribed subspecies
IX	one specimen from pet trade in Germany
X	*E. o. eiselti*

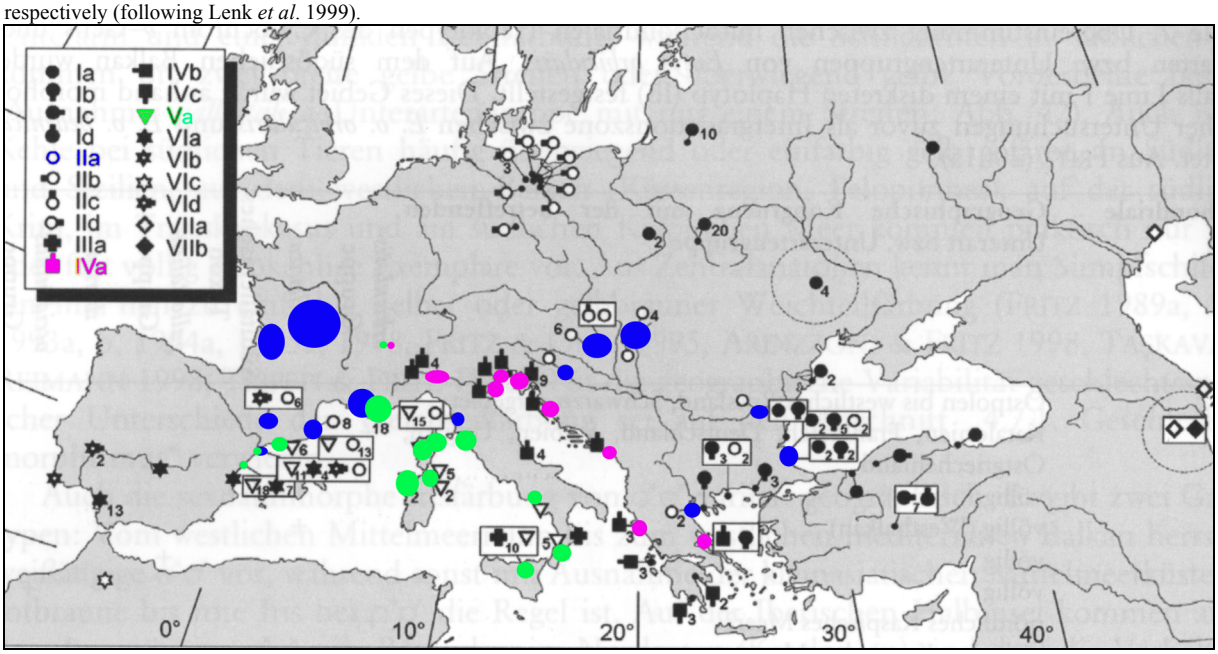
During the Holocene range expansion (-15'000y. BC) northern parts of Western and Central Europe were recolonized by turtles harbouring haploclades I and II from their respective glacial refugia in the Northern parts of the Black Sea / Caucasus region and Southern Balkanic peninsula (Fritz, 2003; Fritz *et al*., 2005b, 2007; Joger *et al*., 2006; Sommer *et al*., 2007, 2009). Indeed, most refuges have not contributed equally to the recolonization of Europe: northwards expansion of lineages III, V and VI was hindered by geographic barriers such as the Alps, the Apennines and the Pyrenees.

On the contrary, lineages I and II recolonized the European biota using biological corridors, such as marshlands and rivers' courses (Fritz *et al*., 2007). Lineage II spread northwards probably along the valleys of the Vardar and Jozna Morava rivers to finally reach the Danube catchment basin, from which Central and Western Europe were then recolonized. On the contrary, Germany and Poland were reached via the Moravian Gate and the Oder river basin (Lenk *et al*., 1999; Fritz, 2003; Fritz *et al*., 2007). Northwards expansion of haploclade IV was blocked by the Alps, but coastal regions are thought to have allowed expansion towards southern France (Fritz *et al*., 2007a).

1.5. Current distribution of haploclades

Because of past restriction and expansion events during the climatic changes durin the Pliocene and the Pleistocene, *E. orbicularis* are currently found from North-Western Africa over a large part of Europe and Asia Minor to the Caspian and Aral Seas (Fritz 2003, Fritz *et al*, 2005b). In Europe, haploclade II (*E. o. orbicularis*) individuals are located in the Danube and Oder rivers catchment basins, as well as in the Balkanic Peninsula, Southern France and Northern Spain (haplotype IIa), and extend to Northern Europe (Lenk *et al*., 1999; Fritz *et al*., 2005b), as illustrated in Figure 1.

Fig 1: Current distribution of the main haplotypes with the repartition of haplotypes IIa, IVa and V highlighted in blue, pink and green respectively (following Lenk *et al*. 1999).



Haplotype Va is considered as one megasubspecies including *E. o. galloitalica* sensu stricto, *E. o. capolongoi* and *E. o. lanzai*. Although no difference on cytochrome *b* gene is detected between them, they differ significantly in 19 morphological characters and habitat choice (Fritz *et al*., 2009). The megasubspecies *E. o. galloitalica* sensu lato is reported on the Western Apenine Peninsula, in Sardinia and in Corsica. Haplotype IV has mainly a circumadriatic distribution on the estern coast of Italy and of the Balkanic Peninsula and is therefore not native in France (Fig. 1).

In France, only haploclades II and IV are considered as native subspecies: haplotype IIa is located in Aquitaine, Centre-Val de Loire and Rhône-Alpes regions and ocurr syntopycally in the hybrid zone of Camargue with haplotype Va (Fritz *et al*. 2005b), which is mainly is restricted to the Mediteranean coast of Southern France and Corsica (Fig. 1).

1.6. Swiss relict populations

Subfossil findings (10'000 years) indicate the presence of *E. orbicularis sp*. in Central Europe (Fritz, 2003; Sommer *et al*., 2007, 2009) and in Switzerland (Stampfli, 1983; Fritz, 2003; Sommer *et al*., 2007). Moreover, historic sources attest the presence of relict populations of this species on the Swiss Plateau until at least the 18th centuries (Fatio, 1872; Fritz, 1995c, 1996; Kinzelbach, 1988). However, later reports (Fischer-Sigwart, 1893, 1896; Göldi, 1914; Fejérvàry 1920; Steinmann, 1923; Zschokke, 1928; Aellen and Perret, 1953; Kramer and Stemmler. 1988) should be taken with caution: number of turtles were already translocated during the Middle Ages from various European regions in Europe as Lent meals and more generally, for Human consumption (Friedel, 1868; Brockmüller, 1876; Dürigen, 1897; Dahms, 1906; Schneeweiss, 1997; Kinzelbach, 1988).

Human's impact has been an important cause of extinction of the native *E. orbicularis sp*. in Central and Western Europe (Fritz, 2001): populations from Northern France, Switzerland, Netherlands, Belgium, as well as from parts of Austria, Germany and Slovakia have certainly become extinct because of a large consumption by Humans (Dürigen, 1897; Schneeweiss, 1997) and because of deterioration and loss of its habitat and of nesting sites (Klemens, 2000; Fritz, 2003; Kovàcs *et al*., 2004), as well as because fishery for large Human consumption. However, populations from Northern parts of Europe (Sweden, Britain and Denmark) are supposed to have collapsed because of local climatic deterioration after the early Subboreal (small ice age, 3750–1750 BC) (Sommer *et al*., 2007, 2009).
Additional threats are its limited capacities to recover high densities of populations after bottlenecks, as well as its delayed sexual maturity and the high juveniles' mortality due to high predation rate (Iverson, 1991; Congdon, 1993; Kovàcs *et al*., 2004; Lanski *et al*., 2006). Pet trade (Klemens, 2000), fire (Cheylan and Poitevin, 1998) and roads (Smith and Dodd, 2003) also seem to may also affect the species in otherparts of the range.

Despite native Swiss populations are supposed to be extinct (Hotz and Broggi, 1992; Grossenbacher and Hofer, 1994; Fritz, 2003; Fritz *et al*., 2004), casual observations are reported in several Swiss regions (on the Southern bank of lake Neuchâtel, in lake Geneva, in Hallwilersee, as well as in cantons Aargau, Thurgau, Tessin and Geneva (Centre Suisse de Cartographie de la Faune, pers. comm.; Monney and Meyer, 2008). Genetic studies previously demonstrated a mixture of individuals in the natural reserve of Moulin-de-Vert (canton Geneva), including allochthonous haploclades IV (*E. o. hellenica*) and V (*E. o. galloitalica*), as well as the autochthonous haplotype IIa (*E. o. orbicularis*). The latter *E. o. orbicularis* subspecies is considered as native in Switzerland Northern of the Alps (Fritz, 2003). Furthermore, individuals from Tessin harbour a mixture of haplotypes IIa, IVa and Va, while their expected native subspecies should harbour haplotype IVa, the same as occurring in the Po plain. However, previous genetic studies (Fritz, pers. comm.) were not able to determine if individuals harbouring haplotype IIa are indigenous or released pet animals.

One method to investigate various origins of individuals harbouring a same haplotype is to combine various genetic markers, including nuclear genetic markers, carrying information on the origins of both parents.

1.7. Genetic markers

Genes' content and order (without any introns) are particularly conserved in mtDNA and are poorly subject to recombination (Avise, 1994; Boore, 1999; Parham *et al*., 2006a). Mitochondrial genes are

present in multiple copies per cell and harbour fast and slowly evolving genes, such as the Cytochrome *b* gene (Cyt *b*) that evolves slowly.
Cyt *b* gene has a very low evolutionary divergence rate of 0.3-0.4% / Myr. in Testudines (depending on the taxa) in comparison to the mtDNA clock of 2% divergence / Myr. described for mammals and birds (Vawter and Brown, 1986 ; Lamb *et al*., 1989; Avise *et al*., 1992; Caccone *et al*.1988).
Mitochondrial DNA (mtDNA) is an important tool to infer phylogeography and taxonomy of reptiles (Brown and Pestano, 1998; Burbrink *et al*., 2000; Surget-Groba *et al*., 2001) and in turtle species (Meyer *et al*., 1990; Norman *et al*., 1994; Osentoski and Lamb, 1995; Walker *et al*., 1998; Lenk *et al*., 1999; Van der Kuyl *et al*., 2002; Austin *et al*., 2003; Fritz *et al*., 2006, 2009; Spinks *et al*., 2010). Because of its only matrilineal inheritance, its use is limited when inferring more complexe evolutionary processes such as hybridization, migration events and genetic diversity. As a consequence the study of such processes requires additionnal markers, including nuclear markers (e.g. microsatellites) that are both maternally and paternally inherited.

Microsatellites (or Simple Tandem Repeat (STR) loci) are parts of the nuclear genome, and are thus bi-parentally inherited markers (except when associated with sexual chromosomes) These codominant markers are non-coding repeated sequences of only a few base pairs have a high mutation rate (Engstrom *et al*., 2007) allowing precise investigation of population structure, especially when inferring intraspecific genetics (Ciofi *et al*., 2002; Kuo and Jensen, 2004; Engstrom *et al*., 2007; Ursenbacher *et al*., 2009), in conservation genetics (Cunningham *et al*., 2002; Velò-Antòn *et al*., 2007, 2008), in paternity and mating systems (Valenzuela, 2000; Roques *et al*., 2004), as well as in interspecific hybridization (Roy *et al*., 1994, 1996; Williams *et al*., 2005), forensic investigations and poaching determination (Manel *et al*., 2002) and to relocate individuals from unknown origin to their supposed native location (Olsen *et al*., 2000; Velò-Anton *et al*., 2007).
Testudines‘ genomes being conservative, primers can be developed for one species and then be used for cross-species amplification (Avise *et al*., 1992; FitzSimmons *et al*., 1995; King and Julian, 2004).

1.8. Aims of the study

As *E. orbicularis sp*. is planned to be reintroduced in several countries such as France, Switzerland and Italy (Cleylan and Mazzotti, pers. comm.), it is crucial to determine if reintroduced individuals will hybridize with other *E. orbicularis sp*. previously released into the wild, or if pre- (mating choice preference, incompatible sexual organs, etc.) or post-copulatory mechanisms (biochemical incompatibilities) are involved in te to avoid hybridization between different *E. orbicularis sp.* However, as hybridization is already reported under captive conditions, it remains unknown if natural populations will have a mating preference for their own subspecies, or if they reproduce with other *E. orbicularis* subspecies.
This study will compare hybridization between subspecies in a natural contact zone (Camargue, France) to hybridization occurring in an artificial contact zone (Moulin-de-Vert, Switzerland). If hybridization occurs between subspecies, care should be made when reintroducing individuals to locations where previously different subspecies had been released in order to avoid potential negative effects of hybridization.

Additionally, a trapping session in various Swiss cantons will be planned to determine if Swiss relict populations of *E. orbicularis sp.* are still present on the Plateau, or if native populations have become extinct. As a few individuals are occasionally observed, and because this species has a very discrete way of life, it remains possible that the few observed European pond turtles are parts of unidentified populations. Several sites with previous observations of the European pond turtle will be thus investigated during several days with the installation of efficient traps in the water.

If relict populations are found, it will be important to not reintroduce *E. orbicularis sp.* in these relict populations, to preserve and manage their Swiss genetic characteristics and their environment. However, is such populations are isolated and harbour only low genetic variability, it will be important to reinforce populations to avoid inbreeding depression in these populations.

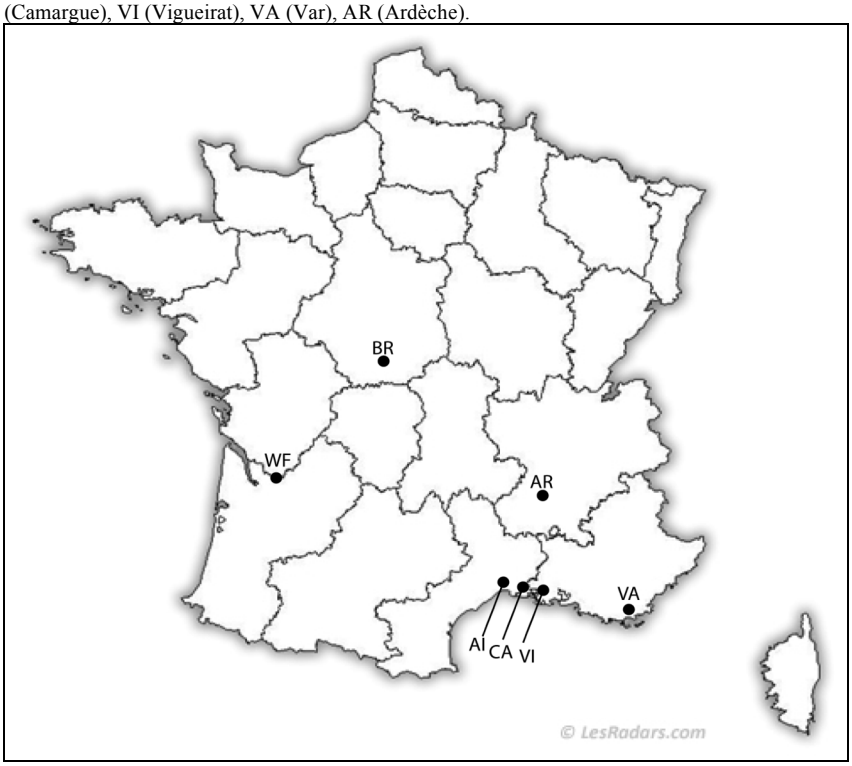
Moreover, several individuals found in various localities in Switzerland during the last decade will be analysed in order to define their origin origin.

2. Materials and Methods

2.1. Sampled sites

Sampled sites in France were located in the regions Brenne and Camargue (Fig. 2).

Fig. 2 : Sampling locations in France: BR (Brenne), WF (Western-France), AI (Aigues-Mortes), CA (Camargue), VI (Vigueirat), VA (Var), AR (Ardèche).



Investigated sites in Switzerland are restricted to natural reserves in the cantons Geneva, Thurgau, Aargau and Bern (Fig. 3). Only one site in canton Geneva harboured potentially favourable environmental conditions with one already-reported population. Sites in cantons Thurgau and Aargau were choosen because of favourable climatic conditions and lower Human density than other cantons of the Swiss Plateau, as well as because of the potentially favourable environmental conditions provided by natural aquatic reserves and the report of isolated individuals. Furthermore, one site in canton Bern was investigated because of potentially favourable environmental conditions and the report of a population of *E. orbicularis* in 1800. General informations (presence of shoreline vegetation and of nesting sites, potential problems and management of the site, potential for a future reintroduction of *E. orbicularis* and durability of populations in this site) were also reported for each

investigated site. However, these general informations are indicative and should not replace precise measures such as temperatures of soil and water, irradiation rate, etc. that should be the subject of an additional research study.

Moulin-de-Vert (46° 10' 30'' N, 4° 26' 0'' O)
This natural reserve is located near Cartigny in the canton Geneva, and occupies a previous oxbow lake of the Rhône river, that was dried out in 1940 during river's rectification. The site has been considered as of national importance since 1956. Numerous species have since been released there, including *E. orbicularis sp*. (Dändiker, pers. comm.) The release of numerous pond turtles from various parts of Europe has led an important artificial contact zone between different subspecies. Investigation of the site was made between the 2nd and the 4th of July 2009.

Bommerweiher (47° 37' 07'' N, 9° 9' 18'' O)
This site is a Pro Natura natural reserve located in canton TG and is constituted of 2 medium-sized ponds. Investigation of the site was made between the 20th and the 23rd of July 2009.

Lengwilerweiher (47° 37' 0'' N, 9° 11' 0'' O)
This site is a Pro Natura natural reserve located in canton TG is constituted of 2 small ponds. Investigation of the site was made between the 20th and the 23rd of July 2009.

Hudelmoos (47° 33' 00'' N, 9° 18' 00'' O)
This site is a Pro Natura natural reserve located in canton TG is constituted of 2 small ponds and was investigated between the 24th and the 25th of July 2009.

Tobelweiher (47° 32' 0'' N, 8° 51' 0'' O)
This site is a group of 3 small-sized ponds located in canton TG, with only few shoreline vegetation and too much shadowed. Investigation of the site was made between the 26th and the 29th of July 2009.

Seebachtal (47° 35' 9'' N, 8° 54' 44'' O)
This site is a Pro Natura natural reserve located in canton TG and englobes 2 large ponds (Hüttwilersee and Nussbaumersee) and 1 medium-sized pond (Hasensee). Investigation of the site was made between the 26th and the 29th of July 2009.

Schinznach am Bad – Brugg (47° 29' 12'' N, 8° 12' 30'' O)
This transect on the Aar river in canton AG was investigated between the 4th and the 6th of July 2009.

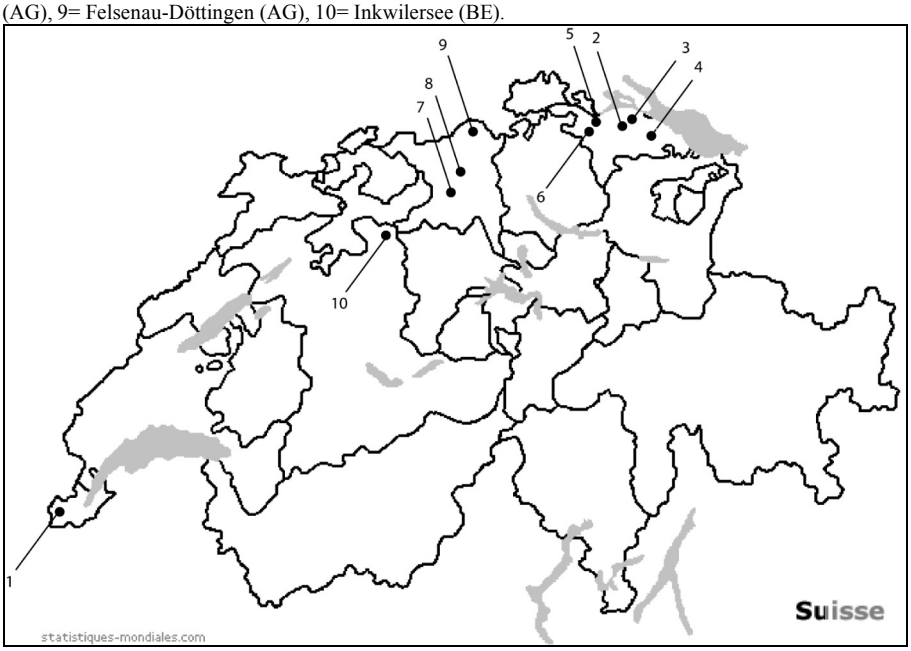
Schinznach am Bad – Wildegg (47° 25' 0'' N, 8° 11' 0'' O)
This transect on the Aar river in canton AG was investigated between the 4th and the 6th of July 2009.

Rohr – Biberstein (47° 24' 55''N, 48° 5' 0'' O)
This renatured transect on the Aar river in canton AG was investigated between the 6th and the 8th of July 2009.

Felsenau – Döttingen (47° 35' 3'' N, 8° 14' 12'' O)
This transect on the Aar river is located at the junction with the Rhin river, at the boundary with Germany. Investigation of the site was made from the 10th to the 14th of July 2009.

Inkwilersee (47° 12' 11'' N, 7° 40' 12'' O)
This site is a medium-sized pond and was investigated between the 17th and the 19th of July 2009.

Fig. 3: Investigated sites in Switzerland: 1= Moulin-de-Vert (GE), 2= Bommerweiher (TG), 3= Lengwilerweiher (TG), 4= Hudelmoos (TG), 5= Seebachtal (TG), 6= Tobelweiher (TG), 7= Rohr-Biberstein (AG), 8= Transects between Schinznach am Bad - Brugg and Schinznach am Bad - Wildegg (AG), 9= Felsenau-Döttingen (AG), 10= Inkwilersee (BE).



2.2. Trapping sessions

Trapping session lasted 2 days in very small sites (Hudelmoos) to 4 days in large sites (Brenne, Camargue, Seebachtal and Felsenau-Döttingen). The use of 29 conical fishing baskets placed perpendicularly to the bank allowed to trap the turtles. Empty plastic bottles were placed in the traps (controlled each day) to prevent them to fall into the water. Animals were photographed, marked with a unique combination of marginal scute notches, following the previous study led in this site (Mosimann, 2002). Following parameters were recorded: weight, plastron length and width, sex and numbers of the trap where the individual has been caught. Animals were then immediately released at their exact site of capture.

2.3. Origins of the samples

Samples come from different populations of *E. orbicularis sp*. from France (Brenne, Western France, Aigues-Mortes, Camargue, Vigueirat, Var and Ardèche), Switzerland (Moulin-de-Vert and founds from different localities) and Hungary (Szeged, coordinates 46° 25’ 5’’ N, 20° 14’ 5’’). All the samples are summarized in Table 4.

2.4. Laboratory analyses

DNA sampling
Sterile buccal swabs (Sarstedt, Italy) were used to obtain oral mucus. This fast and efficient method was set up for tortoises by Poschadel and Möller, 2004 and allows high amounts of mucus to be taken. Cotton swabs are introduced into the oral cavity and spinned at 360 degrees, mainly underneath the

tongue, where mucus pockets are located. Cotton swabs were then stored at 5°C before long-term storage at – 20°C.
Additional samples were provided as blood and claws.

DNA isolation
Total genomic DNA was extracted from blood, buccal swabs and claws after an initial incubation at 56°C in lysis buffer ATL (Qiagen) during 4, 12 and 48 hours resp. (with a subsequent addition during the incubation of 20µl Proteinase K (Qiagen) to improve extraction from claws). Further steps are described in the DNeasy Blood and Tissue Handbook provided by Qiagen (2006). DNA quantities were then estimated with Nanodrop.

PCR conditions
PCR reactions were conducted in 25µl volumes with 2µl DNA template, 8µl sterilized dH_2O, 12.5µl MasterMix (MasterMix Kit, Qiagen) and 1.3µl of each primer. Microsatellites msEo2, msEo21, msEo29 and msEo41 were developed by Pedall *et al*. (2008), while microsatellites GmuB08, GmuD51, GmuD87, GmD93 and GmuD114 were developed by King and Julian (2004). Forward primers were labelled with fluorescent dye. Amplification of Cyt *b* was performed with primers mt-A and H15909 were developed by Lenk and Wink (1997) and Lenk *et al*. (1999), respectively.
Amplification of microsatellites and cytochrome *b* were carried out in PTC100 and Eppendorf thermo-cyclers with following optimized conditions (Table 2).

Table 2: Optimized conditions for microsatellites loci and cytochrome *b* gene, with indications on the number of repeat fragments for each microsatellite and forward and reverse primers' sequences. $T°_D$ refers to the temperature of denaturation, $T°_A$ to the annealing temperature and $T°_E$ to the extension temperature. T_D indicates the time for denaturation, T_A the time for annealing and T_E the time for extension.

Locus	Repeat	Primer	Sequence 5' → 3'	PCR conditions						
				$T°_D$	T_D	$T°_A$	T_A	$T°_E$	T_E	Cycles
msEo2	$(CA)_{15}$	msEo2f	F: TTC AAA CCA ATC CGA TGA GG	94°	45'	58°	45'	72°	60'	37
		msEo2r	R: GCC TTT CTA TGA AAT GCT ACA TG	94°	45'	58°	45'	72°	60'	
msEo21	$(GA)_{11}$	msEo21f	F: GTA GTA ACC CAC TTG ATG AG	94°	45'	58°	45'	72°	60'	37
		msEo21r	R: TTA CCT GGC AAT TAC CTG GC	94°	45'	58°	45'	72°	60'	
msEo29	$(CT)_{14}$	msEo29f	F: ACT TCA TCG GAT GCA TGA AG	94°	45'	58°	45'	72°	60'	37-40
		msEo29r	R: ACT TTT GGA CTA CTG CAG CC	94°	45'	58°	45'	72°	60'	
msEo41	$(ATCT)_{17}$	msEo41f	F: ACT TTT GGA CTA CTG CAG CC	94°	45'	58°	45'	72°	60'	37
		msEo41r	R: AGC CAG AAC TAT GGG GGT G	94°	45'	58°	45'	72°	60'	
GmuB08	$(TAC)_{10}$	GmuB08f	F: CTC TGA GAC CCT TAT TCA CGT C	94°	45'	58°	45'	72°	90'	37
		GmuB08r	R: AGC CTT TGT CTG TAA GCT GTT C	94°	45'	58°	45'	72°	90'	
GmuD51	$(ATCT)_{52}$	GmuD51f	F: GTT GGG CAC TAG ATA GAT TCG	94°	45'	58°	45'	72°	90'	37
		GmuD51r	R: CAT TCA AGT CAA CGG AAA GAC	94°	45'	58°	45'	72°	90'	
GmuD87	$(ATCT)_{22}$	GmuD87f	F: AAA CCC TAA GAC ATC AGA CAG G	94°	45'	58°	45'	72°	90'	37
		GmuD87r	R: CAA ATC CAG TAC CCA GAA AGT C	94°	45'	58°	45'	72°	90'	
GmuD93	$(ATCT)_{18}$	GmuD93f	F: AGA CTC TCT TGA CCA GAT TTT CTC	94°	45'	58°	45'	72°	90'	37-40
		GmuD93r	R: TCT GCC TTC TAT CAC TCT CCT G	94°	45'	58°	45'	72°	90'	
GmuD114	$(ATCT)_{13}$	GmuD114f	F: ATA GAC ATA GTG CAT ATA GAC ATA GCC	94°	30'	58°	30'	72°	30'	37
		GmuD114r	R: ACG TTC TTG CAG GGT CAG AG	94°	30'	58°	30'	72°	30'	
Cyt *b*	-	mt-A	F: CAA CAT CTC AGC ATG ATG AAA CTT CG	94°	30'	60°	30'	72°	45'	37-40
		H15909	R: AGG GTG GAG TCT TCA GTT TTT GGT TTA CAA GAC CAA TG	94°	30'	60°	30'	72°	45'	

2.5. Null alleles

Null alleles in the population have been investigated using the estimator r_c, assuming that null homozygotes are not in the sampling, and the estimator r_b, assuming null homozygotes to be present in the sampling (Chakraborty *et al*. 1992; Brookfield, 1996).

2.6. Observed (Ho) and expected (He) Heterozygosities

Fixation indices F_{IS} and F_{ST} (Wright, 1921) were investigated first with GENALEX 6 (Peakall and Smouse, 2006) with significance of the results tested by the Markov chain method estimating the exact p-value. F_{IS} and F_{ST} were secondly investigated with FSTAT version 2.9.3.2 (Goudet, 2002). Investigation of F_{IS} and F_{ST} were performed in order to determine a deviation of the panmixy within subpopulations (F_{IS}) and within total population (F_{ST}) (Wright, 1921; Frankham, 2002) to measure genetic differentiation between populations by using allele frequencies and identity.

2.7. Allelic richness

Allelic richness was estimated for each locus for each population by counting the alleles and was then analysed with FSTAT version 2.9.3.2 (Goudet, 2002). Differences in allelic richness between populations were tested with an ANOVA in GENALEX 6.

2.8. Mitochondrial (Cytochrome *b* gene) and nuclear (microsatellites) loci analyses

Twenty µl of Cyt *b* PCR products were sequenced on a 3730XL sequencer (Applied Biosystems) and sequences were then aligned by eye and clustered with COLLAPSE v.1.1. This software clusters together individuals with identical DNA sequences (= with the same haplotype). All presently recognized haplotypes were gathered from Genbank and used as a reference (Fritz *et al*., 2005a). Sequences not clustered by COLLAPSE v.1.1 were aligned by eye in order to determine their haplotype.

Microsatellite PCR products were run on an AB3130 sequencer (Applied Biosystems) and allele sizes were scored using the program PEAKSCANNER (Applied Biosystems), and then analysed with STRUCTURE (Pritschard *et al*., 2000) without and with haplotypes. This latter software functions with a Bayesian algorithm that infers population structure and clusters probabilistically individuals to clusters that may form subpopulations at the Hardy-Weinberg equilibrium. It is often used with various genetic markers, including microsatellites (Pritschard *et al*., 2000; Evanno *et al*., 2005). A model was assumed with K unknown subpopulations, characterized by a specific set of allele frequencies. Indeed, ΔK provided by Evanno *et al*. accurates better the true number of subpopulations than K provided by STRUCTURE. Individuals assigned to a population with a probability of assignment (P_{assign}) > 90% are considered to belong to the selected cluster, whereas those with P_{assign} < 90% are considered as putative hybrids between clusters.

2.9. Identification of more complex hybrid individuals

In order to identify more complex hybrids F2 (hybrids between two F1 individuals) and Backckrosses (hybrid individuals between one pure parent and one F1 hybrid) in the reserve of Moulin-de-Vert, the software NEWHYBRIDS version 1.1 beta (Anderson and Thompson, 2002) has been used.

Furthermore, a comparison between the results provided by STRUCTURE in the natural reserve of Moulin-de-Vert and those provided by NEWHYBRIDS version 1.1 beta were performed in order to match the real proportion of pure *E. o. hellenica* and *E. o. galloitalica* previously released in this site. As the software STRUCTURE only provides information about pure and F1 individuals but is unable to identify F2 and Backcrosses individuals. On the contrary, NEWHYBRIDS version 1.1 beta is able to approximate the current proportions of pure individuals, of F1 and F2 hybrids as well as proportions of Backcrosses in the population. Frequencies of both pure *E. o. hellenica* and *E. o. galloitalica* were slightly modified in order to match the same pattern in the graph and to approximate at best the values indicated by NEWHYBRIDS version 1.1 beta.

3. Results

3.1. Sampled sites and presence of relict populations in Switzerland

At the end of the trapping-season, no relict population was detected in the cantons Thurgau, Aargau and Bern while the method was very efficient in the populations from Brenne, Camargue and Moulin-de-Vert (as illustrated by the high number of individuals captured in Moulin-de-Vert, up to 26 individuals in one trap). Characteristics for each site are given in Table 3.

Table 3: Summary of the main characteristics of each sample site, with indication on the presence of shoreline vegetation, of nesting sites, eventual problems occurring in the site, necessary measures of management, possibility for reintroductions and durability of the population.

Site	Vegetation	Nesting sites	Problems	Necessary management	Reintroductions	Durability
Brenne	YES	YES	-	-	-	YES
Camargue	YES	YES	YES	-	-	YES
Moulin-de-Vert	YES	YES	MAYBE	-	-	YES
Bommerweiher	YES	NO	YES	YES	-	-
Lengwilerweiher	-	-	YES	YES	-	-
Hudelmoos (TG)	YES	-	YES	YES	-	-
Tobelweiher	-	-	YES	YES	-	-
Seebachtal	YES	YES	-	-	YES	YES
Schinznach am Bad – Brugg	YES	-	YES	YES	-	-
Schinznach am Bad – Wildegg	YES	-	YES	YES	-	-
Rohr – Biberstein	YES	YES	MAYBE	MAYBE	YES	YES
Felsenau – Döttingen	YES	YES	YES	-	-	YES

Brenne (France)
Located in a continental climate in the French region Brenne that harbours the largest *E. orbicularis* populations (Servan, 2000), the pond Ricot is a large pond with structured shoreline vegetation (mainly *Phragmites sp*.). Around the sites are located a lot of sandy and sunny places for the nesting sites.

None problem was detected for the population that does not require any management. Twelve individuals were obtained during the trapping session and additional samples came later (Table 4).

Camargue (France)
The natural reserve of Pont-de-Gau is composed of shallow ponds and channels, with high connectivity between each other and a Mediterranean climate. Mediterranean shoreline vegetation is present in high quantities, as well as *Phragmites sp*. in low brackisch ponds. Numerous nesting sites have been discovered mainly because of high rates of predation on the nests by one *Vulpes vulpes* individual. However, none management strategy, except the removal of this important predator in the reserve, is required for the middle or long-term survival of this population. Twenty-four individuals were obtained during the trapping session (Table 4).

Moulin-de-Vert (GE)
An important shoreline vegetation including *Nuphar lutea*, *Nymphaea alba*, *Phragmites sp*. and *Potamogeton sp*. are reported in this population. Nesting sites are also present nearby the ponds. Problems may be due to the presence of the invasive and competitive *T. scripta sp*. near the reserve. This population needs neither management measures nor reintroductions to reinforce the population estimated to more than 350 individuals (Mosimann, 2002). Further explanations about the allochthonous population of Moulin-de-Vert will be provided below.

Bommerweiher (TG)
This site is composed of 2 medium-sized ponds, with shoreline vegetation (*Nuphar lutea*, *Nymphaea alba*, *Phragmites sp., Carex. sp*.) and without any nesting site nor small ponds for juveniles. Additional problems are the eutrophication of the water, the presence of agriculture next to the ponds and no connectivity with other locations. This site is as a consequence unsuitable for a long-term survival of the species.

Lengwilerweiher (TG)
This site is constituted of 2 small ponds with little shoreline vegetation (*Phragmites sp*.), without any nesting sites and is located at the edge of the forest, with a large shadowed part of the pond. Additional problems are the absence of connectivity with other aquatic systems, the absence of nesting sites, the low quantity of food and a high human density around the pond.

Hudelmoos (TG)
This site is constituted of 2 small ponds with shoreline vegetation (*Phragmites sp*.), without any nesting sites and is located at the edge of the forest. Additional problems are the very small size of the site, the absence of connectivity with other aquatic systems and the low quantity of food.
Tobelweiher (TG)
This site is composed of 3 small-sized ponds with little shoreline vegetation (*Phragmites sp*.) and a large shadowed part. Furthermore, neither nesting sites nor food are available in this small and isolated group of ponds. No reintroductions should be made in this area with a very high human density.

Seebachtal (TG)
This site englobes 2 large ponds (Hüttwilersee and Nussbaumersee) and 1 medium-sized pond (Hasensee), with structured shoreline vegetation (mainly *Phragmites sp*., *Carex. sp*., *Nuphar lutea*, *Nymphaea alba*), nesting sites and small ponds for juveniles. This site would be suitable for a further reintroduction of *E. orbicularis orbicularis* and a long-term survival of the species in this site (Fig. 4).

Schinznach am Bad – Brugg (AG)
This transect on the Aar river has shoreline vegetation (*Phragmites sp*., *Carex. sp*.), but the presence of dams on the river course, the canalization of the rivers' banks, as well as the absence of nesting sites, a

high human density surrounding the area and risks of overflooding render this site unsuitable for reintroduction *E. orbicularis*.

Schinznach am Bad – Wildegg (AG)
This transect on the Aar river has the same characteristics than the latter site, and is thus unsuitable for *E. orbicularis.*

Rohr – Biberstein (AG)
This renatured transect on the Aar river has open sunny areas (Fig. 5) with permanent wetlands, shoreline vegetation and nesting sites, making this site suitable for reintroductions of *E. orbicularis*. Furthermore, it could act as a source population via the Aar river corridor. This site should however be managed in order to keep this site open and to preserve open dry meadows for nesting sites.

Felsenau – Döttingen (AG)
This transect on the Aar river presents numerous ponds and accesses to the river Aar, with shoreline vegetation (*Phragmites sp.*) and nesting sites (Fig. 6). However, the presence of numerous exotic species (as *T. scripta elegans*) as well as the risks that turtles became lost in the nearby Germany and during overflooding render this site unsuitable for a reintroduction.

Inkwilersee (BE)
This site is a medium-sized pond with important shoreline vegetation, mainly *Phragmites sp., Nuphar lutea, Nymphaea alba* (Fig. 7). Nesting sites and one small pond for juveniles are located next to the main pond. Problems are mainly the absence of connectivity with other aquatic systems, the continuous filling of the pond with organic matter coming from agriculture and decomposition of high densities of *Nuphar lutea*. All these considerations indicate this site as non optimal for a reintroduction.

Fig. 4: Nesting site in Seebachtal (TG).

Fig. 5: Permanent open areas along the transect Rohr - Biberstein (AG).

Fig. 6: Basking sites and shoreline vegetation in the natural reserve of Felsenau - Döttingen (AG).

Fig. 7: Basking sites and shoreline vegetation in the natural reserve of Inkwilersee (BE).

3.2. Summary of captured and sequenced individuals

Number of captured individuals, as well as the number of individuals whose microsatellites were analysed, as well as the number of individuals whose haplotype has been revealed and the total of individuals used in the analyses are represented in Table 4.

Table 4: Number of captured individuals whose microsatellites and cytochrome *b* sequences were analysed (BR = Brenne , WE = Western France, AI = Aigues-Mortes , CA = Camargue, VI = Vigueirat, VA = Var , AR = Ardèche , MDV = Moulin-de-Vert , TI = Tessin and HU = Hungary).

	POPULATIONS										
	BR	WF	AI	CA	VI	VA	AR	MDV	TI	HU	All pop.
$N_{ind.}$ *captured*	36	10	22	24	13	31	5	149	11	24	325
microsatellites	36	10	22	15	13	31	5	149	6	24	311
Cytochrome b	28	10[(1)]	20	17	9	9	0	124	10[(2)]	12	239
used in the analyses	36	10	22	15	13	31	5	149	6	24	311

[(1)] analyses conducted by Fritz and coll., 2005

[(2)] Fritz, pers. comm.

Table 5 represents all individuals whose haplotype is known, partitioned into the three subspecies *E. o. orbicularis* (haplotype IIa), *E. o. hellenica* (haplotype IVa) and *E. o. galloitalica* (haplotype Va) except scattered individuals foud in Switzerland (described later in Table 12). Populations from Brenne and western France harbour only haplotype IIa, while the populations of Aigues-Mortes, Camargue, Var, Moulin-de-Vert and Tessin consist of a mixture of several haplotypes. The population from Vigueirat harbours haplotype Va, while the relict population from Ardèche has no haplotyped individual.

Table 5: classification of 239 cytochrome *b* sequences belonging to the subspecies *E. o. orbicularis*, *E. o. hellenica* and *E. o. galloitalica* (BR = Brenne , WF = Western-France , AI = Aigues-Mortes , CA = Camargue, VI = Vigueirat, VA = Var , AR = Ardèche , MDV = Moulin-de-Vert,, TI = Tessin and HU = Hungary).

SUBSPECIES	POPULATIONS										
	BR	WF	AI	CA	VI	VA	AR	MDV	TI	HU	All pop.
E. o. orbicularis	28	10 [(1)]	3	14	0	5	0	7	8[(2)]	12	88
E. o. hellenica	0	0	11	0	0	0	0	104	1[(2)]	0	116
E. o. galloitalica	0	0	6	3	8	4	0	13	1[(2)]	0	35

Total / population	28	10	20	17	8	9	0	124	10	12	239

(1) analyses conducted by Fritz and coll., 2005

(2) Fritz, pers. comm.

3.3. Null alleles

Presence of null alleles was detected in the loci msEo21 and GmuD93 by relatively high values of the estimators r_c and r_b in all populations. Detailed results are indicated in Annexe 1 for each locus in each population. The probable presence of these null alleles is e.g. due to mutations on the binding site of the primer, thus hindering the binding of the primer and amplification of this allele. As a consequence, false homozygotes will be detected, leading to wrong allele frequencie evaluation and to biased results. This is why both loci were not included in the analyses.

3.4. Hardy-Weinberg equilibrium

Both methods applied to test the Hardy-Weinberg equilibrium indicate the same results (Table 6). While populations from Brenne, Var, Ardèche, Tessin and Hungary show some disequilibrium in some of their loci, all populations except Moulin-de-Vert are not at the Hardy-Weinberg disequilibrium. Populations from Brenne and Ardèche have only 2 loci that are weakly at the disequilibrium, while the population from Var has 3 loci at the disequilibrium. The population from Hungary display one locus at a stronger disequilibrium than the latter 2 populations, while the population from Tessin shows 3 loci with relatively strong disequilibrium. The population from Moulin-de-Vert is however strongly at a disequilibrium, as reflected by the highly significant F_{IS} values for 5 loci.

Table 6: Test of Hardy-Weinberg equilibrium for all populations of *E. orbicularis* (BR = Brenne , WF = Western-France , AI = Aigues-Mortes, CA = Camargue, VI = Vigueirat, VA = Var , AR = Ardèche , MDV = Moulin-de-Vert,, TI = Tessin and HU = Hungary); * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$.

	BR	WF	AI	CA	VI	VA	AR	MDV	TI	HU
msEo2	-0.02 ns	-0.15 ns	0.10 ns	- 0.19 ns	-0.21 ns	0.18*	-	0.02**	0.12 ns	0.08ns
msEo29	0.13*	0.20 ns	-0.02 ns	0.66 ns	0.25 ns	0.29*	0.50*	0.08***	0.05 ns	-0.04 ns
msEo41	-0.02 ns	-0.1 ns	-0.07 ns	0.14 ns	-0.03 ns	0.04 ns	0.64 ns	0.34***	0.33***	0.1**
GmuB08	-0.07 ns	0.03 ns	0.13 ns	0.32 ns	0.02 ns	-0.18 ns	-0.25 ns	0.01**	-0.15*	0.1 ns
GmuD51	0.07 ns	0.19 ns	-0.08 ns	0.07 ns	-0.09 ns	-0.04 ns	0.22*	-0.003 ns	-0.1 ns	0.1 ns
GmuD87	-0.02 ns	0.09 ns	-0.02 ns	0.11 ns	0.08 ns	0.23*	0.5 ns	0.12***	- 0.17 ns	-0.14 ns
GmuD114	0.09*	0.09 ns	0.08 ns	-0.17 ns	-0.11 ns	0.10 ns	-0.11 ns	-0.05 ns	-0.23**	-0.05 ns

3.5. Genetic structure of the populations

The lowest value is 0.039 between the populations from Camargue and Aigues-Mortes which are separated by the Rhône river. Populations from Brenne, Hungary and Camargue also have a low genetic differentiation (F_{ST} = 0.065 between Brenne and Camargue, 0.06 between Brenne and Hungary and 0.064 between Camargue and Hungary) between each other, but differentiation between these populations and the population from Moulin-de-Vert is higher (F_{ST} = 0.094 between populations from Camargue and Moulin-de-Vert, 0.08 between populations from Brenne and Moulin-de-Vert, and 0.097 between populations from Hungary and from Moulin-de-Vert). Interestingly, the population from Tessin has relatively high values of differentiation relative to the other populations. (Table 7).

Table 7:Pairwise Population F_{ST} values (CA = Camargue, BR = Brenne, HU = Hungary, AI = Aigues - Mortes, VA = Var, AR = Ardèche , MDV = Moulin-de-Vert,, TI = Tessin and WF = Western-France).

Populations	CA	BR	HU	AI	VA	AR	MDV	TI	WF
CA	0.000								
BR	0.065	0.000							
HU	0.064	0.060	0.000						
AI	0.039	0.042	0.064	0.000					
VA	0.081	0.093	0.123	0.053	0.000				
AR	0.134	0.156	0.167	0.123	0.137	0.000			
MDV	0.094	0.080	0.097	0.053	0.071	0.154	0.000		
TI	0.107	0.086	0.074	0.061	0.108	0.186	0.078	0.000	
WF	0.039	0.064	0.073	0.038	0.083	0.132	0.092	0.114	0.000

3.6. Observed (Ho) and expected (Hs) Heterozygosities

The average number of alleles per locus, as well as observed (Ho) and expected (Hs) heterozygosities of populations from Camargue, Brenne, Hungary, Var, Ardèche, Moulin-de-Vert, Tessin and Western France are indicated in Table 8. Observed heterozygosity within the populations is in general lower than expected heterozygosity and several populations show the same expected heterozygosity (Brenne, Western France and Camargue). The higher Ho value is given by the population from Moulin-de-Vert, and the higher values of Hs are given equally by this same population and by the population from Tessin. Moreover, the population from Moulin-de-Vert harbours two to four times the average numbers of alleles per locus in the other populations (Moulin-de-Vert: 15.6 and Ardèche: 3.5). More details on the observed and expected heterozygosities, as well as on the number of alleles for each locus are reported in Annexe 1.

Table 8: Ho (observed heterozygozity) and in populations from (BR = Brenne , WF = Western-France , AI = Aigues-Mortes, CA = Camargue, VI = Vigueirat, VA = Var , AR = Ardèche , MDV = Moulin-de-Vert,, TI = Tessin and HU = Hungary).

Population	N alleles / locus	Av. Ho	Av. He
BR	7.6	0.72	0.70
WF	7.4	0.71	0.71
CA	7.3	0.63	0.71
VA	6.9	0.67	0.73
AR	3.5	0.47	0.52
MDV	15.6	0.76	0.82
TI	6.4	0.81	0.75
HU	9.1	0.75	0.74

3.7. Allelic richness

Differences in allelic richness between populations were tested (Table 9). Detailed results for each locus for each population are indicated in Annexe 2. No difference was reported between the Eastern and Western populations from Hungary and Brenne respectively ($p = 0.44$), indicating no difference between easternmost and westernmost populations in the haploclade II. Allelic richness was similar in the populations from Brenne and Hungary (haploclade II) compared to that of Ardèche and Var (haploclade IV) with $p = 0.91$, thus indicating that neiher haploclade II nor haploclade IV have more allelic richness than the other haploclade.

Furthermore, no reduced allelic richness was measurable between populations from Brenne compared to that of the populations from Aigues-Mortes, Camargue and Vigueirat (p = 0.46, 079 and 0.42 resp.). Additionally, these 3 latter populations have only a marginal significant difference in allelic richness compared to the population from Moulin-de-Vert (p = 0.06), as well as between populations from Brenne and Moulin-de-Vert (p = 0.06).

Table 9: Allelic richness of the populations per locus and tested with FSTAT v.2.9.3.2 based on the number of diploid individuals indicated in Table 4.

	CA	BR	HU	VI	AI	VA	AR	MDV	TI	WF	Total
msEo2	3.05	4.01	3.53	3.13	3.84	2.80	2.00	4.92	3.16	3.03	5.28
msEo29	3.57	3.14	4.66	4.85	5.19	3.95	5.00	4.85	6.66	3.69	6.04
msEo41	5.23	5.3	6.80	6.38	6.60	5.55	2.82	5.77	4.85	7.07	7.20
GmuB08	3.38	3.02	2.65	2.88	3.68	2.00	3.67	4.47	3.88	2.98	4.59
GmuD51	7.07	5.61	6.29	7.10	5.05	6.08	4.80	6.59	6.90	5.83	7.20
GmuD87	6.61	5.47	6.08	6.49	5.46	5.39	2.67	7.13	4.45	6.07	7.34
GmuD114	3.93	4.86	5.33	4.95	4.62	5.06	4.50	5.67	4.83	4.98	6.13

3.8. Hybridization between subspecies in a natural hybrid zone (France)

Analyses without haplotypes' information indicates an optimal value of likelihood for K = 2 clades in France: the first includes the populations from Brenne, Western France, Aigues-Mortes, Camargue and Vigueirat, while the other includes both populations from Ardèche and Var. However, analyses including haplotypes' information give an optimal value of likelihood for K = 3 clades: the first genetic group (C1) includes populations from Brenne, Western France and Camargue, the second group (C2) is composed of both populations from Var and Ardèche, while Aigues-Mortes' population is comprised in the third group (C3). Summarized results are indicated in Table 10.

Table 10: Summary of the results obtained from STRUCTURE for each population without and with haplotype information. C1, C2, C3, C4 and C5 correspond to Cluster 1, Cluster 2 and Cluster 3 resp., while IntC1, IntC2 and IntC3 refer to Introgressed Cluster 1, Cluster 2 and Cluster 3, respectively. Pure individuals have a $P_{assign} \geq 90\%$. Introgressed individuals are individuals with a P_{assign} comprised between 50% and 90% (non included). *E.o.o, E.o.h., E.o.g*. correspond to the subspecies *E. o. orbicularis, E. o. hellenica, and E. o. galloitalica* with their corresponding hybrids. Hybrids correspond to the introgressed individuals.

Pop.	STRUCT_without_hapl.	STRUCT_with_hapl.	Subspecies
BR	C1	C1	*E.o.o.*
WF	C1	C1 and Int.C1	*E.o.o.* and hybrid *E.o.o*
AI	C1 and Int.C1	C1, C2 and IntC1, Int.C3	*E.o.o.* and hybrid *E.o.o*
CA	C1, Int.C1, Int.C2	C1, C2 and IntC1, IntC2, IntC3	*E.o.o, E.o.g.* and hybrid *E.o.o., E.o.g. and E.o.h.*
VI	C1 and Int.C1	C1 and Int.C1, IntC2	*E.o.o. and hybrid E.o.o, E.o.g*
VA	C2 and Int.C2	C2	*E.o.g.*
AR	C2	C2	*E.o.g.*
TI	C1	C1	*E.o.o.*
HU	C1	C1	*E.o.o.*

Detailed results for each population are indicated below:

3.8.1. Brenne

a) without the haplotype information (n = 36)
Without the haplotype information, all sampled individuals group together in one cluster (C1) with a $P_{assign} = 99\%$, while no introgressed individual is detected within the population (Fig. 8a).

b) with the haplotype information (n = 36)
The cluster C1 includes only individuals harbouring haplotype IIa corresponding to *E. o. orbicularis* (Table 5).
Including this information in the analyses (Fig. 8b), a slight modification of population structure can be observed: 94% of individuals group in C1 with a P_{assign} = 98%, while two individuals (6% of the population) are introgressed C1. This introgression occurs with C3 (not shown).

Fig. 8a: Genetic clusters without haplotype information in Brenne.

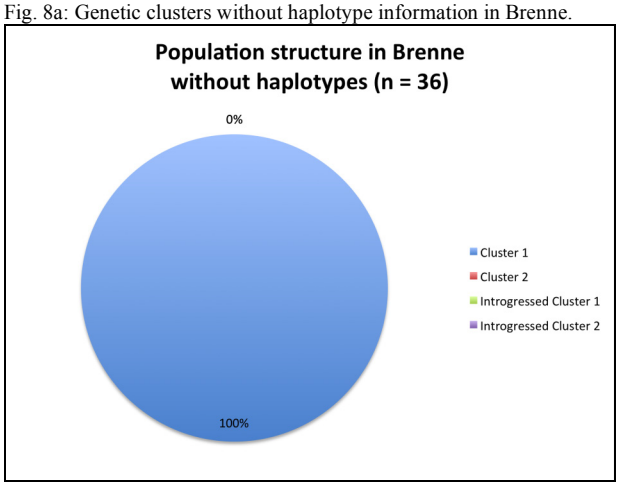
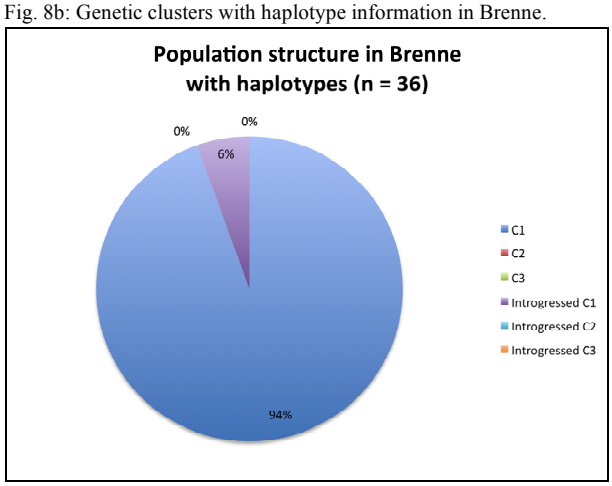


Fig. 8b: Genetic clusters with haplotype information in Brenne.



3.8.2. Western France

a) without the haplotype information (n = 10)
Without the haplotype information, all individuals from this population cluster in C1 with a P_{assign} = 97% , while no individual shows introgression with cluster C2 (Fig. 9a).

b) with the haplotype information (n = 10)
All the sampled individuals harbour haplotype IIa corresponding to the subspecies *E. o. orbicularis* (Table 5).
Including these informations in the analyses (Fig. 9b), 70% of the individuals cluster in C1 with a $P_{assign.}$= 97%, while 30% of the population are introgressed C1(Fig. 9b). This introgression occurs with C3 (not shown).

Fig. 9a: Genetic clusters without haplotype information in Western France.

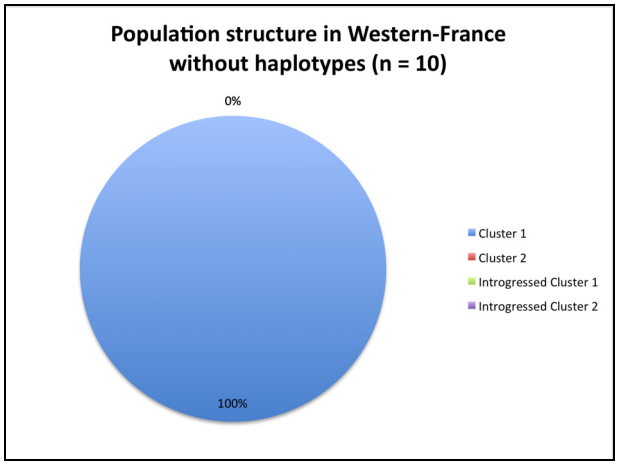
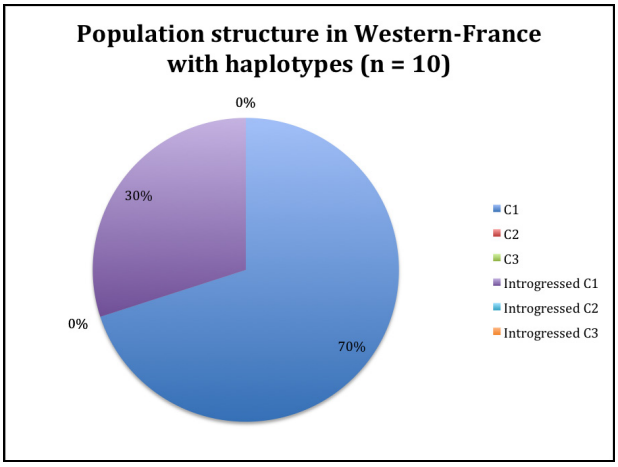


Fig. 9b: Genetic clusters with haplotype information in Western France.



3.8.3. Aigues-Mortes

a) without the haplotype information (n = 22)
Without the haplotype information, 86% of individuals cluster in C1 with a P_{assign}= 98%, while 14% (3 individuals) show introgression with C2 (Fig. 10a).

b) with the haplotype information (n = 22)
Only 15% of the population harbours haplotype IIa (*E. o. orbicularis*), while 55% and 30% of the population harbour haplotypes IVa (*E. o. hellenica*) and Va (*E. o. galloitalica*) respectively (Table 5).
Including these informations in the analyses, results are strongly modified: none individual clusters in C1 or C2, while 75% of the population clusters in C3 with a P_{assign}= 97%. Twenty-five percent hybrids between C1, C2 and C3 (Introgressed C1, Introgressed C2 and Introgressed C3) are reported in the population (Fig. 10b). C3 individuals harbour haplotypes IVa and Va, while hybrid individuals harbour haplotypes IIa, IVa and Va.

Fig.10a: Genetic clusters without haplotype information in Aigues-Mortes.

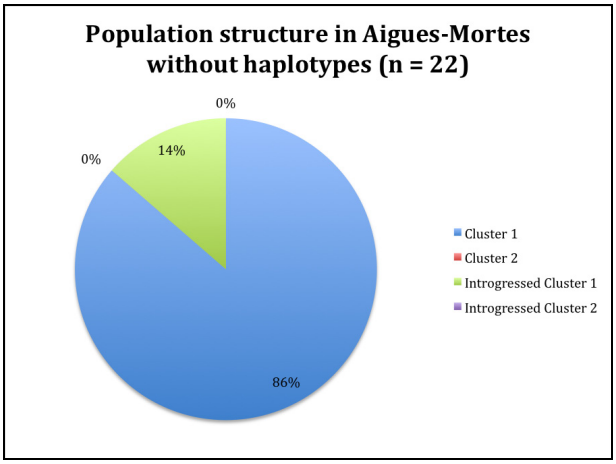
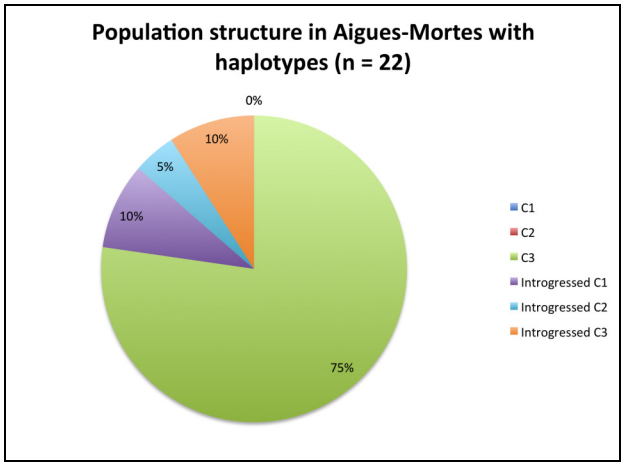


Fig.10b: Genetic clusters with haplotype information in Aigues-Mortes.



3.8.4. Camargue

a) without the haplotype information (n = 15)
Without the haplotype information, 86% of the population clusters with a P_{assign}= 97% in C1, while 2 hybrids (7% Introgressed C1 and 7% Introgressed C2) are reported in the population (Fig. 11a).

b) with the haplotype information (n = 15)
Seventy-three and 20% of the population harbours haplotype IIa (*E. o. orbicularis*) and Va (E. o. galloitalica) respectively (Table 5). Seven percent did not reveal their haplotype.
Including this information in the analyses, 72% of the individuals cluster in C1 (P_{assign}= 97%) and only 7% with C2, which consists of only one individual harbouring haplotype Va. Individuals clustered in C1 harbour only haplotype IIa. The presence of C3 individuals in this population is not reported.
On the 3 hybrids (21% of the population), one in an introgressed C1, the second is an Introgressed C2 and the third is an Introgressed C3 (Fig. 11b).

Fig. 11a: Genetic clusters without haplotype information in Camargue.

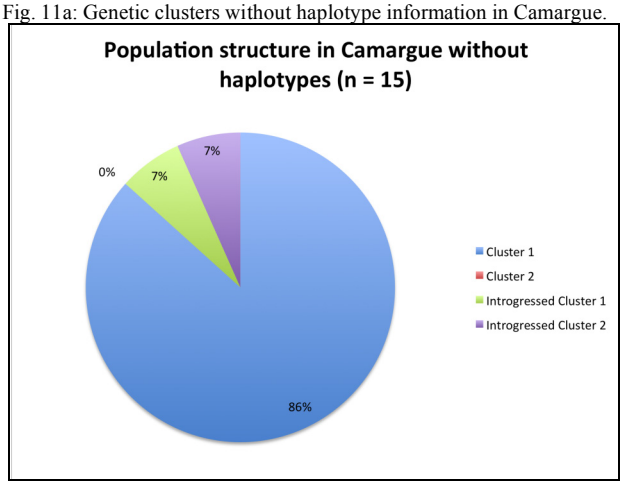
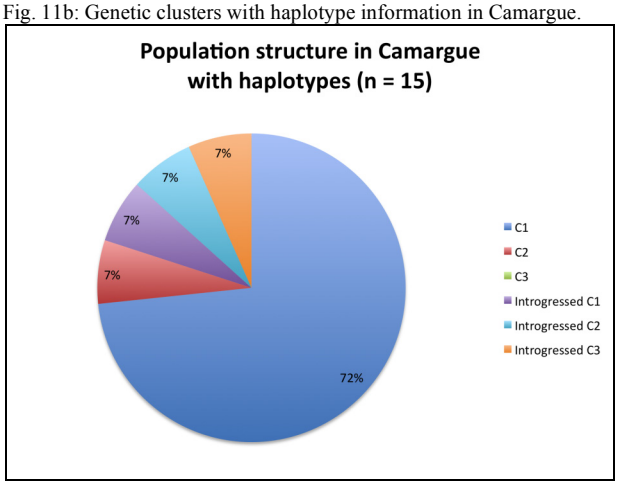


Fig. 11b: Genetic clusters with haplotype information in Camargue.



3.8.5. Vigueirat

a) without the haplotype information (n = 13)
Without the haplotype information, 12 individuals (92%) cluster in C1 with a P_{assign}= 98%, while one hybrid (8% of the population) is reported (Fig. 12a).

b) with the haplotype information (n = 13)
All individuals harbour haplotype Va corresponding to *E. o. galloitalica* (Table 5).
Including this information in the analyses, 39% of the population were assigned to C1 with a P_{assign}= 98%, while neither C2 nor C3 individuals are detected. Eight hybrids are reported between C1 and C2: three hybrids (22% of the population) are Introgressed C1 and 5 hybrids (39% of the population) are Introgressed C2 (Fig. 12b).

Fig. 12a : Genetic clusters without haplotype information in Vigueirat.

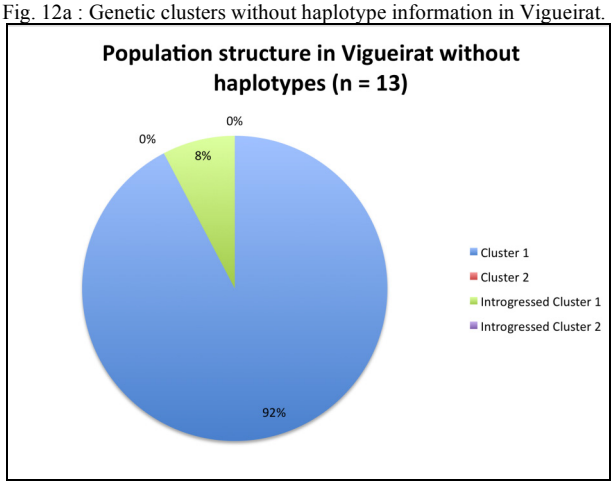
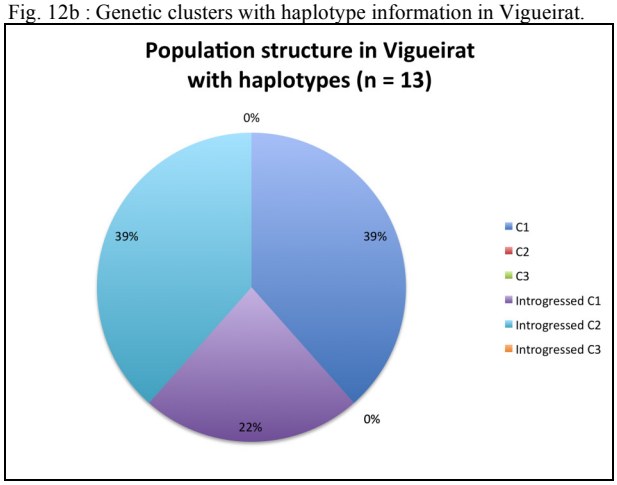


Fig. 12b : Genetic clusters with haplotype information in Vigueirat.



3.8.6. Var

a) without the haplotype information (n = 31)
Without the haplotype information, individuals cluster in C2 with a P_{assign}= 99%, while no C1 individual is detected. However, one hybrid individual (3% of the population) is an Introgressed C2 (Fig. 13a). This introgression occurs with C1.

b) with the haplotype information (n = 31)
Fifty-six percent of the sampled individuals harbour haplotype IIa corresponding to the subspecies *E. o. orbicularis* while 44% bear haplotype Va corresponding to the subspecies *E. o. galloitalica* (Table 5).
Including these informations in the analyses, 100% of the individuals are clustered in C2 with a P_{assign}= 98%, while no hybrids are reported (Fig. 13b).

Fig. 13a: Genetic clusters without haplotype information in Var.

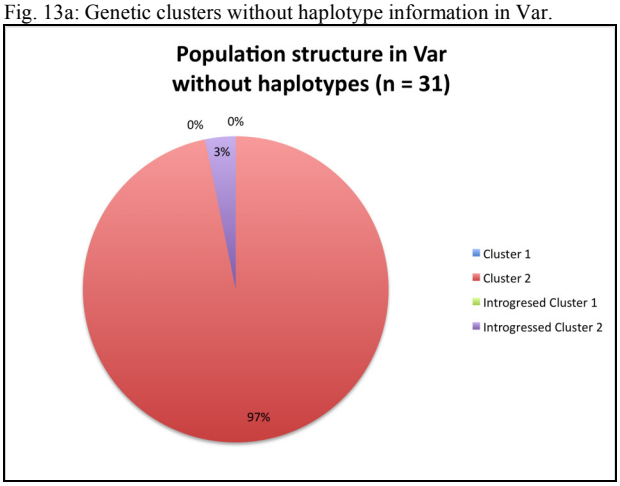
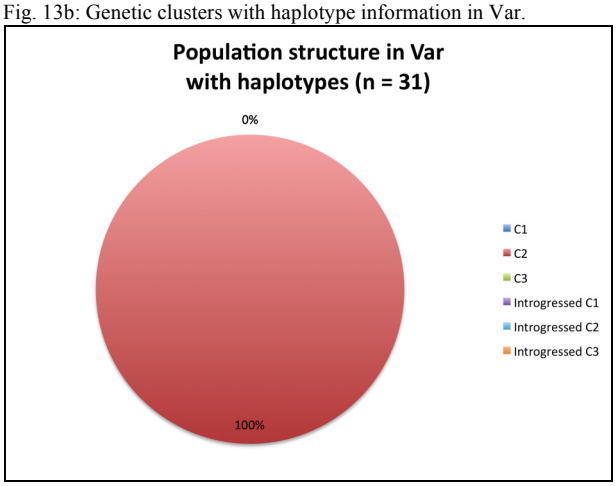


Fig. 13b: Genetic clusters with haplotype information in Var.



3.8.7. Ardèche

a) without the haplotype information (n = 5)
Without the haplotype information, all individuals cluster in C2 with a P_{assign} = 98% without any record of C1 or hybrids individuals (Fig. 14a).

b) with the haplotype information (n = 5)
No individual from Ardèche has a known haplotype (Table 5). However, when this population is included in the analyses with other haplotyped individuals, all individuals cluster in C2 with a P_{assign} = 98% without any C1 nor hybrid individuals reported in the population (Fig. 14b).

Fig. 14a: Genetic clusters without haplotype information in Ardèche.

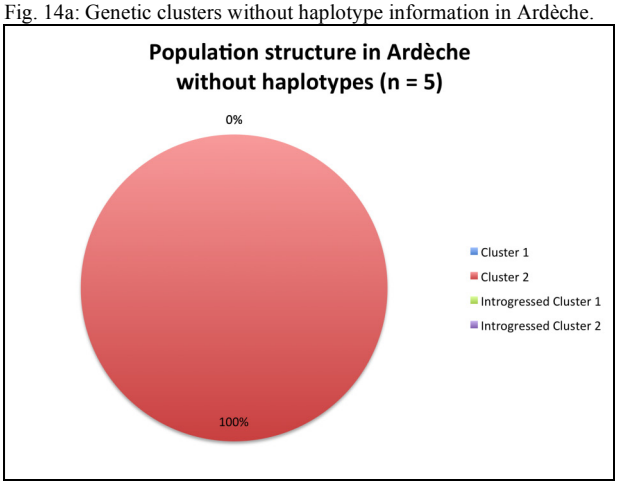
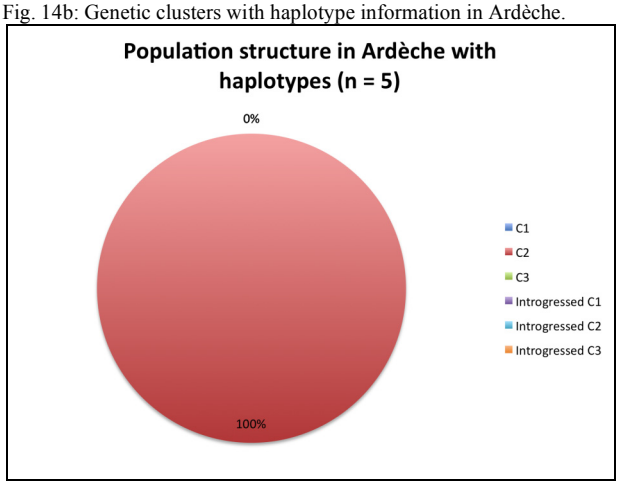


Fig. 14b: Genetic clusters with haplotype information in Ardèche.



3.9. Hybridization between subspecies in an artificial hybrid zone (Moulin-de-Vert, Geneva)

The study in France was led on various populations in order to define hybridization levels between different subspecies in a natural contact zone. This chapter focuses on the contrary on only one population harbouring different subspecies, in an artificial contact zone. Without the haplotype information, analyses give a ΔK of 5 genetic groups, while including haplotypes reveal a ΔK of 3 genetic genetic groups in the artificial population of Moulin-de-Vert (Table 11). The different clusters below do not correspond to which previously used in the study in France.

Table 11: Summary of the results obtained from STRUCTURE for the population from Moulin-de-Vert without and with haplotypes. C1, C2, C3, C4 and C5 correspond to Cluster 1, Cluster 2, Cluster 3, Cluster 4 and Cluster 5 respectively. *E.o.o, E.o.h., E.o.g.* correspond to the subspecies *E. o. orbicularis, E. o. hellenica, and E. o. galloitalica* with their corresponding hybrids.

Pop.	STRUCT_without_hapl.	STRUCT_ with_hapl.	*Subspecies*
MDV	C1, C2, C3, C4, C5, Int.	3 other clusters C1, C2, C3	*E.o.o, E.o.h., E.o.g. and hybrids E.o.o., E.o.h. and E.o.g.*

a) without the haplotype information (n = 149)

Without the haplotype information, 9% of the individuals are clustered in C1 with a P_{assign} = 98%, 5% of the individuals are clustered in C2 with a P_{assign} = 93%, 5% of the individuals are clustered in C3 with a P_{assign} = 93%, while 4% of the individuals cluster in C4 with a P_{assign} = 94% and 5% in C5 with a P_{assign} = 94% (Fig. 15a).

The remaining 72% are hybrids between the 5 clusters and are partitioned into 5 different classes called Hybrids C1-C5, which name indicates the largest part of their genome (an individual from the class Hybrids C1 has a higher proportion of C1 genes and is introgressed by the other clusters). Twenty-two percent of hybrids cluster in the hybrid class Hybrids C1, 9% of the population clusters within Hybrids C2 and 23% in Hybrids C3, while 23% and 25% are partitioned in the hybrid classes Hybrids C4 and Hybrids C5 respectively. Interestingly, the class Hybrids C2 has about 3 times lower introgression than the other 4 clusters (Fig. 15b).

Fig.15a: Genetic clusters without haplotype information in Moulin-de-Vert.

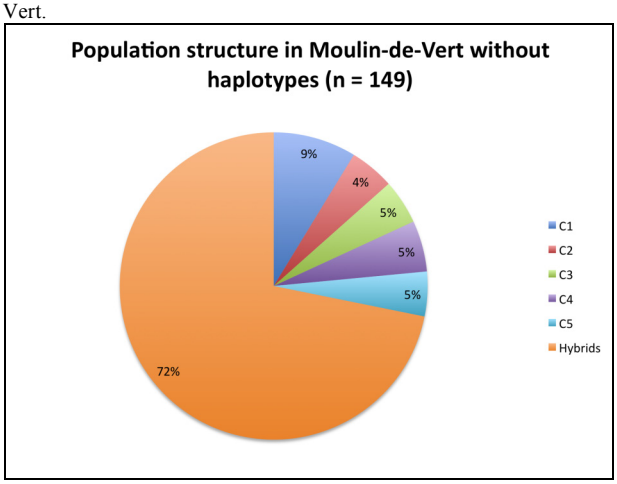
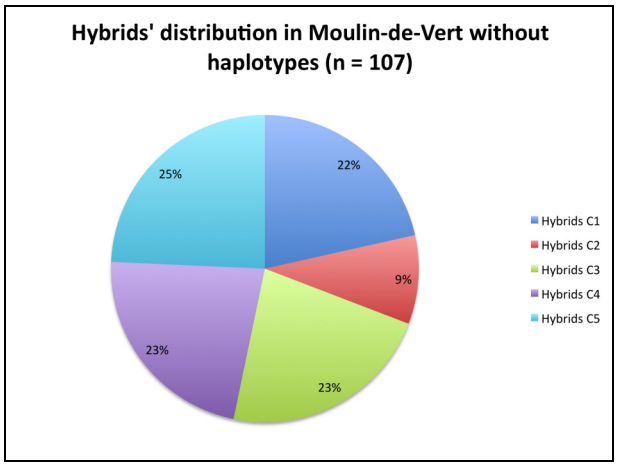


Fig. 15b: Repartition of hybrids into 5 different classes.



b) with the haplotype information (n = 149)

Haplotypes reveal the presence of 7 (6%) of *E. o. orbicularis*, 104 (84%) of *E. o. hellenica* and 13 (10%) of *E. o. galloitalica* (Table 5) in the natural reserve of Moulin-de-Vert.
Including these informations in the STRUCTURE analyses, 3% of individuals are considered as pure *E. o. orbicularis* with a P_{assign} = 99%, 57% as pure *E. o. hellenica* with a P_{assign} = 95% and 6% as pure *E. o. galloitalica* with a P_{assign} = 96%, while hybrids account for 34% of the sampled population (Fig. 16a).
In hybrid classes, 23% are hybrid *E. o. orbicularis*, 61% are hybrid *E. o. hellenica* and 16% are hybrid *E. o. galloitalica* and are partitioned into 3 different classes (Fig. 16b).
Interestingly, previous identified clusters (when haplotypes were excluded from the analyses) consisting of 5 clusters. Cluster C1 and C2 comprise both individuals harbouring haplotypes IIa and IVa corresponding to the subspecies *E. o. orbicularis* and *E. o. hellenica* respectively. Cluster C3 comprises only individuals harbouring haplotype Va corresponding to *E. o. galloitalica*. Furthermore, clusters C4 and C5 consist both of only haplotype IVa individuals corresponding to *E. o. hellenica*.

Fig.16a: Genetic clusters with haplotype information in Moulin-de-Vert.

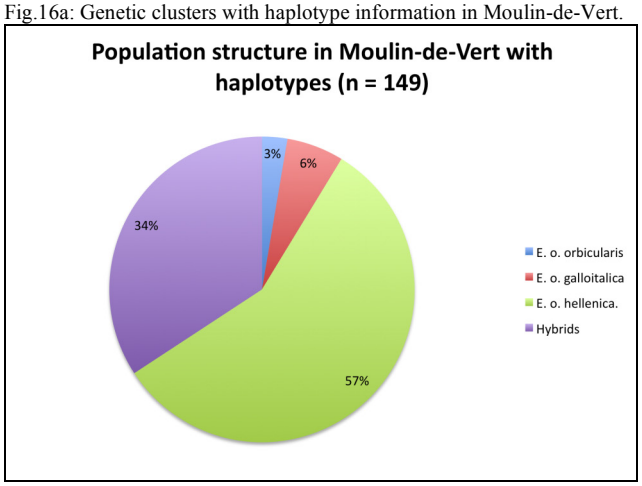
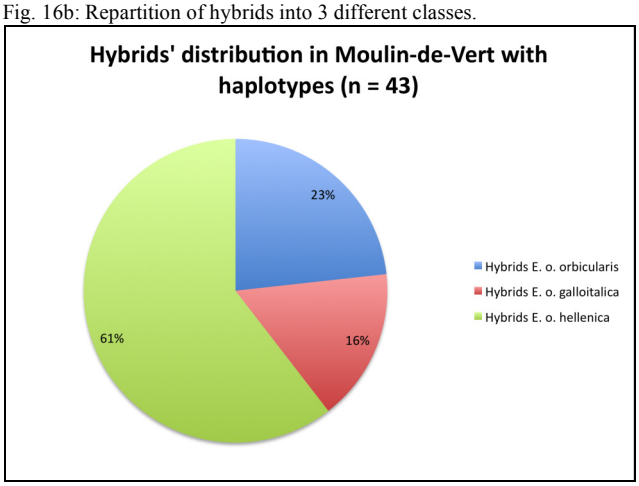


Fig. 16b: Repartition of hybrids into 3 different classes.



3.10. Assignment of Swiss samples collected in several locations during the last years to their putative location of origin

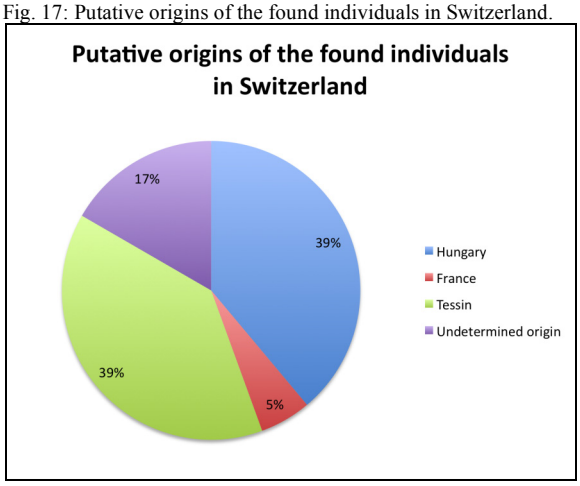
Several individuals collected in different locations in Switzerland harboured haplotype IIa. As this haplotype is considered as native in Switzerland, they could be Swiss relict individuals. This is why microsatellites were surveyed in order to compare them with those from other populations harbouring haplotype IIa. This comparison allows to discriminate between potential Swiss relict individuals and introduced individuals (Table 12).

Table 12: Description of the individuals found in Switzerland harbouring haplotype IIa and their putative location of origin.

Individual	Site of capture	Putative origin	Probability of assignment to the putative origin
DG15	Aar (AG)	Undetermined	80.5%
DG16	Reusstal (AG)	Eastern Europe	91.4%
DG17	Wynau (AG)	Eastern Europe	98.0%
DG18	Reusstal (AG)	Eastern Europe	94.5%
DG24	Dottikon (AG)	Eastern Europe	95.4%
DG26	Switzerland	Central France	90.3%
HPS3	Switzerland	Undetermined	73.7%
HPS5	Künten (AG)	Undetermined	68.9%
HPS6	Küttigen (AG)	Eastern Europe	91.3%
MW1	Lauerzensee (SZ)	Eastern Europe	90.4%
MZ1	Pra Vicc (Genestrerio)	Tessin	95.5%
TM1	Lauerzensee (SZ)	Eastern Europe	94.4%
UF4	Bolle di Magadino (TI)	Tessin	94,4%
UF5	Bolle di Magadino (TI)	Tessin	98.9%
UF6	Bolle di Magadino (TI)	Tessin	98.5%
UF7	Bolle di Magadino (TI)	Tessin	98.3%
UF8	Bolle di Magadino (TI)	Tessin	99.0%
UF9	Bolle di Magadino (TI)	Tessin	95.5%

Individuals found in Switzerland and harbouring haplotype IIa cluster together with populations from Hungary (DG16-DG24, HPS6, MW1 and TM1) and Brenne (DG26). Tessin's individuals cluster in another group than Brenne's and Hungarian clusters. Three individuals (DG15, HPS3, HPS4) could not be assigned to their population of origin (P_{assign} = 80.5%, 73.7% and 68.9% resp.) and show some intermediate state between Brenne's and Hungarian populations (Table 12; Fig.17).

Fig. 17: Putative origins of the found individuals in Switzerland.

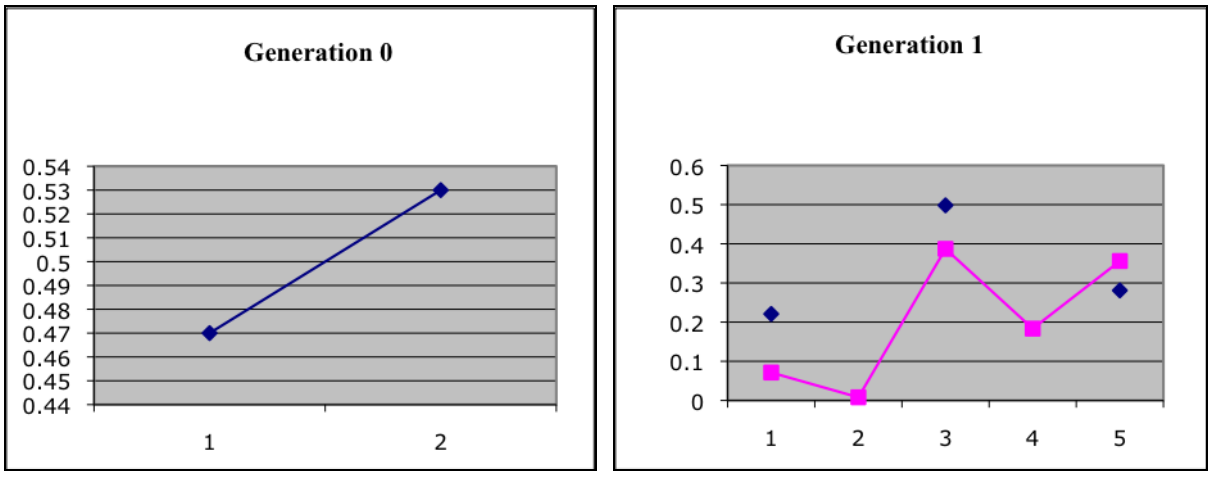


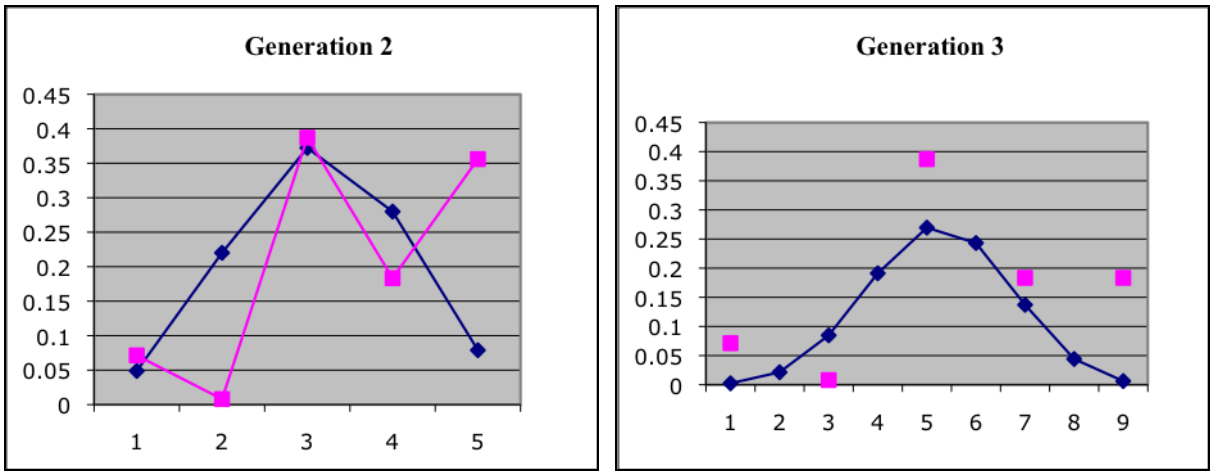
3.11. Identification of complex hybrids in the population of Moulin-de-Vert

Proportions indicated by NEWHYBRIDS at Generation 3 (2010) reflect a current proportion of 7% and 35% of pure individuals of the subspecies, while F1 and F2 hybrids are reported with 0.6% and 38% respectively. Backcrosses 1 (between the first pure population and hybrids F1), as well as Backcrosses 2 (between the second pure population and hybrids F1) are reported in the population with 0.8% and 18% respectively.

As NEWHYBRIDS gives an insight into complex hybrids such as F2 and Backcrosses individuals, its use for the study of the initial proportions of released *E. o. hellenica* and *E. o. galloitalica.* Four generations (Generations 0 to 3) reflecting a time generation of 19 years each and are represented in the Figure 17. Generation 0 indicates when both subspecies met in this artificial contact zone in 1953. Following generations indicate the approximated proportions of both pure and hybrid individuals after 19 years (1972), after 38 (1991) and 57 years (2010) respectively. Values obtained by NEWHYBRIDS were included in the equation from Hardy-Weinberg equilibrium developed on 3 generation times and were variated in order to match at best the current percentages of pure *E. o. hellenica* and *E. galloitalica* indicated by STRUCTURE (the low proportion (35) of pure E. o. orbicularis in the population was neglected).
Both softwares STRUCTURE and NEWHYBRIDS version 1.1 approximate better when population 1 (*E. o. galloitalica*) has an initial proportion of 47% and population 2 (*E. o. hellenica)* an initial proportion of 53%.

Fig. 18: Hardy-Weinberg equilibrium on different generation times in frequency provided by STRUCTURE (in blue) and NewHybrids (in pink). Numbers 1 to 9 describe the different levels of hybridization in the population in Moulin-de-Vert. Generation 0 harbours only pure individuals (classes 1 and 2). Generation 1 and 2 harbour pure individuals (classes 1 and 5), hybrid F1 (class 3) and backcrossed individuals (classes 2 and 4). Generation 3 harbours pure individuals (classes 1 and 9) and different levels of hybrid individuals (classes 2 to 8).





4. Discussion

4.1. Suitable sites for future reintroductions

As chosen sites were natural reserves, aquatic habitats were highly preserved with important shoreline vegetation and probably enough food. However, only a few sampled localities have nesting sites, which are considered to be the limiting factor in many turtles' species (Fritz, 2001). Indeed, potential sites with the required specificities for the European pond turtle are the Seebachtal (TG) and the transect Rohr-Biberstein (AG). All other sampled locations were unsuitable for various raisons (Table 3). The Seebachtal region consists of 2 large- and one medium-sized ponds around which are located several nesting sites and shallow ponds, where juveniles may grow until they reach a sufficient size to be safe from predators. Another potential site is located in a recently renatured transect of the Aar river between Rohr und Biberstein (AG), and comprises permanent ponds, large and sunny nesting sites, as well as the potential of widespread down- or upstream the Aar river, and could act as a biological corridor for further recolonization of the river and of further localities. Although this site has been recently renatured, management of its habitats should be done in order to preserve these habitats, especially those with nesting sites.

4.2. Presence of relict populations in Switzerland

Trapping sessions were conducted in sites where no previous study was made and where individuals had been occasionally recorded until the 1990's (Centre Suisse de cartographie de la faune, CSCF, Neuchâtel, pers. comm.). The absence of any recorded population in these last potential sites for harbouring relictual Swiss *E. orbicularis* populations leads to the conclusion that native *E. orbicularis* populations have definitively become extinct in eastern Switzerland.

However, despite no relict Swiss population still exists in Switzerland, one large population and 2 small-sized groups of allochthonous individuals are known to live in the country: the first is located in Moulin-de-Vert (GE), the second in Hallwilersee (AG) and the third in Tessin. The population of Moulin-de-Vert will be discussed further in the discussion. Previous genetic studies had already indicated a genetic mixture of haplotypes Ia, IIa, IVa and VIIIa in Hallwilersee, suggesting that IIa individuals are also allochthonous (Dusej, pers. comm.). Furthermore, as Human impact in and around the lake is important, as illustrated by numerous and diverse exotic species occurring in this lake

(Huber, pers. comm.), it remains unlikely that individuals harbouring haplotype IIa are native at this location. In Tessin, 2 groups of *E. orbicularis sp*. are reported: the first one in the Colomberra pond (Stabio), and the second one in Bolle di Magadino. Already tested individuals in Bolle di Magadino indicate a mixture of haplotypes Ia, IIa, IVk and Va (Fritz, pers. comm.) but no individual harboured the expected haplotype IVa (*E. o. hellenica*). Swiss populations southern of the Alps should indeed harbour the same haplotype than populations in the Delta Po river in Italy, mainly haplotype IVa (*E. o. hellenica*). Furthermore, as Tessin populations are in high human densely areas, this could bring additionnaly evidence for pets release. Moreover, as mountain chains are important barriers to turtles' spread (Fritz *et al*., 2006), no gene flow should have occurred between northern of the Alps IIa native populations and southern of the Alps IVa populations, thus indicating an allochthonous origin of the Tessin population.

As a consequence, no relict population is reported to live in Switzerland, and the occurrence of a few observed individuals is due to released individuals that survive in the wild.

4.3. Hardy-Weinberg equilibrium

The significant negative F_{IS} values of the population of Moulin-de-Vert indicate an excess of heterozygotes, what could be expected in this young contact zone between 2 subspecies. On the contrary, old hybrid zones, as in the populations of Aigues-Mortes, Camargue and Vigueirat are all at the Hardy-Weinberg equilibrium suggesting that they reproduce without a mating choice towards their own subspecies. This may indicate that the population from Moulin-de-Vert may also tend towards this equilibrium.

4.4. Genetic structure of the populations

Small F_{ST} values displayed (lower than 10% between sites distant from more than 2000km) indicate that only slight differentiation exists between the populations, as well as between subspecies, what may indicate important gene flow and introgression between the populations. Furthermore, F_{ST} is lower than 10% between sites

4.5. Ho, Hs and allelic richness

The population harbouring the higher level of heterozygosity is the Moulin-de-Vert. As this site possesses individuals from various origins, it is thus not surprising to find in this population more heterozygosity than in the other populations (including the old hybrid zones). Furthermore, this high level of heterozygosity corroborates the excess of heterozygotes found by the Hardy-Weinberg disequilibrium of this population.

4.6. Assignment of found individuals to their putative location of origin

Individuals found in Switzerland are surely released or escaped pets that may survive in the wild if environment is favourable (presence of permanent aquatic habitats, basking sites and food) and may not form a viable core population as they are scattered from each other (what was not the case in

Moulin-de-Vert, where more than 50 individuals were released in a restricted area (Dändiker, pers. comm.)). Found individuals come mainly from Eastern-Europe, what can be corroborated by the large importations of European pond turtles from former Yougoslavia (H.-P. Schaffner, pers. comm.).
Only one individual (DG26) comes from Central France, while the whole population from Tessin forms a distinct cluster indicating a possible differentiation of this population from Brenne's and Hungarian populations. This may be due to genetic drift and inbreeding in this small population. However, as allelic richness was not lower than in the other populations (Table 9), populations seem not to be inbred. On the contrary, it harbours more observed heterozygosity than the young population from Moulin-de-Vert Table 8), what may be due to released individuals from various locations, thus resulting in very high genetic diversity.
As 3 individuals (DG15, HPS3 and HPS5) cluster as hybrids between populations from Hungary and Brenne, it remains possible that they originate from a region between both Eastern and Western European populations, thus making them suitable Swiss individuals. However, as these 3 individuals lack genetic data (33%, 44% and 55% resp.), care should be taken with this conclusion and further analyses with microsatellites must be done to determine their origin.

As a consequence, no relict Swiss individual is presumably identified within these found individuals, and the 3 "potential" Swiss individuals must be further analysed, in order to test if they harbour a real Swiss origin. Analysing the control region of DNA will allow to investigate their genetic diversity and to test if it corresponds to which expected in Switzerland.

4.7. Hybridization in a natural hybrid zone between subspecies (France)

The population from Brenne consists of only one genetic group, harbouring haplotype IIa and corresponds to the subspecies *E. o. orbicularis*. This corroborates previous studies (Lenk *et al*., 1999; Fritz *et al*., 2005b) who also found only one haplotype in this region. However, as they only used mtDNA as genetic markers, they were not allowed to investigate more precisely the genetic structure of the population. On the contrary, combination of mtDNA (haplotypes) and nDNA (microsatellites) information highlights some introgression with individuals located on the French Mediterranean coast, what may be due to weak gene flow between both populations. Gene flow between populations from Brenne and Southern France may occur via the river courses Rhône, Gardon and Allier northwards to the Loire basin and the Brenne region.

In the population from Western-France, all sampled individuals harbour haplotype IIa and correspond to the subspecies *E. o. orbicularis*. However, one individual with haplotype IIh was previously reported in this region, within a large sampling of more than 150 individuals (Fritz *et al*., 2005b). The occurrence of this new haplotype should be observed with caution and may be due to a single mutation event rather than to populations harbouring 2 distinct haplotypes. Such an event may due to the extreme location of the Western French population at the border range of the species, where genetic drift can be more severe than in the center of the species' range. Microsatellite data indicate a large proportion of individuals clustering with population from Brenne, indicating that Western France population are pure *E. o. orbicularis*. However, including haplotypes in the analyses reveals some introgression with other *E. o. orbicularis* populations from Southern France. The Dordogne, the Tarn, the Aveyron, the Lot, the Allier, the Gardon and the Rhône rivers systems may therefore act as biological corridors to gene flow between Western and Southern *E. o. orbicularis* populations living on the French Mediterranean coast.

In the population of Aigues-Mortes, the presence of the subspecies *E. o. hellenica* (haplotype IVa) was not expected because it originates from the Adriatic coasts of Italy and of the Balkans. As a consequence, the high proportion of *E. o. hellenica* may indicate the release of this subspecies in the region. Furthermore, introgression between *E. o. hellenica* and both *E. o. orbicularis* and *E. o. galloitalica* subspecies has been found in adult specimens, what may indicate the presence of this allochthonous subspecies in the region for several years.
On the contrary, both *E. o. orbicularis* and *E. o. galloitalica* subspecies are considered as native in this region described as an old contact zone dating from the Holocene expansion (Fritz *et al*., 2005b). The unexpected absence of pure *E. o. orbicularis* and *E. o. galloitalica*, as well as the low proportion of genes from both native subspecies (only reported in 5 hybrids) and the presence of numerous allochthonous individuals may reflect a high human impact in this region.
The absence of pure *E. o. orbicularis* and *E. o. galloitalica* may indicate that the old native populations decreased before the arrival of *E. o. hellenica* in the region, allowing an important introgression of the genomes of both native subspecies by *E. o. hellenica*. Another explanation is that *E. o. hellenica* survives or competes better than the 2 other subspecies, and that it outcompetes both subspecies. Moreover, it may be supposed that the large parts of *E. o. hellenica*'s genome found in hybrids lead to a possible genetic domination of *E. o. hellenica* on the genomes of other subspecies. However, additional data relative to the sampled site should be acquired in order to determine if both native subspecies could have decreased before or after the arrival of *E. o. hellenica*.
Despite only 2 hybrids between *E. o. hellenica* and *E. o. galloitalica* are reported in this study, the Grand-Rhône river does not seem to be a barrier to gene flow between populations living on both sides of the river, as it is reflected by the low F_{ST} value (F_{ST} = 0.039) between populations from Aigues-Mortes and Camargue.

In Camargue, only subspecies *E. o. orbicularis* and *E. o. galloitalica* are reported, while no *E. o. hellenica* are detected. The absence of this allochthonous subspecies in Camargue (compared to its high presence in Aigues-Mortes), as well as the high proportion of native *E. o. orbicularis* and *E. o. galloitalica* subspecies in Camargue's population (compared to absence in the latter population of Aigues-Mortes) argues for a less important human impact in Camargue than in Aigues-Mortes.
The high proportion (73%) of *E. o. orbicularis* relative to the lower proportion of *E. o. galloitalica* may be due to an important gene flow from Central and Western France via the Rhône corridor. A regular addition of gene flow from IIa and IVa haplotypes downwards the Rhône river may therefore occur continuously towards Camargue, possibly playing the role of a sink population for *E. o. orbicularis*.
The presence of *E. o. galloitalica* in Camargue indicates that the eastern Petit-Rhône river is not a barrier to its western dispersal, and thus gene flow may occur between both subspecies in Camargue. However, the report of only one pure *E. o. galloitalica* individual, as well as the low proportion of hybrids (21%) may suggest that the Rhône river brings a lot of gene flow from upstream regions (where *E. o. orbicularis* is abundant) and that a weak gene flow may occur in Camargue from eastwards regions harbouring *E. o. galloitalica*.
Microsatellites indicate that the Camarguan population is composed of pure haplotype IIa *E. o. orbicularis* with only 2 haplotype IIa *E. o. orbicularis* hybrids. On the contrary, when taking into account the haplotype information in the analyses, 72% of pure *E. o. orbicularis*, 7% *E. o. galloitalica* and 21% hybrids are reported in the population. This may indicate that the unique *E. o. galloitalica* individual has a strong introgressed nuclear genome with *E. o. orbicularis*.

In the population of Vigueirat, all haplotyped individuals correspond to *E. o. galloitalica*. However, despite mitochondrial data consider all individuals as *E. o. galloitalica*, no individual is clustered within cluster C2 where are assigned all the pure *E. o. galloitalica* (either by including or not haplotypes in the analyses): when haplotypes are not included in the analyses, 92% of the population clusters within C1 (the cluster including all IIa individuals from Brenne).

However, when haplotypes are included in the analyses, 39% are always included in C1, despite they harbour haplotype Va. This discordance means that nuclear genomes of these discordant inividuals *E. o. galloitalica* are strongly introgressed by nuclear genomes of *E. o. orbicularis*. This is consistent with the transmission rule of nDNA and mtDNA where nDNA is subject to introgression, while mtDNA does not recombine and is only maternally inherited. This is why all haplotyped individuals harbour haplotype Va corresponding to *E. o. galloitalica* while their nuclear genome corresponds to *E. o. orbicularis*. The large presence of hybrids between *E. o. orbicularis* and *E. o. galloitalica* argues for an important hybrid zone between both subspecies in Vigueirat, with a larger introgression of *E. o. orbicularis* into the genome of *E. o. galloitalica*. As Camargue and Aigues-Mortes are not far from Vigueirat, but that hybridization is less important in these latter 2 populations, the Petit-Rhône rivers may thus act as an important barrier to gene flow between *E. o. orbicularis* and *E. o. galloitalica* western of the Petit-Rhône rivers.

The population from Var is composed of 56% haplotype IIa (*E. o. orbicularis*) and only 44% haplotype Va (*E. o. galloitalica*) However, all individuals cluster with a high probability of assignment (98%) to C2 when including haplotypes in the analyses. When haplotypes are excluded from the analyses, all individuals cluster separately from the cluster C1 (including all the individuals from Brenne and harbouring haplotype IIa). This discordance allows to argue that the Var region is an important hybrid zone between *E. o. galloitalica* and *E. o. orbicularis* with a larger introgression of *E. o. galloitalica* into the genome of *E. o. orbicularis*. Such gene flow may occur via the Verdon and Durance rivers to the Rhône river.
The population from Var seems therefore to be genetically isolated from other Southern French populations and shares no or little gene flow with them, what can be due to its physical isolation in a valley with surrounding mountains and debouching in the Mediterranean sea far western of the Rhône Delta.

Genetic information about the relict population from Ardèche should be observed with caution, as it is considered as the unique relict population of *E. orbicularis sp.* in Ardèche. Despite the small sampling size of this population due to severe repeated bottlenecks and the absence of known haplotypes, this relict group of individuals indicates a pure *E. o. galloitalica* population. Absence of gene flow with *E. o. orbicularis* populations may be explained by the isolated situation of this population in a small area in the Cévennes massif with no possible gene flow from the Rhône nor Gardon rivers. This population should be haplotyped and monitored carefully in order to prevent and identify inbreeding depression. Such monitoring should be done to estimate the size of the remaining population, and possible reinforcements with *E. o. galloitalica* may be necessary to guarantee long-term survival of this population.

4.8. Hybridization between subspecies in an artificial hybrid zone (Moulin-de-Vert, GE)

Depending on the consideration or not of haplotypes in the analyses, different proportions of pure compared to hybridized individuals are detected.
When haplotypes'data are not considered in the analyses, the population of Moulin-de-Vert seems to be composed of 5 subpopulations, while including both nuclear and mitochondrial data suggests only 3 subpopulations. This different repartition of individuals may be due to an important impact of haplotypes data.

Without taking into account haplotypes in the analyses, a low proportion of pure individuals (28% of the population) is recorded in the population compared to the high proportion of hybrids (72%). This high rate of hybrids relative to "pure" individuals is the result of an important hybridization between the 3 subspecies present in Moulin-de-Vert.

When haplotypes are known but not included in the analyses, clusters C1 and C2 are observed to consist of only individuals harbouring either haplotype IIa (*E. o. orbicularis)* either haplotype IVa (*E. o. hellenica)* and cluster C3 is observed to consist of only haplotype Va (*E. o. galloitalica)*, while the 2 last clusters C4 and C5 consist both of haplotype IVa (*E. o. hellenica)*.

Including haplotypes in the analyses reveals a higher proportion of pure *E. o. hellenica* (57%) and low proportions of pure *E. o. orbicularis* and *E. o. galloitalica* (9% and 4% without haplotypes and 3% and 6% with haplotypes for both subspecies, resp.). Only 34% of the individuals are reported to be hybrids, with a higher proportion of introgressed *E. o. hellenica* (61% of the population) relative to introgressed *E. o. orbicularis* and *E. o. galloitalica* (23% and 16% of the population resp.).

The hybrid class Hybrids C2 was previously reported to have a lower hybridization level than the other 4 hybrid classes (9% compared to 22%, 23%, 23% and 24% corresponding to the classes Hybrids C2, Hybrids C1, Hybrids C3, Hybrids C4 and Hybrids C5 resp.). These Hybrids C2 harbour haplotype Va corresponding to the subspecies *E. o. galloitalica*. This low level of hybridization in this subspecies may be due to the lower number of detected *E. o. galloitalica* (with and without haplotypes), or to the fact that this subspecies is more sensitive to introgression than the other subspecies. Interestingly, relatively large amounts of *E. o. galloitalica* were released since the 1950's in Moulin-de-Vert (Dändiker, pers. comm.), so the low proportion of pure or hybridized *E. o. galloitalica* is unexpected and could be explained by several causes, as a non-adaptation of this subspecies to the Swiss environment, or to an inexact number of released individuals (comprising for example more *E. o. hellenica* females than expected and transmitting their haplotype to a more numerous offspring*)*, or to a high sensitivity of *E. o. galloitalica*'s genome to repeated hybridization. This may also be reflected in another hybrid population (Vigueirat, France), where all individuals harbour haplotype Va, but are considered as pure *E. o. orbicularis* because of strong introgression with this latter subspecies. Furthermore, the very low proportion of *E. o. galloitalica* in contact zones (Aigues-Mortes: 0% pure and 0% introgression; Camargue: 7% pure and one introgression; Vigueirat: 0% pure and 39% introgression) may corroborate this hypothesis in Moulin-de-Vert. However, the absence of *E. o. galloitalica* in Aigues-Mortes may also be due to Human impact, and to low natural genetic flow in Camargue and Vigueirat.

The presence of *E. o. orbicularis* in Moulin-de-Vert does not allow to assign the individual to a native population, because they are located in a population with a very high proportion of allochthonous individuals (95%) and because this site was created in 1953. In a consequence, it remains unprobable that these pure *E. o. orbicularis* individuals are native to this region. Further analyses on the genetic diversity of the control region could perhaps clarify their genetic status.

When more complex hybrids are investigated, such as F2 (hybrid between 2 F1 individuals) and Backcrosses (hybrid between one pure parent and one F1 parent), only 7% of the individuals are considered as pure *E. o. galloitalica* (as also indicated by STRUCTURE), while 36% of the individuals are considered as pure *E. o. hellenica* (a lower proportion relative to which previously indicated by STRUCTURE: 57%) and 34% as hybrids. Results provided by NEWHYBRIDS indicates however 57% of hybrids, including F1 (0.6%) and complex F2 (38%) and Backcrossed hybrid 0.8% for Backcrosses 1 (which pure parent is *E. o. galloitalica*) *and* 18% for Backrosses 2 (which pure parent is *E. o. hellenica*)).

However, when results from STRUCTURE and NEWHYBRIDS are combined, an initial proportion of 47% *E. o. galloitalica* and 53% *E. o. hellenica* can be estimated, what approximates very well the known reports of released individuals since 1953 (45% *E. o. galloitalica* and 55% *E. o. hellenica*.

As both subspecies would have been equally represented in the initial population, the higher current proportion of *E. o. hellenica* may be due to a better survival of this subspecies, or may be due to a more sensitivity of *E. o. galloitalica* to hybridization than other subspecies. However, these results should be taken with caution, as high proportions of pure *E. o. hellenica* are detected compared to other subspecies, what could be due either to a better survival of the subspecies or to the presence of old pure individuals. As a consequence, analyses should be performed with individuals aged of approximately 10-15 years with more than 7 microsatellites and coalescence analyses should be led in order to confirm these first results.

In a conclusion, even if the artificial contact zone of Moulin-de-Vert between 3 subspecies is young (less than 60 years), relatively high and complex levels of hybridization can be observed between the 3 subspecies.
Furthermore, the demography of this population having increased rapidly, these hybrids individuals could harbour a sufficient genetic diversity to adapt rapidly to new environment.

4.9. Impact of the invasive *Trachemys scripta sp.*

Competition with exotic species will be an important challenge for native species. As animals and plants are released or escape from gardens, they can spread into sensitive habitats and affect evolutionary communities. As exotics will become more numerous the next decades (esp. because of global trade and travels, as well as climate changes), indigenous species will face this new threat.
The invasive Red-Eared Slider (*Trachemys scripta sp*.) was imported in high quantities during the last decades to fulfil pet trade, and finally was released by privates into the wild. *T. scripta sp*. has already been recorded in many parts of Europe as in France, Latvia, Switzerland (Cadi *et al*., 2004, Polo-Cavia *et al*., 2007; Pupins, 2007; Outerbridge, 2008; Hohler, pers. comm.) and breeds successfully in southern Europe (Martinez-Silvestre *et al*., 1997; Quesada, 2000; Cadi *et al*., 2004). This species is highly competitive against *E. orbicularis* for basking sites (Cadi and Joly, 2000) and transmits pathogens to *E. orbicularis* (Cadi and Joly, 2004). Furthermore, a higher mortality of the native *E. orbicularis* is measured when grouped with *Trachemys scripta sp*. (Cadi and Joly, 2004). It is thus important to remove all the individuals from the wild to avoid acclimatation and adapted populations.
This turtle species is however not imported anymore and the occurrence of *Trachemys scripta sp*. in the wild should decrease dring the next decades, except if acclimated individuals form adapted populations.

4.10. Perspectives and conservation strategies

Conservation plans are implemented for *Emys orbicularis* in several countries (France, Italy, Austria, Spain), as this species is declining in most of its distribution range. Important for species' survival are: habitat preservation, restoration and management (especially of nesting sites), creation of an optimal 1000-1500m buffer zone around the wetland (depending on land structure and use) to assure terrestrial movements, connectivity between populations and wetlands, and reintroduction of suitable individuals for populations reinforcement and reintroduction.

As the taxonomic differentiation within *E. orbicularis sp*. is highly structured, it needs specific conservation strategies to preserve subspecies and genetic biodiversity of this species. In Switzerland, both native *Emys o. orbicularis* (northern of the Alps) and *E. o. galloitalica (*southern of the Alps*)* subspecies should be reintroduced. Furthermore, some behavioural characteristics may change even within a same subspecies.

Indeed southern populations are known to lay 2 small clutches (one at the beginning and the other at the end of summer) while norther populations are reported to lay only one big clutch size. As the native northern of the Alps *E. orbicularis* is known to live on southern Mediterranean coats, as well in more northern parts of Europe, this subspecies may have developped two behavioural strategies for clutch sizes. It is therefore crucial to introduce the correct subspecies and to select ecotypes living naturally in the same environment and under similar conditions than in Switzerland.

However, in the artificial allochthonous population of Moulin-de-Vert, high reproduction rate occurs to the presence of a core population restricted to a small area, with favourable microclimate, food quantity as well as numerous basking and nesting sites. In the contrary, non-native *E. orbicularis* individuals are often released in permanent ponds or lakes but may not form a core population and may lack favourable nesting sites to reproduce. Because of poor favourable conditions in many places where they have been released, allochthonous pond turtles do not form viable populations, except in the reserve of Moulin-de-Vert, and maybe in Tessin. Furthermore, poor favourable conditions in many locations in Switzerland have not allowed relict populations to survive until now.

Furthermore, individuals may harbour different behaviours relative to settled way of life. When some individuals may have a more sedentary way of life, others may harbour a more exploratory behaviour. The first individuals would be better suitable for the formation of a core population, while the addition of further more exploratory individuals may accelerate colonization of new aquatic systems.

When introducing individuals from another location or from farms into a novel environement, adaptation and imprinting to the site should be made in order to avoid a rapid loss of the individuals in neighbouring regions. Previous experiments showed however that reintroduced individuals try to escape the novel environment, what was maybe due to homing behaviour well known in many turtle species (Smar and Chambers, 2005; Formia *et al*., 2006). These individuals came however again towards the pond, but in Switzerland, where Human density is very high (128 hab. /km^2, Brookfield University), such a dispersion may result in death on numerous neighbouring roads and fields, and finally to the loss of these inividuals for reintroduction.

4.11. Future studies

As hybrids have been reported between *Emys orbicularis*, *E. o. hellenica* and *E. o. galloitalica* and that individuals from Moulin-de-Vert harbour a rapid growth rate, it would be interesting to compare growth rates, dispersal and survival rates of hybrids relative to that of pure individuals, to determine if hybrids have a better fitness than pure individuals. This could be explained by a positive effect of the new combination of genes between subspecies. This study may be conducted on the population of Moulin-de-Vert with already collected data. However, dispersal was not tested yet and should be done with radiotelemetry on both hybrids and pure individuals.

As subfossils dating from 10'000 years have already been found in Switzerland, it may be interesting to investigate the presence of old DNA with specially dedicated technologies and laboratories in order to define the old native subspecies in prehistoric times.

The presence of different *Emys orbicularis sp.* in ponds and lakes may be investigated by the use of environmental DNA. This new technique allows for definig genetic taxa in environmental samples, such as water, faeces and soil (Ficetola *et al*., 2008). Such method would rapidly and efficiently discriminate between the presence of different subspecies in water systems and would avoid to disturb ecosystems with trapping sessions.

Reintroduction of *Emys orbicularis* should be made with caution. Indeed, a study on the impact of released individuals should be made before and after such a reintroduction. The original community of animals and plants should be observed before and after several years following the release of *Emys orbicularis* in such a pond. Furthermore, the species on which they feed in the pond should be investigated by analysing DNA present in the faeces and by comparing it with sampled plants and animals present on the site, in order to determine if they feed on rare and endangered species or if they feed on any species.

5. Conclusion

Despite ancient native Swiss populations should have harboured haplotypes IIa and IVa (northern and southern of the Alps respectively), an individual found in nature with this "native" haplotype does not necessarily mean it is autochthon. The reason is that number of individuals have been translocated in Europe for centuries (Friedel, 1868; Brockmüller, 1876; Dahms, 1906) and imported in Switzerland as pets from various European countries, especially from Danube plain and former Yougoslavia (Fritz, 2004). Importations ceased with the federal law on the trade of species and only occasional individuals can be nowadays imported with specialized federal authorizations. As a consequence an individual found northern of the Alps and harbouring haplotype IIa may come from other parts of the range of *E. orbicularis*, and thus may have an allochthonous origin.

No relict population having been detected when using the efficient trapping method of the conical fishing baskets in the last non-sampled potential sites (natural reserves in cantons Thurgau, Aargau and Bern), it can be argued that no relict population has survived on the Swiss Plateau. This extinction in Switzerland may be explained by the high human density on the Swiss Plateau, by direct destruction of populations by fishery and meat consumption, by the canalizations of water systems, draining of wetlands and enrichment of dry meadows for agriculture leading to the loss of the nesting sites. Historical sources and subfossil findings dating of 10'000 years demonstrate the historical presence of *E. orbicularis* in Switzerland (Stampfli, 1983; Fritz, 2003; Sommer *et al*., 2007) until the 18th century (Fatio, 1872; Fritz, 2001).

Population structure is strongly influenced by past (glaciations constrictions and interglacial expansions, local extinctions) and present (direct or indirect human impacts) events. Because of old Pliocene and Pleistocene events, the European pond turtle *E. orbicularis sp.* shows a highly fragmented taxonomy, with haploclades I-X and numerous haplotypes.

When haplotypes are included in the analyses on hybridization, results may be slightly (population structure from Brenne) or strongly (population structure from Aigues-Mortes) modified. For a consequence, it appears that haplotypes have a strong influence on the results by reinforcing maternal information. When hybridization occurs, nuclear alleles are more subject to introgression and genetic drift than mitochondrial DNA. As a consequence, repeated introgressions with another subspecies may lead to the loss of alleles belonging to the first subspecies. Hybrids can thus have mitochondrial genes from one subspecies, while harbouring introgressed nuclear genes belonging to the other subspecies. It is thus important to combine both mtDNA and nDNA to reveal hybrids in a population.

Syntopic occurence of different haploclades probably reflects Holocene expansion and are restricted to narrow contact zones in the northern Iberian Peninsula, southern Apenine and Balkanic Peninsulas and in Southern France. In this natural hybrid zone, between *E. o. orbicularis* and *E. o. galloitalica* subspecies, the Grand-Rhône river does not seem to allow gene flow between populations located on both sides of the river, as reflected by the low levels of hybridization on the western side of the river.

However, an important Human impact may also have led to the decrease of the native populations situated on this side of the river. On the contrary, evidence is shown that a hybrid zone is located between both populations from Vigueirat and from Var, with a more important introgression of *E. o. galloitalica* by *E. o. orbicularis* on the western side of the Petit Rhône river, while a more important introgression of *E. o. orbicularis* by *E. o. galloitalica* occurs on the eastern side of the Petit Rhône river. This indicates that this river allows some gene flow between populations situated on both sides of the river.

In the artificial hybrid zone of Moulin-de-Vert (GE) allochthonous individuals are knpown to have been released since 1953 (Dändiker, pers. comm.). Pure and hybridized (or introgressed) individuals live syntopically in the reserve, and are partitioned into 3 subspecies: *E. o. orbicularis* (haplotype IIa), *E. o. hellenica* (haplotype IVa) and *E. o. galloitalica* (haplotype Va). Only *E. o. orbicularis* is considered as native northern of the Alps and individuals belonging to this subspecies could belong to relict native individuals. However, results demonstrate that they are either hybrids with allochthonous subspecies, either released individuals from Southern France.

Taxonomic differentiation should not be based exclusively on one character, but should include several data, especially in natural or artificial hybrid zones. Indeed, morphological features in turtles (especially shell characters) are often subject to homoplasy, intraspecific polymorphism, and selective pressure. Phenotypic plasticity in Testudines can thus lead to incongruence between morphological and molecular phylogenies (Barth et al., 2002; Honda et al., 2002; Van der Kuyl et al., 2002; Fritz et al., 2008, 2009; Velò-Anton *et al*., 2008; Spinks and Schaffer, 2009). When inferring taxonomy and reconstructing phylogenies of species, it is therefore crucial to consider morphological, behavioural, ecological and genetic data. Furthermore, new nuclear and mitochondrial markers need to be developped to infer more precisely phylogeography and genetics of species and populations, in order to define and delineate more precisely distinct management units.

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8. Annexes

Annexe 1: characteristics of 9 microsatellite DNA loci in 10 populations of *Emys orbicularis sp.*, where Na indicates the number of alleles in the population and Nind. the number of individuals which allele size was used in the analyses. Hs and Ho correspond to expected and observed heterozygosity, resp. r_c and r_b are indicators of null alleles fequency.

Locus	**Population**									
	BR	WF	EB	CA	VA	AR	MDV	HU	TI	HU
msEo2										
Na (Nind.)	5 (38)	5 (21)	7 (35)	8 (21)	2 (27)	3 (5)	11 (166)	6 (23)	4 (11)	6 (23)
Hs	0.730	0.415	0.651	0.440	0.576	0.180	0.770	0.674	0.591	0.674
Ho	0.750	0.500	0.657	0.530	0.484	0.200	0.755	0.636	0.545	0.636
r_c	-0.014	-0.093	-0.005	-0.093	0.087	-0.053	0.010	0.028	0.040	0.028
r_b	-0.012	-0.060	-0.004	-0.063	0.058	-0.017	0.008	0.022	0.029	0.022
msEo21										
Na (Nind.)	6 (38)	5 (21)	7 (35)	5 (12)	5 (30)	3 (6)	9 (158)	6 (22)	4 (11)	6 (22)
Hs	0.718	0.725	0.696	0.691	0.707	0.681	0.687	0.740	0.690	0.740
Ho	0.543	0.800	0.571	0.667	0.452	0.167	0.518	0.818	0.455	0.818
r_c	0.139	-0.049	0.098	0.018	0.220	0.607	0.140	-0.050	0.206	-0.050
r_b	0.102	-0.043	0.073	0.014	0.149	0.306	0.100	-0.045	0.139	-0.045

msEo29										
Na (Nind.)	4 (34)	8 (12)	9 (34)	5 (14)	3 (11)	4 (5)	15 (163)	7 (17)	8 (7)	7 (17)
Hs	0.568	0.653	0.822	0.681	0.718	0.620	0.784	0.772	0.837	0.772
Ho	0.500	0.571	0.765	0.25	0.533	0.200	0.721	0.824	0.857	0.824
r_c	0.064	0.067	0.036	0.463	0.147	0.512	0.042	-0.033	-0.012	-0.033
r_b	0.043	0.049	0.031	0.256	0.107	0.259	0.035	-0.029	-0.011	-0.029
msEo41										
Na (Nind.)	11 (35)	13 (14)	14 (34)	8 (16)	3 (30)	10 (6)	16 (168)	13 (21)	6 (11)	13 (21)
Hs	0.801	0.870	0.892	0.793	0.828	0.403	0.816	0.880	0.769	0.880
Ho	0.829	1.000	0.941	0.714	0.806	0.333	0.544	0.810	0.545	0.810
r_c	-0.017	-0.070	-0.027	0.052	0.013	0.094	0.200	0.042	0.170	0.042
r_b	-0.015	-0.070	-0.026	0.044	0.012	0.050	0.150	0.037	0.126	0.037
GmuB08										
Na (Nind.)	4 (36)	14 (23)	5 (34)	4 (22)	2 (30)	2 (6)	6 (174)	4 (23)	5 (11)	4 (23)
Hs	0.631	0.648	0.639	0.656	0.487	0.500	0.782	0.353	0.682	0.353
Ho	0.686	0.667	0.588	0.467	0.581	0.667	0.779	0.318	0.818	0.318
r_c	-0.042	-0.014	0.042	0.168	-0.088	-0.143	0.002	0.052	-0.091	0.052
r_b	-0.034	-0.011	0.031	0.114	-0.063	-0.111	0.002	0.026	-0.081	0.026
GmuD51										
Na (Nind.)	13 (36)	9 (9)	14 (35)	11 (20)	6 (30)	12 (6)	21 (168)	13 (22)	10 (10)	13 (22)
Hs	0.789	0.806	0.853	0.890	0.857	0.778	0.886	0.863	0.870	0.863
Ho	0.743	0.714	0.914	0.857	0.903	0.833	0.892	0.773	1.000	0.773
r_c	0.030	0.060	-0.035	0.019	-0.026	-0.034	-0.003	0.055	-0.070	0.055
r_b	0.026	0.051	-0.033	0.018	-0.025	-0.031	-0.003	0.048	-0.070	0.048
GmuD87										
Na (Nind.)	10 (35)	9 (16)	10 (34)	12 (22)	3 (30)	9 (6)	26 (171)	9 (23)	6 (10)	9 (23)
Hs	0.831	0.830	0.853	0.869	0.817	0.292	0.909	0.862	0.740	0.862
Ho	0.857	0.800	0.853	0.800	0.645	0.167	0.799	1.000	0.900	1.000
r_c	-0.016	0.018	0.000	0.041	0.118	0.273	0.065	-0.074	-0.098	-0.074
r_b	-0.014	0.016	0.000	0.037	0.095	0.097	0.058	-0.074	-0.092	-0.074
GmuD93										
Na (Nind.)	6 (36)	6 (11)	11 (28)	6 (11)	4 (25)	10 (6)	16 (167)	13 (21)	7 (8)	13 (21)
Hs	0.720	0.805	0.830	0.777	0.859	0.722	0.751	0.900	0.797	0.900
Ho	0.629	0.100	0.286	0.273	0.231	0.333	0.548	0.900	1.000	0.900
r_c	0.068	0.779	0.488	0.480	0.577	0.368	0.156	0.000	-0.113	0.000
r_b	0.053	0.391	0.298	0.284	0.338	0.226	0.116	0.000	-0.113	0.000
GmuD114										
Na (Nind.)	9 (38)	8 (18)	9 (35)	7 (20)	4 (30)	8 (6)	18 (170)	10 (22)	6 (10)	10 (22)
Hs	0.753	0.730	0.823	0.662	0.813	0.625	0.811	0.808	0.780	0.808
Ho	0.694	0.700	0.800	0.800	0.742	0.833	0.852	0.864	1.000	0.864
r_c	0.041	0.021	0.014	-0.094	0.046	-0.143	-0.025	-0.033	-0.124	-0.033
r_b	0.034	0.017	0.013	-0.083	0.039	-0.128	-0.023	-0.031	-0.124	-0.031

Annexe 2: Allelic diversity of each locus for each population with the number of analysed individuals given in brackets.

	BR	WF	EB	CA	VA	AR	MDV	Tessin	HU
msEo2	5 (38)	5 (21)	7 (35)	8 (21)	3 (27)	2 (4)	11 (166)	4 (11)	6 (23)
msEo21	6 (38)	5 (21)	7 (35)	5 (12)	4 (30)	3 (5)	9 (158)	4 (11)	6 (22)
msEo29	4 (34)	8 (12)	9 (34)	5 (14)	4 (11)	3 (4)	15 (163)	8 (7)	7 (17)
msEo41	11 (35)	13 (14)	14 (34)	8 (16)	10 (30)	2 (5)	16 (168)	6 (11)	13 (21)
GmuB08	4 (36)	4 (23)	5 (34)	4 (22)	2 (30)	2 (5)	6 (174)	5 (11)	4 (23)
GmuD51	13 (36)	9 (9)	14 (35)	11 (20)	12 (30)	4 (5)	21 (168)	10 (10)	13 (22)
GmuD87	10 (35)	9 (16)	10 (34)	12 (22)	9 (30)	1 (5)	28 (171)	6 (10)	9 (23)
GmuD93	6 (36)	6 (11)	11 (28)	6 (11)	10 (25)	3 (5)	16 (167)	7 (8)	13 (21)
GmuD114	9 (38)	8 (18)	9 (35)	7 (20)	8 (30)	3 (5)	18 (170)	6 (10)	10 (22)