

# Distribution of mtDNA haplotypes (cyt *b*) of *Emys orbicularis* in France and implications for postglacial recolonization

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**Abstract.** The European pond turtle, *Emys orbicularis*, is a wide ranging species, distributed from Northwest Africa over a large part of Europe and Asia Minor to the Caspian and Aral Seas. For 106 pond turtles from France mtDNA sequence variation has been assessed, using a 1031 bp portion of the mitochondrial cytochrome *b* gene. Three of nine haploclades currently known from the entire species' range were found in France. One clade (II) is represented with four very similar haplotypes, differing by one mutation, and the two other clades (V, VI) are represented with one haplotype each. A syntopic occurrence of clades II and V is reported for the first time for the Camargue. Besides, clade II occurs in the French regions Aquitaine, Centre-Val de Loire, and Rhône-Alpes. Outside of France, it is found mainly in the catchment areas of the Danube and Oder rivers and in the Balkans. Haploclade V, which is also known from the Apennine peninsula, Sardinia, and the northern Mediterranean coast of Spain, is restricted in France to Corsica and the Provence-Alpes-Côte d'Azur region. A single individual bearing a haplotype of an Iberian and North African clade (VI) was found in Aquitaine near Pau. This could indicate gene flow between the Iberian peninsula and West France, if the specimen is native. The distribution of the distinct haploclades in France probably reflects Holocene range expansions, especially of haploclade II turtles. In the postglacial, haploclade II terrapins arrived from the east and spread over the Rhône corridor to the Mediterranean coast. In the southern Rhône area they met and hybridized with haploclade V turtles. Further research is needed to clarify whether this hybridization is a locally restricted phenomenon.

## Introduction

The distribution of the European pond turtle, *Emys orbicularis* (L., 1758), covers Northwest Africa north of the Atlas Mts., a fair part of Europe south of Scandinavia and Asia Mi-

nor and reaches eastwards to the Caspian and Aral Seas (Fritz, 2003). For decades, *E. orbicularis* was thought to be a textbook example of a wide ranging monotypic species (e.g., Boulenger, 1889; Wermuth and Mertens, 1961, 1977; Ernst and Barbour, 1989). However, recent research demonstrated that it is one of the most fragmented and structured reptile taxa of the western Palearctic. Currently, 13 morphologically distinctive subspecies are recognized, which largely correspond, as far as studied, with mtDNA lineages or haplotypes (review in Fritz, 2003). Our ongoing investigations of the mitochondrial phylogeography of *E. orbicularis* (Lenk et al., 1998, 1999; Fritz et al., in press) led to a considerable refinement of the understanding of the zoogeography of the species (Fritz, 2003). Until now only 31 specimens from six localities in France have been studied genetically (Bouches du Rhône: 1, Haute Corse: 2 specimens from 2 localities, Indre: 9, Rhône: 1, Var: 18; Lenk et al., 1999), although many French populations are currently monitored for ecological and morphological research (Sauret and Ri-

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chon, 2002; Cadi, 2003; Olivier, 2003). Here we report results of mitochondrial haplotyping of *E. orbicularis* from France and raise the number of studied specimens and localities considerably. We compare these data with earlier morphological findings and put them into a zoogeographic scenario for West Mediterranean *E. orbicularis* to provide a basis and stimulus for further research.

## Materials and methods

Blood samples of 106 *Emys orbicularis* from the French regions Aquitaine (14), Centre-Val de Loire (18), Corse (4), Provence-Alpes-Côte d'Azur (53), and Rhône-Alpes (17) have been obtained and stored as described in Haskell and Pokras (1994) and Arctander (1988). Exact localities are as follow:

AQUITAINE: *Dordogne*: Montpon-Ménéstérol (2); *Gironde*: Cadaujac (1), Le Haillan (5), Martignas-sur-Jalle (1); *Landes*: Gaillères (1); *Pyrénées Atlantiques*: Bassussary (2), Lescar, Serres-Castet (2).

CENTRE-VAL DE LOIRE: *Indre*: four ponds in Brenne (18).

CORSE: *Haute Corse*: Biguglia (2), Bravone (1), Fango (1).

PROVENCE-ALPES-CÔTE D'AZUR: *Bouches du Rhône*: Mas Thibert (2), Tour du Valat (31); *Var*: Plan de la Tour (2), Ramatuelle (15), St. Tropez (3).

RHÔNE-ALPES: *Ain*: Saint André de Corcy (2); *Isère*: Isle Crémieu, Morestel (14); *Rhône*: Lyon (1). The specimens from Saint André de Corcy and Lyon were perhaps released there as no other European pond turtles have been recorded at these localities for many years.

Total genomic DNA was extracted following standard proteinase K and phenol-chloroform protocols (Sambrook et al., 1989). PCR and sequencing are explained in detail in Lenk et al. (1999). Our target sequence is the mitochondrial cytochrome *b* gene (cyt *b*). Of the 1031 aligned sites, 69 are variable. 63 substitutions are transitions, and six are transversions; 41 sites are parsimony informative. 13 sites are variable at the first, eight at the second, and 48 at the third codon position. For each sequence, variable sites are checked individually to prevent sequencer output errors. We define haplotypes and haplotype clades according to individual mtDNA sequences (Lenk et al., 1999). Haplotype nomenclature follows Lenk et al. (1998, 1999). Newly identified haplotypes belonging to one of the previously known clades are bearing the Roman numerals for lineages of Lenk et al. (1998, 1999) and Fritz et al. (in press) and are specified by the addition of consecutive letters. For EMBL accession numbers see table 1.

To reveal how all haplotypes and lineages are related, we calculated a minimum spanning network with the program Arlequin (Schneider et al., 2000). In this network presentation all 44 haplotypes have been included which were

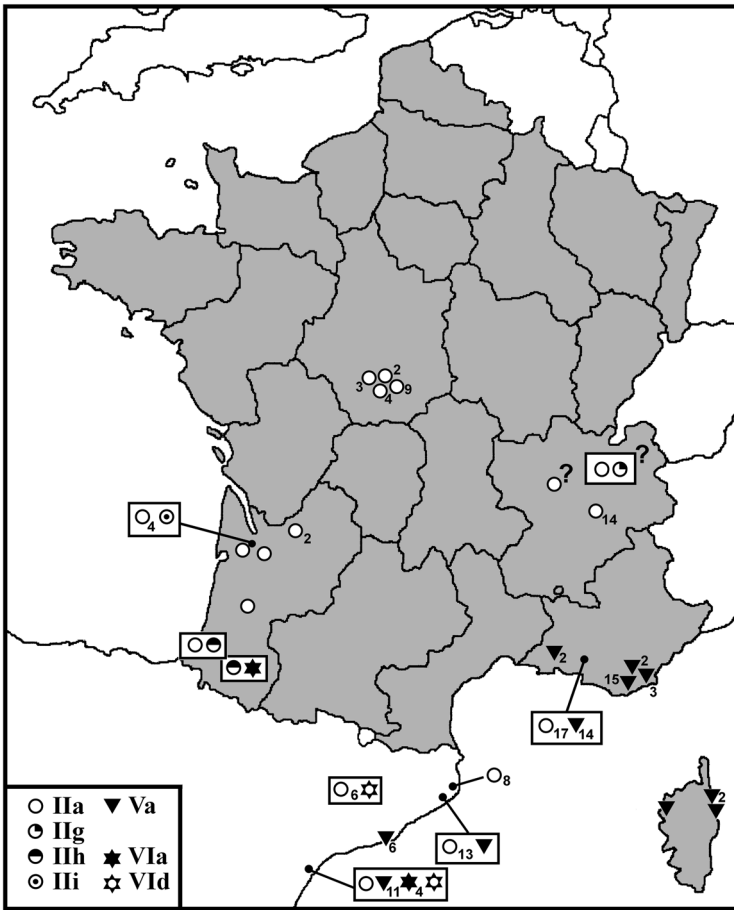
**Table 1.** EMBL accession numbers of *Emys orbicularis* mtDNA haplotypes.

Haplo-type	Accession Number	Haplo-type	Accession Number
Ia	AJ131407	IVa	AJ131417
Ib	AJ131408	IVb	AJ131418
Ic	AJ131409	IVc	AJ131419
Id	AJ131410	IVd	AY652871
Ie	AY652865	IVe	AY652884
If	AY652866	IVf	AY652872
Ig	AY652867	IVg	AY652873
Ih	AY652868	IVh	AY652874
Ii	AY652879	Va	AJ131420
Ij	AY652880	Vb	AY652875
Ila	AJ131411	Vc	AY652876
Ilb	AJ131412	VIa	AJ131421
Ilc	AJ131413	VIb	AJ131422
Ild	AJ131414	VIc	AJ131423
Ile	AY652869	VI d	AJ131424
Ilf	AY652881	VIe	AY652877
Ilg	AY652870	VIIa	AJ131425
Ilh	AY652888	VIIb	AJ131426
Ili	AY652882	VIIIa	AY652878
Ilj	AY652889	IXa	AY652887
IIk	AY652883		
IIIa	AJ131415		
IIIb	AJ131416		
IIIc	AY652890		

identified in the course of our ongoing investigations on the systematics and phylogeography of *E. orbicularis*, based on more than 950 samples from many parts of the species' range (Lenk et al., 1999; Fritz et al., in press). In the network, haplotypes are connected in the most parsimonious way in that the overall number of putative mutations leading from one haplotype to another is minimized. In contrast to phylogenetic trees, networks allow for persistent ancestral nodes and reticulations. Thus, a network is able to demonstrate alternative evolutionary pathways at the same time. The occurrence of reticulations visualizes ambiguous or uncertain domains. In haplotypic data, loops may also indicate the occurrence of reverse or parallel mutations. Moreover, the position of a haplotype in a network implies some information about its age. Older haplotypes are thought to have a greater likelihood of becoming interior in a network (Posada and Crandall, 2001).

## Results

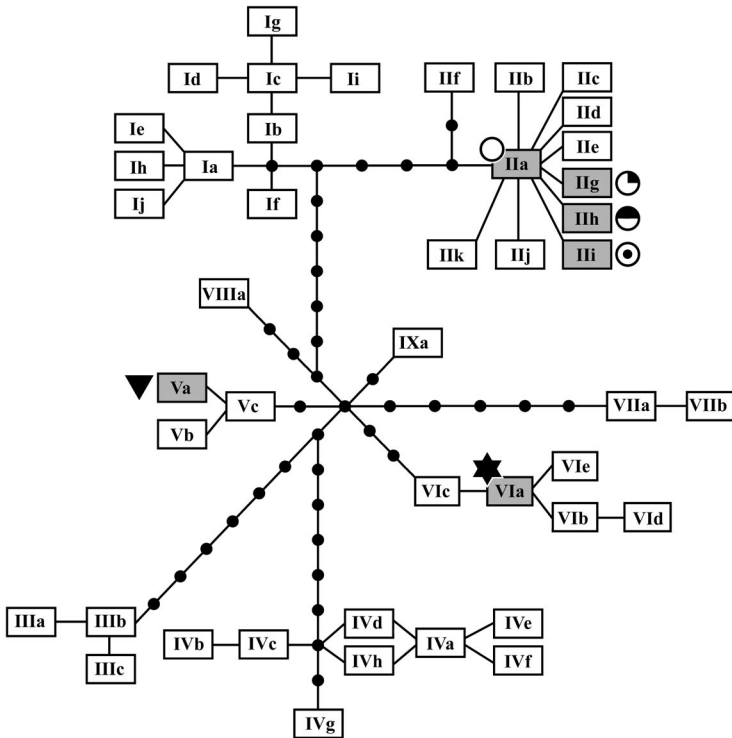
We discovered six haplotypes in France, belonging to lineages II, V and VI of Lenk et al. (1998, 1999). Lineage II occurs with four individual haplotypes, differing by one mutation (IIa, IIg, IIh, Ili), and lineages V and VI are represented with one haplotype each (Va, VIa;



**Figure 1.** Distribution of mtDNA haplotypes (*cyt b*) in France (gray) and adjacent Spain. Borders within France are administrative regions. Numbers by the symbols indicate the number of specimens bearing this haplotype, symbols in boxes stand for the syntopic occurrence of different haplotypes. The question marks denote two French localities (Saint André de Corcy, Lyon), which could represent allochthonous specimens. More west- and southwards, the Iberian peninsula is inhabited exclusively by haploclade VI turtles. Data for Spain from Lenk et al. (1999).

figs 1-2). Three of the haplotypes of clade II (IIg, IIh, IIi) were identified for the first time and have not been found in other parts of the species' range. The network (fig. 2) reflects the nine previously identified major phylogeographic lineages of *Emys orbicularis* (Lenk et al., 1999; Fritz et al., in press); only one loop is present within haplotypes of a lineage not occurring in France (lineage IV, corresponding to *E. o. hellenica*). According to the network, the French haplotypes IIg, IIh and IIi are derived from the ancestral and geographically widespread haplotype IIa. Besides continental France it is distributed in the catchment areas of

the Danube and Oder rivers, in the southeastern Balkans, and in northeastern Spain (Lenk et al., 1999). Also an introduced population on Mallorca harbors this haplotype (Fritz et al., 1998; Lenk et al., 1999). Haplotype Va is confined in France to Corsica and the Provence (fig. 1). All four samples from Corsica and all 20 samples from the Department Var represent haplotype Va, and the same is true for two samples from the environs of Mas Thibert (Bouches du Rhône). However, we provide for the first time evidence that haplotype Va occurs syntopically with haplotype IIa in the Camargue. Among 31 samples from the Tour du Valat area (Camar-



**Figure 2.** Minimum spanning network of all 44 hitherto detected *Emys orbicularis* mtDNA haplotypes (cyt *b*). Each dot or box symbolizes an identified or missing haplotype, a line between two dots or boxes a single mutation step. Gray boxes with symbols indicate haplotypes found in France. The symbols are the same as for these haplotypes in figure 1.

gue), 17 correspond to haplotype IIa and 14 to haplotype Va. Otherwise, haplotypes of lineage II are found in the west and in the more northern regions of France (Aquitaine, Centre-Val de Loire, Rhône-Alpes). All 18 specimens from Brenne (Centre-Val de Loire) have the same haplotype (IIa), while in Aquitaine among 13 lineage II samples ten represent haplotype IIa, two represent IIIh, and one represents IIIi. A further sample from Aquitaine, from a locality close to the Spanish border (Serres-Castet, near Pau), represents our sole record for haplotype VIa. Among the 17 lineage II samples from the Rhône-Alpes region, 16 correspond to haplotype IIa and one to haplotype IIg.

### Zoogeography and discussion

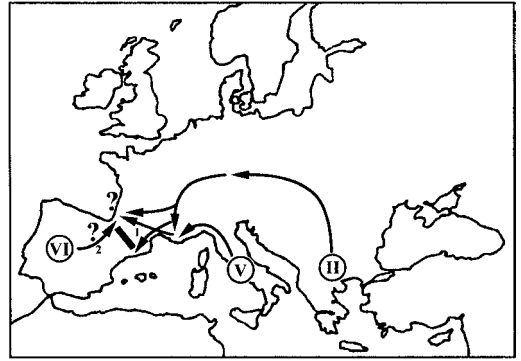
The distribution of *Emys orbicularis* mtDNA haplotypes in France matches the general zoo-

geographic and phylogeographic picture drawn by Fritz (1996, 2003), Lenk et al. (1999), and Mascort et al. (2000): During the last glacial *E. orbicularis* was forced to retreat into refugia which were positioned, among others, along the northern Mediterranean coast. In the glacial refugia populations survived which represented already different taxa. Based on more than 400 specimens of a fair part of the species' range, Lenk et al. (1999) reported for the mtDNA lineages of *E. orbicularis* a star-like phylogeny with seven main lineages (haploclades), and Fritz et al. (in press) added two further lineages. The molecular clock estimation for the origin of most haploclades presented by Lenk et al. (1999) is 3.0-4.1 million years; i.e., the initial split occurred in prequaternary times (Early to Middle Pliocene). Only the separation of lineages I and II is likely to be the result of a Pleistocene vicariance event. This is in line with our minimum spanning network (fig. 2) without

reticulations or loops connecting the mtDNA lineages, mirroring their long separation and distinctiveness.

After the Holocene climatic warming, most *E. orbicularis* subspecies expanded their ranges only moderately, due to physiographic barriers (e.g., mountain chains) and most likely also due to ecological adaptations to a mild, Mediterranean climate (Fritz, 1996). The more northern parts of West Europe and parts of Central Europe were colonized exclusively from a Balkanic refugium via the Danube river, and these new invaders were pond turtles bearing haplotypes of lineage II (Lenk et al., 1999). Generally, this lineage corresponds to a certain subspecies of the *orbicularis* subspecies group of *E. orbicularis*. Due to nomenclatural problems, Fritz (2001, 2003) applied to this taxon the provisional name *Emys orbicularis orbicularis* II. Lineage II is distributed today mainly in the catchment areas of the Danube and Oder rivers, in the Balkans and central France. In addition, turtles with haplotype IIa have been found along the northern Mediterranean coast of Spain and on Mallorca, where they occur syntopically with specimens bearing other haplotypes (Lenk et al., 1999). While this has been interpreted as a postglacial range expansion of haplotype II turtles through the Rhône corridor for northeastern Spain (Lenk et al., 1999; Mascort et al., 2000; Fritz, 2003), the syntopic occurrence of haplotypes IIa and Va on Mallorca is thought to be the result of an introduction of pond turtles by man (Fritz et al., 1998; Lenk et al., 1999).

In this study we report for the first time the presence of lineage II turtles in West France and the syntopic occurrence of haplotypes IIa and Va in the Camargue. Haplotype Va belongs to a clade (V) corresponding to the *galloitalica* subspecies group of *E. orbicularis* (Lenk et al., 1999; Fritz, 2003). Besides Mallorca and Menorca, two islands with allochthonous pond turtle populations, haplotype V is confined nearly entirely to the coastal parts of northeastern Spain, the French Mediterranean coast, the



**Figure 3.** Holocene range expansion scenario for *Emys orbicularis* mtDNA lineages in West Europe. Roman numerals refer to mtDNA lineages (haploclades) and indicate very approximately the position of the respective glacial refugia. 1: Range expansion of lineage II (*Emys orbicularis orbicularis* II) and V turtles (*Emys orbicularis galloitalica*) to the northern Mediterranean coast of Spain and perhaps to Aquitaine; 2: lineage VI turtles (*occidentalis* subspecies group) perhaps arrived in Aquitaine from the Iberian peninsula bypassing the Pyrenees in the west. The black bar symbolizes the Pyrenean mountain barrier.

western Apennine peninsula, Corsica, and Sardinia (Lenk et al., 1999; this study). On the European continent, the distribution of this haploclade matches the range of *E. o. galloitalica*.

Our discovery of a pond turtle bearing a haplotype of a North African and Iberian clade (VI) in southwestern France, from a locality close to the Pyrenees (Serres-Castet, near Pau), might represent the first proof for a natural occurrence of haploclade VI outside the Iberian peninsula in Europe. Not far away from Pau also the stripe-necked terrapin (*Mauremys leprosa*), which is widely distributed on the Iberian peninsula, has been recorded (Nive and Adour rivers, Espelette; Keller and Busack, 2001). This suggests that the haploclade VI *Emys* might be native in Aquitaine and could indicate gene flow between the Iberian peninsula and West France. Haploclade VI corresponds otherwise to the Ibero-Magrhebinian *occidentalis* subspecies group of *E. orbicularis* (Lenk et al., 1999; Fritz, 2003).

For the area under consideration, the zoogeographic scenario may be summarized as in figure 3: It is supposed that *E. o. galloitalica*

survived the last glacial along the Italian west coast and perhaps, at least in warmer phases, its refugium included also the French Mediterranean coast (Fritz, 2003). Mascort et al. (2000) believe that also on the northern Mediterranean coast of Spain relict populations of *E. o. galloitalica* persisted. In contrast to that, lineage II turtles are thought to be Holocene, i.e., postglacial immigrants from a refugium in the southern Balkans (Lenk et al., 1999; Mascort et al., 2000; Fritz, 2003). The hypothesis that the central French pond turtle populations belong to a southeast European radiation was put forward for the first time by Fritz (1992). Later, Fritz (1995, 1996) hypothesized that the populations from the southern Rhône basin, including the Camargue, and of western France are the result of an intergradation of *E. o. galloitalica* and immigrating *E. o. orbicularis* as some specimens from these areas had intermediate morphological characters and others looked like “pure” *E. o. galloitalica* or *E. o. orbicularis*. Fritz (1995, 1996) even believed that pond turtles from central France (Brenne) exhibit a certain influence of the southern subspecies in being smaller and somewhat lighter colored than *E. o. orbicularis* from the more eastern part of the subspecies’ range (from Poland eastwards). Since then it turned out that the reason for the morphological differences between the central French and more eastern populations is a taxonomic differentiation (Lenk et al., 1998, 1999; Fritz, 2001), i.e., that the eastern and French populations represent different taxa (*E. o. orbicularis* I and *E. o. orbicularis* II in the terminology of Fritz, 2001, 2003). However, the original hypothesis, that the southern Rhône basin is a contact zone between *E. o. orbicularis* and *E. o. galloitalica*, is supported by our finding of the syntopic occurrence of haplotypes IIa and Va there. On the other hand, in western France (Aquitaine) only haploclade II and the above mentioned specimen with haplotype VIa have been recorded. The following alternative hypotheses could explain this finding:

- (a) western France was colonized only from *E. o. orbicularis* II and the haplotype VIa specimen was introduced from Spain,
- (b) western France represents a natural contact zone between *E. o. orbicularis* II and Iberian pond turtles (haploclade VI),
- (c) western France is a natural contact zone between *E. o. orbicularis* II and *E. o. galloitalica*; the haplotype VIa specimen was introduced from Spain, or
- (d) western France is a natural contact zone between *E. o. orbicularis* II, Iberian pond turtles (haploclade VI), and *E. o. galloitalica*.

If hypothesis (a) is correct, the morphological characters of western French pond turtles interpreted by Fritz (1995, 1996) as indicating an intergradation between *E. o. orbicularis* and *E. o. galloitalica* (smaller size, lighter coloration, distal blotches as plastral pattern) would have another reason, e.g., clinal or ecologically caused variation. If hypothesis (b) is correct, the morphological characters of pond turtles from western France would be the result of an amalgamation of *E. o. orbicularis* II and an Iberian taxon (and not *E. o. galloitalica*). If hypotheses (c) or (d) are correct, haplotypes of clade V are yet to be discovered or must have been lost in Aquitaine. Animal mitochondrial genes have a distinctly smaller effective population size of approximately one quarter of nuclear genes as they are haploid and, as a general rule, inherited only maternally. Therefore, mitochondrial genes are more susceptible to genetic drift and introgression. In migration events, like range-expansions, mtDNA is stronger influenced by founder effects than nuclear DNA, leading to the elimination of mitochondrial gene diversity whereas nuclear diversity is distinctly less affected (Wilson et al., 1985; Birky, 1991; Hay et al., 2003; Ballard and Whitlock, 2004). Such a situation could have led to the loss of clade V haplotypes in Aquitaine pond turtle populations. On the Balearic islands exists a model for this hypothesis: On Mallorca and Menorca occur introduced

*E. orbicularis* populations. While on Mallorca haplotypes (Ia, Va) of two distinct lineages persist until today, the turtles on Menorca are bearing exclusively haplotype Va. Interestingly, Menorcan turtles are morphologically similar to specimens from Mallorca. Except their smaller size, turtles from both islands resemble in gross morphology normally native haplotype Ia populations, but differ significantly from pure *E. o. galloitalica*. This provides evidence for a loss of haplotype Ia on Menorca (Braitmayer et al., 1998; Fritz et al., 1998; Lenk et al., 1999; Fritz, 2003).

Additional morphological and nuclear DNA data are needed to understand the situation in the south and west of France better as mtDNA cannot answer some of the most urgent questions, like: (1) Is the hybridization of the different taxa a locally restricted phenomenon, as implied by the syntopic occurrence of haplotype Ia and Va only in the Camargue area, and if so, (2) could the population there represent a hybrid swarm of two biological species, i.e., without far reaching gene introgression?

Regarding haplotype II it is remarkable that we found in the most northern localities (Brenne) exclusively haplotype Ia, whereas in the more southern regions Aquitaine and Rhône-Alpes other variants (IIg, IIh, IIi) were detected. On the other hand, also in the Camargue, where haplotype Va occurs syntopically, only haplotype Ia was encountered. This finding is difficult to interpret at the moment but we wish to point out that for Brenne a genetic impoverishment seems unlikely. This region harbors the largest known *E. orbicularis* population of France with estimated more than 50,000 specimens (Servan, 2000). Taking this into account, a founder effect seems more probable as explanation, and this could be also true for the Camargue.

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