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Chapter 4
Genetic Diversity and Structure at Different Spatial Scales in the Processionary Moths

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1 Introduction

This chapter presents the evolutionary history of *Thaumetopoea* species associated with pines, as revealed by molecular markers at different temporal and spatial scales. This field of research has remained unexplored in this genus until the beginning of the 2000s, and all the data and results presented here correspond to

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relatively recent discoveries and ongoing works using both sequencing technologies and population genetics tools. Most of the subchapters focus on the winter pine processionary moths, known as the *T. pityocampa*/*T. wilkinsoni* species complex. We will first present a phylogenetic perspective of the evolution of the genus (including *Thaumetopoea* species associated with pines, cedars and broad-leaved plants), which allows to build hypotheses about the evolutionary pathways of main biogeographical and ecological traits. The second subchapter is restricted to the phylogeographical patterns of the winter pine processionary moth over the Mediterranean basin, and shows where the populations survived past climate changes, and how they managed to recolonize northern and altitudinal biotas during interglacials, eventually forming contact zones of divergent lineages.

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The third subchapter specifically addresses the question of the most recent geographical expansions, from the last glacial maximum to the fairly recent expansion that is being observed for the last decades. It opens perspectives of genetic studies developed at a local spatial scale (landscape genetics). Then the fourth subchapter gives an overview of the evolutionary history of the northern pine processionary moth, *T. pinivora*, which occurs in Europe as scattered populations and has experienced a recent outbreak on the Swedish island of Gotland. Both global and local genetic structures are studied for this species to elucidate its evolutionary history as well as its contemporary distribution dynamics. Last, we present the genetic and ecological characteristics of a very peculiar population of *T. pityocampa*, discovered in Portugal in 1997, which has a shifted phenology compared to all other populations of this species, including a sympatric one. The results suggest that this population diverged recently, and already shows signs of adaptive ecological differentiations. In brief, this chapter presents results obtained through molecular approaches that give insights about the effects of past (Quaternary) climate changes on the populations of winter and northern pine processionary moths in different regions, and allow to study the contemporary changes affecting present-day populations in front of the present climate warming.

2 Evolutionary History of the Genus *Thaumetopoea*

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2.1 Introduction

The evolutionary history of insects largely depends on a number of abiotic and biotic factors, and from their interactions. Climate changes for example can cause expansion, contraction, or fragmentation in the geographical distribution of many insect species (Hewitt 2000) determining isolation of interbreeding populations and possibly leading to allopatric speciation. Among biotic factors, host plant is probably the main source of diversification in herbivorous insects (Mitter et al. 1988). Lepidoptera developed a variety of methods to circumvent plant defences that include both behavioural and biochemical/physiological adaptations (Dussourd 1993). These adaptations in turn limit host shifts of phytophagous insects only between closely related host plants (same genus/ family) (Lopez-Vaamonde et al. 2003) or between unrelated plants with similar secondary metabolic compounds (Ehrlich and Raven 1964; Strong et al. 1984).

Another biotic factor favouring species diversification is represented by natural enemies that can promote diverse evolutionary paths toward the acquisition of enemy-free space (Singer and Stireman 2003). In particular defensive mechanisms could play an important role in the niche definition and in speciation of herbivorous insects.

Processionary moths are an interesting case to better assess the role of these main evolutionary forces in the speciation process. They are all included in the genus *Thaumetopoea* Hubner, (described in Chap. 2) (Lepidoptera Notodontidae), even though a recent revision suggested to split it into three genera (namely *Traumatocampa*, *Thaumetopoea* and *Heliantocampa*, de Freina and Witt 1982, 1987). They occur mainly in the Mediterranean and Iranoturanic regions (Agenjo 1941; Kiriakoff 1970). Larvae attack trees and shrubs of very different families (Anacardiaceae, Cistaceae, Fagaceae, and Pinaceae). They are protected against vertebrate predators attacks by urticating setae, and thereby represent a sanitary risk for humans and domestic animals (Battisti et al. 2011).

In this subchapter, we present the main results of a phylogenetic study we carried on the genus *Thaumetopoea* (Simonato et al. 2013) in order to clarify the evolution of some morphological and phenological traits. This approach is useful to address some taxonomic problems, evaluating whether the morphological characters used to define taxa agree with the phylogenetic relationships found or are rather convergent. The study covered the most common species of the genus *Thaumetopoea*, while a few rare species were not available (*T. apologetica* (Africa), *T. cheela* (India), *T. jordana* (Jordan) and *T. dhofarensis* (Oman)).

2.2   Approach

The list of the *Thaumetopoea* species considered as well as the moth species used as outgroups is provided in Table 4.1, together with information on biogeography, host plant, ecology, and morphology.

One individual per species was used in the genetic analysis. Three portions of mitochondrial DNA and three nuclear genes were sequenced for each species of the *Thaumetopoea* genus. For the outgroup taxa *Ochrogaster lunifer* (Notodontidae), *Lymantria dispar* (Lymantriidae) and *Hyphantria cunea* (Arctiidae), information was obtained from both GenBank and *de novo* sequencing. Phylogenetic trees were inferred using both a Bayesian inference (BI) and maximum likelihood (ML) methods (Felsenstein 2004; Ronquist and Huelsenbeck 2003). Posterior probabilities were calculated for each node of the BI trees. Nonparametric bootstrap tests (Felsenstein 1985) were performed to assess the robustness of ML tree topologies. All further details about sequencing and data analysis can be retrieved in Simonato et al. (2013).

Several traits listed in Table 4.1 and based on literature were used to discuss the molecular phylogeny. Biogeographic traits were based on classical zoogeographic categories and on specific areas of occurrence. Individual reports of species occurrence were checked for consistency with the most recent taxonomic position of taxa, and doubtful cases were excluded. The knowledge about host plants range, in particular for those species with a widespread and continuous distribution over large areas, was useful to define the general distribution of taxa. For each host-plant genus we assessed the presence/absence of resins, defined as hydrocarbon secretions produced and transported in specific resin ducts of plants (Kramer and Kozlowski 1979). As one prominent characteristic of the processionary moth is sociality at larval stage, four traits associated with this behaviour were identified and used in the analysis. First, foraging type was classified into three levels according to Fitzgerald (1993), i.e. nomadic (larvae moving from one patch of food to another), patch-restricted (larvae living directly on their food source), and central place (fixed nesting site from which larvae move to get food). Second, the presence/absence of a conspicuous silk tent where the larvae rest when they are not feeding was recorded. Third, gregariousness of larvae was classified as present throughout the whole stage or only in the early instars. Fourth, the procession behaviour, consisting of head-to-tail line of larvae moving in the host plant to feed or to the pupation site, was recorded as presence/absence. Two more traits associated with life history and relevant for ecological adaptation were used. They are pupation site (silk tent, litter, soil) and overwintering stage (egg, larva, prepupa/pupa) of the typical univoltine cycle of all species of *Thaumetopoea*. The frequent possibility to enter prolonged diapause over 1 or more years was not considered because of lack of precise information in several species.

The morphological traits of eggs, larvae and adults, used for taxa identification were also classified for their presence/absence or status in the different species. This was the case for the front of the adults (crested or smooth) and for the occurrence of a prominent spine on the foreleg tibia (present/absent) (Agenjo 1941; de Freina and Witt 1982, 1987). The presence/absence of scales covering the egg clusters was evaluated as well as their shape, classified into combined categories of length (short: 0.7–0.8, medium: 1.4–1.9, long: 2.3–3 mm) and width (narrow: 0.2–0.5, medium: 0.7–0.9, wide 1.6–1.7 mm) (Agenjo 1941). The colour of the scale was also considered in previous taxonomic work but could not be used in the analysis because of lack of precise information in several species. The presence/absence of urticating setae on the larvae was evaluated based on the numerous reports summarised by Kiriakoff (1970) and de Freina and Witt (1987). For the case of *T. herculeana*, reported to be non-urticating (Agenjo 1941), the larvae used for the genetic analysis were inspected. The traits were mapped on the molecular phylogeny to trace their evolution within the *Thaumetopoea* genus. The ancestral character states were reconstructed by applying the parsimony algorithms implemented in MacClade 4.08 (Maddison and Maddison 2005). The analysed characters were treated as binary or multistate and as unordered.

Table 4.1 List of *Thaumetopoea* species and traits used in the analysis

Trait	*T. bonjeani*	*T. herculeana*	*T. ispartaensis*	*T. libanotica*	*T. pinivora*	*T. pityocampa*
Biogeography						
Distribution	Localized	Localized	Localized	Localized	Localized	Widespread
Region	NW Africa	W Mediterranean	E Mediterranean	E Mediterranean	N Europe, W Europe	S Europe, NW Africa, W Asia
Zoogeography	Mediterranean	Mediterranean	Mediterranean, Iranoturanic	Mediterranean, Iranoturanic	Eurosibiric	Mediterranean, Eurosibiric
Temperature	Meso	High	Meso	Meso	Meso, low	High, meso
Humidity	Low	Low	Low	Low	Low	Low, meso
Host plant						
Host group	Gymnosperm	Angiosperm	Gymnosperm	Gymnosperm	Gymnosperm	Gymnosperm
Host family	Pinaceae	Cistaceae	Pinaceae	Pinaceae	Pinaceae	Pinaceae
Host genus	*Cedrus*	*Helianthemum*, *Cistus*	*Cedrus*	*Cedrus*	*Pinus*	*Pinus*, *Cedrus*
Anti-herbivore chemicals	Present	Present	Present	Present	Present	Present
Eaten foliage	Mature	Young	Mature	Mature	Mature	Mature
Ecology						
Foraging	Patch-restricted	Nomadic	Patch-restricted	Patch-restricted	Patch-restricted	Central place
Silk tent	Scarce	Scarce	Scarce	Scarce	Scarce	Abundant
Gregariousness as larva	Present	Present	Present	Present	Present	Present
Procession	Present	Present	Present	Present	Present	Present
Pupation	Soil	Soil	Soil	Soil	Soil	Soil
Overwintering	Egg	Egg	Egg	Egg	Egg	Larva
Diapause	Present	Present	Present	Present	Present	Present
Morphology						
Front of adult	Crested	Crested	Crested	Crested	Crested	Crested
Fore tibia	With spine	With spine	With spine	With spine	With spine	With spine
Egg scales	Present	Present	Present	Present	Present	Present
Scale length mm	1.91	1.5	1.7	1.74	1.62	2.94
Scale width mm	0.72	0.34	0.94	0.85	0.81	1.63
Scale type	Medium/medium	Medium/narrow	Medium/medium	Medium/medium	Medium/medium	Long/wide
Urticating setae on larvae	Present	Present	Present	Present	Present	Present
Urticating setae on moth	Absent	Absent	Absent	Absent	Absent	Absent

T. pityocampa ENA	T. processionea	T. solitaria	T. wilkinsoni	O. lunifer	H. cunea	L. dispar
Widespread	Widespread	Localized	Widespread	Localized	Widespread	Widespread
NE Africa	Europe	E Mediterranean	E Mediterranean	Australia	N America	Europe, Asia
Mediterranean	Mediterranean, Eurosibiric	Mediterranean, Iranoturanic	Mediterranean	Australian	Nearctic	Eurosibiric
High	Meso	High	High	High	Meso	Meso, low
Low	Meso	Low	Low	Low	Meso	Meso, high
Gymnosperm	Angiosperm	Angiosperm	Gymnosperm	Angiosperm	Angiosperm	Angiosperm, Gymnosperm
Pinaceae	Fagaceae	Anacardiaceae	Pinaceae	Leguminosae	Polyphagous	Very polyphagous
Pinus, Cedrus	*Quercus*	*Pistacia*	*Pinus, Cedrus*	*Acacia*	Polyphagous	Very polyphagous
Present	Absent	Present	Present	Present	Absent	Absent
Mature	Young	Young	Mature	Young	Young	Young
Central place	Central place	Patch-restricted	Central place	Central place	Nomadic	Nomadic
Abundant	Abundant	Scarce	Abundant	Abundant	Abundant	Absent
Present	Present	Present	Present	Present	Absent	Absent
Present	Present	Present	Present	Present	Absent	Absent
Soil	Nest	Litter	Soil	Litter	Litter	Litter
Larva	Egg	Egg	Larva	Prepupa	Pupa	Egg
Present	Present	Present	Present	Absent	Absent	Absent
Crested	Smooth	Smooth	Crested	Smooth	Smooth	Smooth
With spine	Without spine	Without spine	With spine	Without spine	Without spine	Without spine
Present	Present	Present	Present	Present, diverse	Absent, hairs	Absent, hairs
2.88	0.73	1.5	2.38	1.4	Not applicable	Not applicable
1.67	0.15	0.47	1.59	0.2	Not applicable	Not applicable
Long/wide	Short/narrow	Medium/narrow	Long/wide	Medium/narrow	Not applicable	Not applicable
Present	Present	Present	Present	Absent	Absent	Absent
Absent	Absent	Absent	Absent	Present	Absent	Absent

2.3　Phylogeny of the Thaumetopoea Genus and Evolution of the Main Traits

The phylogenetic tree obtained from the molecular data showed that *Thaumetopoea* species were split in three well supported clades named A, B, and C (Fig. 4.1).

Clade A included *T. herculeana*, *T. processionea* and *T. solitaria*. Clade B contained *T. pityocampa* ENA, *T. pityocampa* and *T. wilkinsoni*. Clade C encompassed *T. bonjeani*, *T. ispartaensis*, *T. libanotica* and *T. pinivora*. Clades B and C were grouped as sister taxa with very high BI/BT support, while clade A represented an earlier branching off within the *Thaumetopoea* genus. The evolution of several traits, ranging from biogeography, host plants, ecology, and morphology
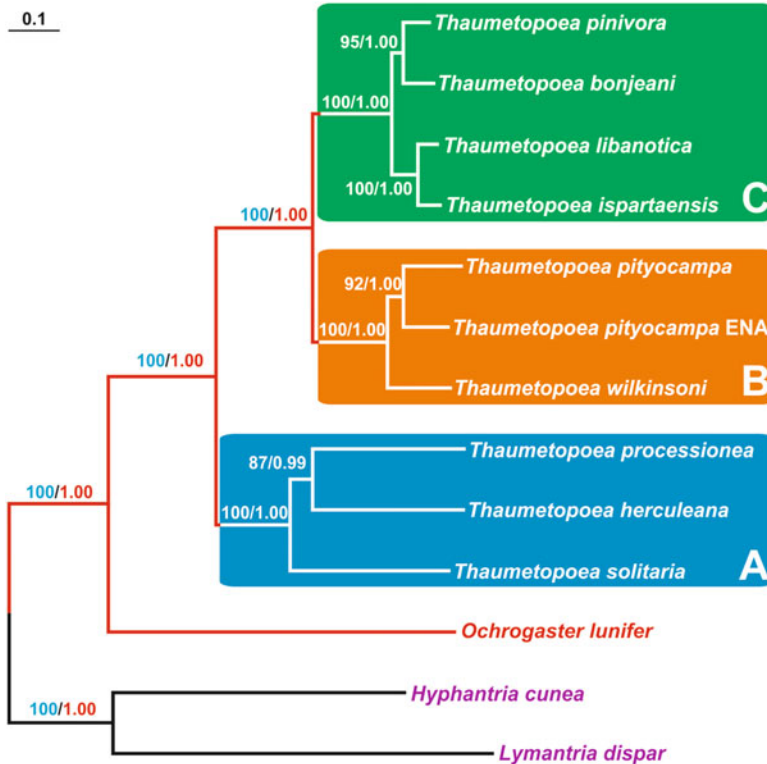
Fig. 4.1 Maximum likelihood tree inferred from all the available genes (10,357 bp) for the 13 species considered in this study. The analysis was performed by applying the evolutionary model and the partitioning scheme described in Simonato et al. (2013). The statistical support of the clustering is indicated by bootstrap/posterior probabilities figures. The bootstrap values are expressed as percentage while the posterior probabilities, computed through Bayesian inference, are presented as fraction of 1. The maximum support to the clustering is represented respectively by 100 and 1. The scale bar represents 0.1 substitutions per nucleotide site (Figure modified from Simonato et al. 2013)

was traced on the reference tree. This approach allowed to identify some of the features that characterized the common ancestor of *Thaumetopoea* moths or represented novelties restricted to some species of this genus (Fig. 4.2).

2.3.1 Biogeography

The analysis of the biogeographic traits (Fig. 4.2a) indicated that the ancestor of the genus had a Mediterranean distribution, with at least one species with such a distribution in each of the three clades of *Thaumetopoea*. The ancestor of *Thaumetopoea* had a biogeographic distribution centred in the Mediterranean basin, and it very likely derived from a stock of taxa living in the African continent where several Thaumetopoeinae genera occur, including one species of *Thaumetopoea* (*T. apologetica*) (Kiriakoff 1970). Successively, a shift to regions with continental climate (higher latitudes in Europe) occurred independently in each clade for *T. pinivora*, *T. processionea* and *T. pityocampa*, respectively. The occurrence of species with similar distribution in each of the clades excludes the possibility that geography has driven the split of the main clades within the genus. The spread outside the Mediterranean basin for *T. processionea, T. pityocampa* and *T. pinivora* was probably linked to the range expansion of their respective host plants after the last Quaternary glaciation, and occurred independently in each clade.

The confinement to separated areas is a very likely factor speeding up the speciation process, through geographic isolation, inside two clades: range fragmentation due to biogeographical events and climatic changes during the late Tertiary, influencing host availability, has been invoked to explain allopatric speciation in clade B (Kerdelhué et al. 2009) and may also explain speciation in clade C. In addition, the species of clade B have responded to the recent climate change by a rapid expansion to higher elevation and latitude (Battisti et al. 2005; Kerdelhué et al. 2009; Rousselet et al. 2010), while potential climate-driven range shifts expected in clades A and C have been hypothesized (Aimi et al. 2008; Groenen and Meurisse 2012).

2.3.2 Host Plants

Shifts in host plant use seem indeed to be the main factor that determined speciation within the genus. The analysis of the host plants shows that the genus *Thaumetopoea* is associated with four plant families, one in the Gymnosperms (Pinaceae) and three in the Angiosperms (Anacardiaceae, Cistaceae, Fagaceae) (Fig. 4.2b). All species feeding on Pinaceae belongs to clades B and C, while all species feeding on Angiosperms are grouped in clade A. Species in clades A and B appear to be oligophagous, being able to feed on more than one genus in each host plant family, while specialization toward monophagy is observed in clade C, with species associated with *Pinus* (*T. pinivora*) or *Cedrus* (*T. bonjeani*, *T. ispartaensis*, *T. libanotica*). The last common ancestor of *Thaumetopoea* moths exploited
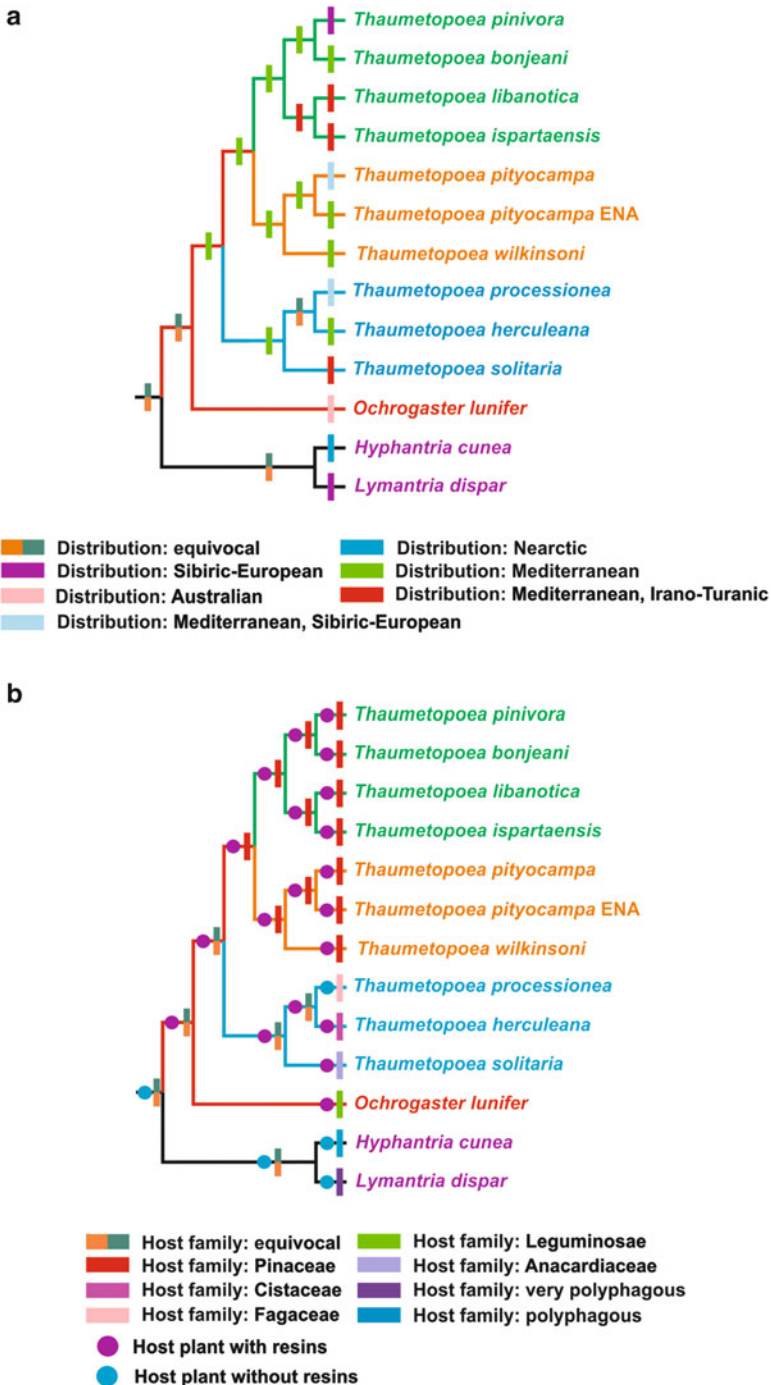
Fig. 4.2 Evolution of some characters in the *Thaumetopoea* genus. The evolutionary pathways of the investigated traits (indicated as *coloured bars* and *circles*) were reconstructed on the reference tree by applying a parsimony algorithm as reported in Simonato et al. (2013). (**a**) Evolution of biogeographic distribution; (**b**) Distribution of resins in plants and host plant family; (**c**) Foraging strategy and type of foliage eaten; (**d**) Life stage and location of overwintering; (**e**) Evolution of adult and eggs morphological features (Figure modified from Simonato et al. 2013)
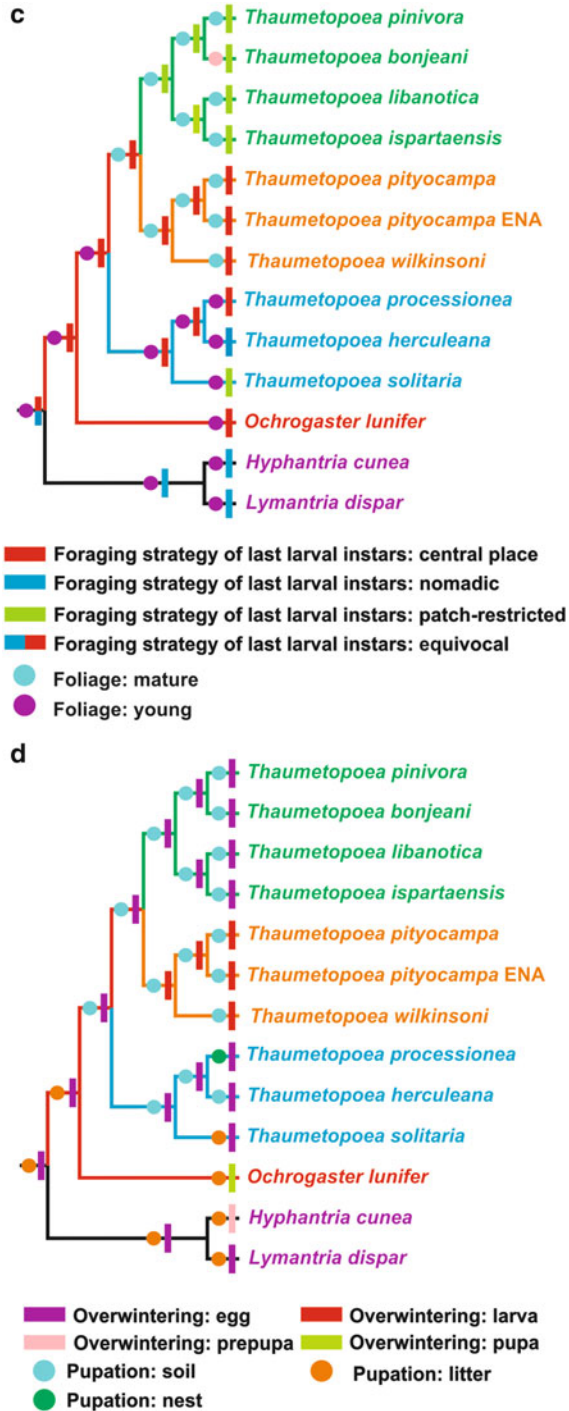
Fig. 4.2 (continued)
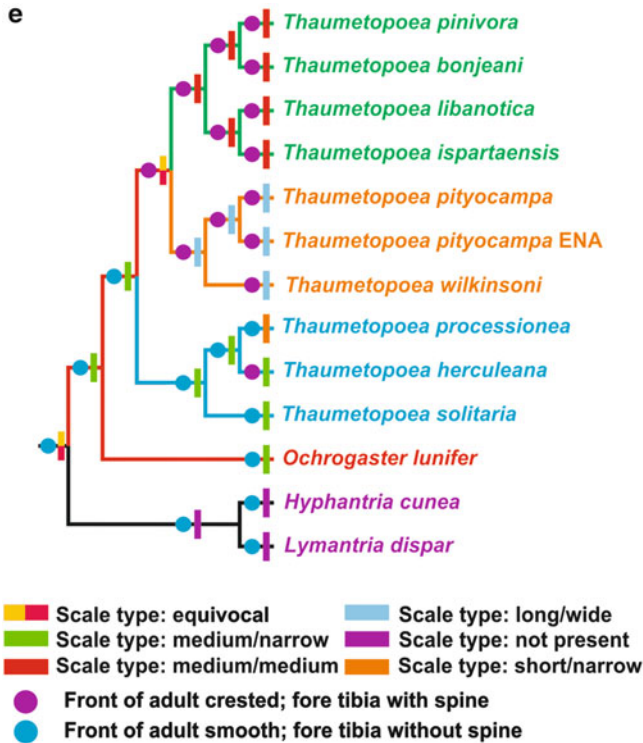
Fig. 4.2 (continued)

Angiosperm plants as larval food, but current evidence does not allow to unambiguously identify the exact family. Presence of resin canals is common to all host plants of *Thaumetopoea*, with the only exception of *T. processionea*, which feeds on Fagaceae (mainly *Quercus* spp.).

The lack of specialization suggests that the common ancestor had the capability to cope with a broad array of host plant quality, and this could have been important in the splitting and radiation of the *Thaumetopoea* moths. The shift to Gymnosperms occurred just once and it may have been facilitated by similar characteristics in morphology (for example, foliage toughness) and phytochemistry (resins) of these plants with the ancestral broad-leaved host plants. Moreover, ancestral and new host plants belong to the same ecological association of plants adapted to poor soils and first colonizers of disturbed areas.

The shift from broadleaf host plants to Pinaceae was associated with a dramatic loss of nutritional power, as pine needles contain at least three times less nitrogen than Angiosperm leaves (Kramer and Kozlowski 1979). The shift from Angiosperms to Gymnosperms corresponded also to a change of feeding from young to mature leaves: larvae preferably feed on mature leaves on Gymnosperms (clades

B and C) and on young leaves on Angiosperms (clade A). Such a change has been possible because Pinaceae retain leaves for 3 or more years but also because the mature leaves contain less defensive resin compounds than young leaves (Kramer and Kozlowski 1979). In addition, mature needles contain slightly more nitrogen than young needles (Battisti 1988). It is interesting to observe that in the species of clade C the preference for mature needles has been maintained in spite of a spring development of the larvae (Aimi et al. 2008), exactly when the young needles become available, indicating the avoidance of leaves with higher resin content.

Within Pinaceae, the species of clade B maintain a certain degree of polyphagy, being able to exploit host plants in different genera (Stastny et al. 2006; Paiva et al. 2011a), while in clade C there is a specialization for either *Pinus* (*T. pinivora*) or *Cedrus* (the other three species). The shift on *Cedrus* could not be established before the Miocene, provided that this plant genus was not present in the Mediterranean basin before (Qiao et al. 2007). Here we were unable to estimate divergence times due to the absence of calibrating points and we rely only on indirect evidence for our discussion. Irrespective of the exact time of the shift on Pinaceae, its occurrence further favoured the process of differentiation among *Thaumetopoea* moths. Indeed *Pinus* and *Cedrus* became successively isolated in the mountains of the Mediterranean region, during both glacial and interglacial periods.

2.3.3   Life History Adaptations

The shift to Pinaceae also probably led to a longer feeding and larval developmental time in order to compensate for the lower food quality. Thus the developmental time of the ancestor lineage, typically taking 2–3 months in spring, had to extend into summer, i.e. under the high temperatures that occur in the Mediterranean region and that can be fatal to larvae (Santos et al. 2011a). There are two ways by which the species associated to Pinaceae have responded to this strong constraint. The first is the change to the winter feeding observed in clade B, and the second is the extension of the developmental cycle to another year, associated with the retraction of the range to upper elevation and latitude observed in clade C. Both adaptations have a cost consisting in a longer exposure to natural enemies, and in protection from low temperature for species of clade B (tent construction, adoption of a central place foraging strategy), but also an indirect benefit because a longer developmental time is associated with higher body size of the moths, in agreement with the temperature-size rule (Zuo et al. 2012). Actually the species of clade B are those reaching the highest size in the genus, followed by those of clade C and A, respectively (Agenjo 1941; de Freina and Witt 1987). In addition, the shift to winter feeding in clade B may have contributed to reduce the competition with other pine defoliating insects, especially sawflies, which are generally active in spring. As a further matter of facts, the reversion from winter to summer feeding recently observed in a population of coastal Portugal of *T. pityocampa*, and in which a higher tolerance of the larvae to high temperature was demonstrated (Santos et al. 2011a), indicate that the trait is still

under selection and likely driven by climatic factors. The shift to winter feeding could also explain the larger egg scales of clade B, as they are functional to achieve an earlier hatching by increasing the egg temperature by several degrees (Milani 1990).

The shift to winter feeding in clade B could be also interpreted as an escape from natural enemies, especially predators and parasitoids active during spring and summer. Although winter colonies are conspicuous and exposed during a long time to generalist predators such as insectivorous birds (Barbaro and Battisti 2011), the mortality is low because of the presence of urticating setae that protect the larvae from vertebrate predators (Battisti et al. 2011). The co-occurrence of social behaviour and defensive urticating setae in the *Thaumetopoea* larvae set them apart from the juvenile stages of all other Lepidoptera species. The advantages of sociality have been described for a number of organisms, including Lepidoptera (Costa 2006). In the case of the processionary moths, it has been shown that not only sociality per se but group size matters for colony performance and survival (Pérez-Contreras et al. 2003; Aimi et al. 2008; Ronnås et al. 2010).

Foraging in group may help circumvent the host plant resistance (e.g. by weakening its defenses) and at the same time lower the probability of being attacked by a predator (Costa 2006); this is particularly true for the pine processionary moth (Fitzgerald and Panades I Blas 2003). Moreover it could represent a further defensive strategy as aposematic signal for vertebrates (Battisti et al. 2011). In the *Thaumetopoea* genus, central place species are always associated with a large tent, which may be used for several months in species overwintering as active larvae (clade B) (Fig. 4.2c); this latter condition resulted to be an evolutionary novelty characterizing clade B. In the other species only temporary tents are produced at the time of moulting, or no tent is produced in species losing gregariousness in later instars (*T. herculeana*). The central place type foraging strategy seems to be an ancestral trait that characterized the *Thaumetopoea* species (Fig. 4.2c). This status was reverted to a patch-restricted foraging strategy, which emerged independently in clade C and in *T. solitaria*. A further shift to the nomadic foraging strategy occurred in clade A for *T. herculeana,* the only species showing this foraging strategy at the larval stage in the genus and, noteworthy, also the less urticating species (Agenjo 1941). Colony movement in head-to-tail lines is instead a typical feature of all larval instars during foraging bouts on the host plant, and it occurs for most species when searching for pupation sites (Fig. 4.2d).

The evolutionary success was further enhanced by the pupation strategy that occurs in the soil in most of the species, while it is performed in the litter in *T. solitaria* or in a well-protected nest in *T. processionea*. This trait was involved for the different *Thaumetopoea* species in protection against vertebrate predation. The pupation habit appears to be linked with major morphological traits of adult moths, as those pupating in the soil have crested front and a prominent spine on the foreleg tibia (Fig. 4.2e). These traits do not seem to be the ancestral trait of the group (Fig. 4.2e). According to the transformation pathway shown in the tree, both characters represent derived traits, that co-evolved independently twice within the

genus (clade B+C *vs. T. herculeana*). Species that have switched to larval overwintering (clade B) show larger egg scales than those overwintering as an egg, which seems to be the ancestral trait (Fig. 4.2d, e). All larvae of *Thaumetopoea* carry urticating setae on the abdominal tergites starting from the third larval instar, including those of *T. herculeana*.

2.3.4 Taxonomic Implications

The agreement between molecular phylogeny and the most recent taxonomic revision of the former genus *Thaumetopoea*, resulting in three genera (de Freina and Witt 1982, 1987), is high but not complete. While the genus *Traumatocampa* matches perfectly with clades B and C, which include the processionary moths feeding on Pinaceae in winter and summer, respectively, the other two genera, *Thaumetopoea* (corresponding to *T. processionea* and *T. solitaria*) and *Heliantocampa* (including the single species *T. herculeana*), are intermixed within clade A.

Based on a good correspondence between host plant taxonomy and the placement of the species in the identified clades, we may propose hypotheses about the position of taxa that for various reasons were not included in the analysis. Indeed all taxa feeding on Gymnosperms (Pinaceae) form the monophyletic group B+C, while the species associated with Angiosperms are included in clade A. Thus we hypothesize that *T. jordana* and *T. cheela*, both feeding on Anacardiaceae, belong to clade A, as they use the same host plant family as *T. solitaria* (Trougth 1954; Furth and Halperin 1979). The same could be true for *T. dhofarensis*, found in a habitat similar to that of *T. jordana* (Wiltshire 1980) and for *T. apologetica*, restricted to Eastern Africa where there are no native Pinaceae. Conversely, the two recently described species (*T. sedirica* and *T. torosica*, that feed on Pinaceae) are predicted to belong to either clade B or clade C.

Our molecular phylogenies strongly support a parallel evolution of the morphological traits used to divide *Thaumetopoea* in the three distinct genera *Traumatocampa, Heliantocampa* and *Thaumetopoea sensu stricto* (de Freina and Witt 1982, 1987). This result leads us to suggest that all species (included or not in the present work) should be treated as members of a single genus *Thaumetopoea sensu lato*. Further systematic revision should be delayed until a complete taxonomic coverage becomes available and the phylogenetic relationships among all species are fully resolved. There is also a need for identifying new morphological synapomorphies that support the three identified clades, as those characters more commonly used (presence of the crest on the front of the adult and of the spine on the foreleg tibia, size of the egg scales) show evidence of convergent evolution. Indeed those characters are more related to ecological functions such as the emergence from the soil (Démolin 1969) and egg thermoregulation (Milani 1990), and they are present in all clades.

2.4 Summary

Comparing biological traits and distribution of most *Thaumetopoea* species through phylogenetic reconstruction, we outlined the main evolutionary factors leading to speciation in this taxon. The main split inside the genus is associated with the host plant shift from Angiosperms to Pinaceae. This host-plant shift probably implied a longer larval developmental time because of the lower nutritional quality of the leaves, thus leading to different evolutionary responses to this constraint. One of the two clades associated with Pinaceae switched the larval feeding to the cold season (winter pine processionary moths), while the other retracted its range to high elevation and latitude with a development cycle extended over 2 years (summer pine processionary moths). Moreover, recent global warming is affecting all species and seems able to further shape the evolution of the group.

   In conclusion, the split in the three major clades inside the genus can be viewed as the result of an adaptive radiation, made possible by an array of life history traits, which allowed a successful evolutionary response to changes in host use and climate. At the present state of knowledge, it is not possible to give full support to the division of the genus into three genera. More taxonomic work and the inclusion of other species will certainly help to solve this issue.

3 Phylogeography of the Pine Processionary Moth Complex: Genetic Structure and Potential Contact or Hybrid Zones

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Christian Burban, Jérôme Rousselet, Lorenzo Zane, Zvi Mendel,
and Andrea Battisti

3.1 Introduction

Past climate changes, in particular the Quaternary climatic oscillations, have had dramatic impacts on the geographic distribution, demography, and thus the evolution of species. The contemporary distribution of genetic diversity cannot be understood without studying how organisms responded to climate over geological times. The distribution ranges of temperate species were restricted during glacial maxima to a few glacial refugia and the organisms re-colonized northwards, tracking newly suitable habitats, during interglacial periods characterized by the retreat of the ice core. In this case, genetic diversity is supposed to have been retained in the glacial refugia, and to be reduced in the northern, recently colonized areas. Some re-colonization routes could be blocked by geographical barriers or by expansion

routes from other lineages (Hewitt 1996). The suture zones formed by lineages originating from different refugia and coming into secondary contact are expected to show higher genetic diversity than other geographic regions (Petit et al. 2003). The "genetic legacy of the Quaternary ice ages" (Hewitt 2000), i.e. the genetic footprint of species' responses to glacial-interglacial successions, has been extensively studied on many species in Europe and North America, that is, in the geographical regions where glaciations were most intense (Hewitt 2004; Schmitt 2007).

The effects of climatic changes on the effective population sizes were more dramatic in northern than in southern regions, meaning that northern populations should bear the signature of a rapid demographic expansion following the climate amelioration, whereas southern populations should evidence marks of more stable, long-term effective population sizes. In most cases, refugial areas were localized in the southernmost regions. Yet, cold-tolerant species may also have survived in northern or altitudinal refugia (Stewart and Lister 2001), such as the Alps, or central, eastern and northern Europe. Moreover, some species strictly depend on other organisms (hosts, mutualists, symbionts, etc.) for their development or dispersal. In that case, the phylogeographic pattern of the dependent species can be influenced by that of its partner. All these characteristics dramatically affected the present-day distribution of genetic diversity and may influence the ability of species to cope with the current global changes.

We here review the data and knowledge concerning the phylogeographic patterns of the pine processionary moth species complex (*T. pityocampa/T. wilkinsoni*, i.e., within clade B identified in the previous subchapter) over its whole geographical range, that is, around the Mediterranean basin. The aim of these studies was to characterize the geographic limits of the previously identified clades, and to analyse within-clade genetic structure. As the intensities of the climatic oscillations varied among the different regions, we expect to identify different patterns of genetic diversity. Quaternary climatic variations were most intense in Western Europe, where classical phylogeographic patterns probably occur, while the populations may have been maintained over much longer time spans in the southern rim of the Mediterranean basin as well as in the Middle East. We moreover present new evidence of a hybrid zone occurring in Turkey along a contact region between *T. pityocampa* and *T. wilkinsoni*.

3.2 Phylogeographic Patterns Over the Mediterranean Basin

3.2.1 Three Main Clades Within the Complex

To understand the geographical patterns of the distribution of genetic diversity over the whole range of the species complex, 732 individuals were sampled from 74 localities on various host species. Part of the COI and COII mitochondrial genes were analysed for each individual by SSCP and sequencing. Phylogenetic
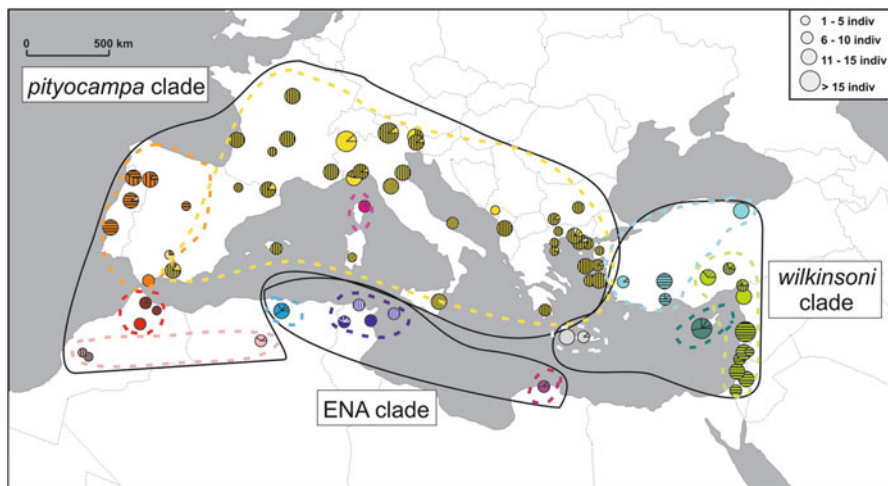
Fig. 4.3 Geographic distribution of the haplotypes found in Simonato et al. (2007) and Kerdelhué et al. (2009), showing the limits of the main clades and sub-clades identified by the phylogenetic analyses and haplotype networks

trees of haplotypes were constructed using a Bayesian approach, and statistical parsimony networks were computed within clades. All details can be found in Simonato et al. (2007) and Kerdelhué et al. (2009).

The results showed a very clear geographical pattern, with three main, parapatric mitochondrial clades (Fig. 4.3). The first clade encompasses all European localities (from the Iberian Peninsula to Greece), Morocco and one Algerian site; it will hereafter be referred to as the *pityocampa* clade, as it probably corresponds to *T. pityocampa sensu stricto*. The second clade occurs in the Middle East (Turkey, Lebanon, Israel and Cyprus), and also includes the island of Crete. It will here be called the *wilkinsoni* clade. A third clade appears to be the sister group of the *pityocampa* clade and is restricted to Eastern North Africa (part of Algeria, Tunisia and Libya). It was named the ENA clade (Kerdelhué et al. 2009).

A Bayesian analysis allowed to estimate the date of the main phylogenetic splits. Even though molecular dating using only mitochondrial markers and without calibration points should be considered with caution, it is interesting to note that the most recent common ancestor (MRCA) of all three clades could date back to 7.5 million years (Myrs) on average, which could correspond to the full opening of the Aegean trench. The existence of the ENA clade was unexpected, and its geographical range does not correspond to any obvious geographical barrier that could have hampered gene flow. It was hypothesized that a conjunction of events may explain this pattern, such as a major biogeographical event (the rise of the Tellian Atlas) combined with the late Tertiary climatic change that could have caused a gap in host availability due to arid phases (Kerdelhué et al. 2009). It also

means that the pine processionary moth species complex is ancient, and predates the Quaternary period. Moreover, each clade has diverged before the Quaternary oscillations, and thus independently experienced these past climate changes.

3.2.2   A Strong Within Clade Geographical Structure, with Contrasting Patterns

Each of the three identified clades showed a strong phylogeographical structure (Kerdelhué et al. 2009), and was composed of 3, 4 or 5 well-differentiated sub-clades (Fig. 4.4). With the notable exception of the European range (except a part of the Iberian Peninsula), each sub-clade was restricted to a rather narrow geographical region (Fig. 4.3). However, one should keep in mind that the sampling available in North Africa was too loose to definitely conclude about the exact
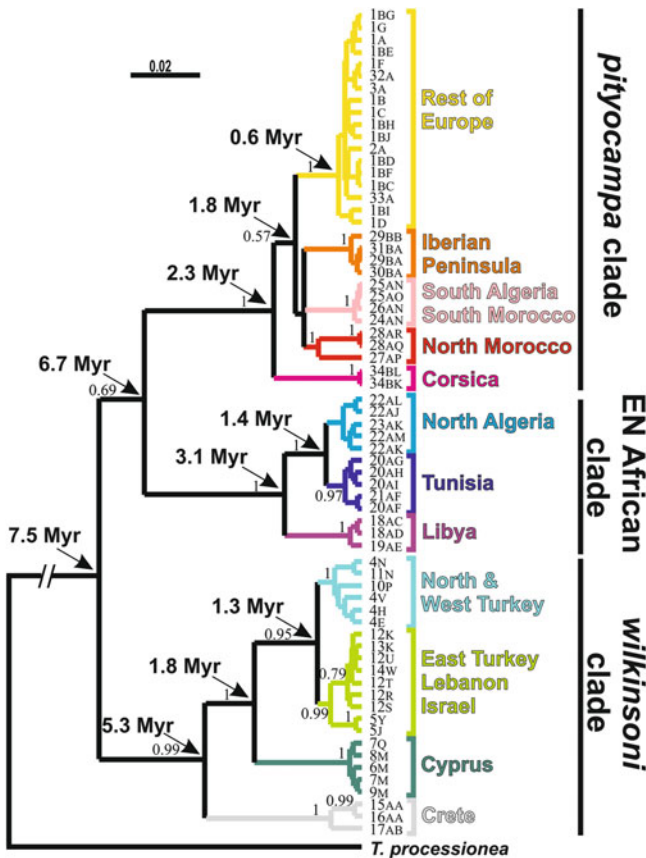
Fig. 4.4 Bayesian phylogenetic tree of haplotypes showing the main geographical clades and sub-clades. Estimated ages of MRCA and Bayesian supports over 0.5 are given (Modified from Kerdelhué et al. 2009)

geographical limits of the identified groups. Interestingly, a vast majority of haplotypes were endemic to one single population, and only five were found in three or more populations. Thus, the pine processionary moth exhibits an extreme spatial structure and a highly reduced mitochondrial female gene flow. Physical barriers as well as ecological characteristics (phenology of reproduction, that is known to differ depending on local climatic conditions (Huchon and Démolin 1970)), or local host adaptations, which in some case can represent a high selective pressure (Zovi et al. 2008) probably recurrently acted to maintain and reinforce the genetic structures. Drift associated to low female migration lead to an increase of the genetic differentiation with time, and to the existence of different mitochondrial lineages in different regions. Moreover, still with the exception of Europe, a majority of the sampled populations in the natural range of the species showed some genetic diversity, even at the rear edge of the distribution. These results suggest that populations were maintained over long periods and demographic changes allowed to retain genetic diversity despite cyclic gradations. The occurrence of potential prolonged diapause in pine processionary moth (i.e. the possible emergence of adults of the same generation over several years) probably further reduces the risk of local extinction and loss of genetic diversity. Such patterns are consistent with the fact that the Quaternary climatic oscillations had only limited effects in these regions. However, only one haplotype was found throughout Lebanon and Israel. This unexpected loss of genetic diversity is due to a very recent origin of the moth in this region, as pine trees were planted there during the afforestation process of the 1910s. The moth populations actually expanded from eastern Turkey during the twentieth century, from a single source of migrant females (Simonato et al. 2007).

In each of the three main clades, the most divergent sub-clades occur in "islands". The Corsican haplotypes were the most differentiated within the *pityocampa* clade, the Cretan and the Cypriot individuals formed the two sister-groups of all other sub-clades within the *wilkinsoni* clade, and the highly isolated moths of Cyrenaica (that can be considered as an "island" in Libya, isolated by the absence of pine hosts in the surroundings) were most divergent in the ENA clade. Based on the estimated ages of their divergence, we can suggest that the PPM survived locally on these remote islands without female exchanges from the continent during few glacial cycles. Thus, they had to evolve locally to cope with at least some Quaternary oscillations and environmental changes (Coope 2004). Yet, the estimates for the age of MRCA within islands were quite recent, and could be due to a founder effect followed by strong genetic drift in small populations. In contrast with the high genetic differentiation of Corsica, Crete and Cyprus, some islands harboured populations of moths that were genetically very close, or even similar, to their closest continental neighbours. This is not surprising for islands that are located very close to the continent, like most Greek islands or Sicily, as they can probably be recurrently colonized from mainland sources. In contrast, in spite of a high distance from the continent, the populations of Sardinia, Pantelleria, or the Balearic Islands bear haplotypes found in Tuscany, Tunisia and eastern Spain respectively, suggesting a recent accidental introduction of the moth.

The genetic patterns found in Europe were drastically different from the results obtained in all other regions (Kerdelhué et al. 2009). Most of the European range, except for the western and central part of the Iberian Peninsula, corresponded to a single sub-clade. Moreover, only one haplotype was found in most of the sampled localities (Fig. 4.3), and occurred from the Atlantic coast to Greece. Further analyses showed that the genetic patterns were consistent with a past demographic expansion. European populations underwent bottleneck events due to the consecutive glaciations and then recurrently expanded after the retreat of the ice. Such results are classically found for temperate and cold-sensitive species in this region (Schmitt 2007). The spatial distribution of the rare haplotypes helps localizing putative refugial areas where the moths survived the glaciations, and possibly also the interglacials as this Mediterranean species is susceptible to both winter cold and summer heat and aridity. Not surprisingly, such refugia were identified in southern Spain, in the Balkans and in Italy, which is typically found for European temperate species. The results also suggested that some genetic diversity was retained in the Pyrenees and in the Alps.

To decipher the role of topography in the past history of the pine processionary moth, Rousselet et al. (2010) designed a specific study in Western Europe, based on a dense sampling including the main mountainous ranges and on the sequencing of a longer fragment of the mitochondrial COI gene. This work showed that the geographical limit between the Iberian and the European sub-clades was not localized along the Pyrenees, but rather occurred in the Ebro basin (on the southern side of the Pyrenean range), where a contact zone was identified, and along the Mediterranean coasts of Spain. Moreover, it showed the existence of three lineages within the European sub-clade. One of them was restricted to the eastern Pyrenees (Pyrenean lineage), one was found in eastern Spain, southern Pyrenees and western France (Spanish-French lineage), while the third was located in Italy, the Alps as well as eastern and central France (eastern lineage, see Fig. 4.8 in the following subchapter). Interestingly, the two main lineages present in France were not separated by the Massif Central as could be expected, but a contact zone was identified on the western slopes of this mountainous region. Similarly, the Alps did not separate different lineages, but all slopes were occupied by a single haplogroup. Moreover, in each region, maximal haplotypic diversity was found at moderate elevations. All patterns thus suggest that mountainous areas did not play a role of physical barrier to dispersal, but rather provided refugia during the Quaternary climatic oscillations, allowing up- and down-movements along the slope to cope with climate change. The Pyrenean lineage was "trapped" and survived locally on montane pines without contributing to the colonization of northern territories after the last glacial maximum. On the other hand, the two other European lineages recently expanded northwards. Details about expansion routes from the main refugial areas are given in the following subchapter.

3.2.3 Nuclear and Mitochondrial Data Are Not Always Consistent

Most of the works reviewed above were based on mitochondrial data, and thus only referred to the maternal lineages. In some cases, limited nuclear data were also available for some regions, and confirmed the patterns identified with the mito-chondrial sequences. For instance, ITS1 sequences presented in Santos et al. (2007) showed a differentiation between Iberian and French populations that could reflect the mitochondrial data. Preliminary data using microsatellites in France suggested a clear differentiation between the eastern and the western populations (Kerdelhué et al. 2006), as was suggested above by the existence of two mitochondrial lineages.

Yet, in some cases, we could identify significant discrepancies between markers, which is most probably due to sex-biased dispersal (Petit and Excoffier 2009). Within *T. wilkinsoni*, the mitochondrial data suggested 4 sub-clades, namely [Crete], [Cyprus], [West-Turkey] and [East Turkey, Lebanon and Israel]. In particular, Cyprus was highly divergent from the continental clades (Fig. 4.4). Simonato et al. (2007) genotyped the same populations using 4 microsatellite loci and AFLP markers. The patterns identified were consistent between microsatellites and AFLP, but very different from the results found with the mitochondrial sequences as concerned the Cypriot populations. Nuclear results clearly placed Cyprus as a closely related sister group of southern Turkey, showing genetic similarity between the populations sampled in the island and the closest continental populations (Fig. 4.5).
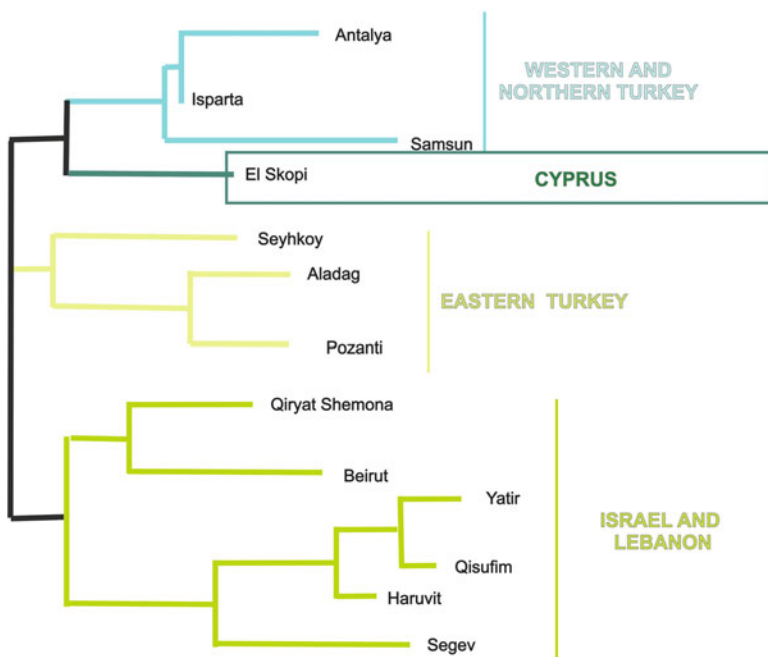
Fig. 4.5 Phylogenetic tree of populations obtained from nuclear markers (AFLP and microsatellites)

Such a pattern can occur if recurrent male gene flow occurred between the island and the continent whereas the female gene pool remained isolated in Cyprus, which is consistent with known differences in males and females dispersion capacities in the pine processionary moth. Similarly, the same study showed that nuclear genetic diversity was found in expanding populations in Israel in which mitochondrial diversity was null. Again, it was supposed that recurrent male gene flow allowed a certain recovery of genetic diversity following site colonization (Simonato et al. 2007).

3.3 Identification and Characterization of a Contact Zone Between T. pityocampa and T. wilkinsoni in Turkey

Ranges of *T. pityocampa* and *T. wilkinsoni* meet in Anatolia, as shown in Fig. 4.3. Given that these species exhibit similar habitat requirements, reproductive behaviour, and pheromone chemicals (Frérot and Démolin 1993), the existence of a potential hybrid zone can be hypothesized. Can pine processionary moth be a model organism to study interspecific hybridization, which is currently a popular topic in ecology and evolution? We here present unpublished data that encourage further research about this question (İpekdal 2012). Our objective was twofold: (1) to characterize finely the potential contact zone between both species in Turkey; (2) to compare the patterns brought by a mitochondrial and a nuclear gene to look for traces of interspecific introgression.

Pine processionary moth individuals were sampled either as larvae or male adults from various regions in Turkey, Bulgaria and Greece, including some of the Greek islands close to Turkey. For each sampled locality, the DNAs of 4–10 individuals originating from different nests were extracted, and two genes were amplified and sequenced for each individual, namely the second half of the mitochondrial COI gene (using the primer pair C1-J-2183 (Jerry) and TL2-N-3014 (Pat), Simon et al. 1994), and an exon of a nuclear gene coding for photolyase, hereafter *Pho* (Simonato et al. 2013). Mitochondrial differentiation between both species was found to be highly consistent with the phylogenetic data shown in Fig. 4.4. Mean genetic distance between *T. pityocampa* and *T. wilkinsoni* COI sequences was 0.07 (Maximum Composite Likelihood Model calculated using MEGA 5, Tamura et al. 2011). For nuclear sequences, divergence between *T. pityocampa* and *T. wilkinsoni* alleles was lower than for COI, but interspecific distances were still higher than intra-specific variability. The most parsimonious *Pho* network of alleles is provided in Fig. 4.6. Hence, it was possible for both markers to assign unambiguously each haplotype to one of the two species. We thus could identify (i) individuals characterized by both "*pityocampa*" COI and *Pho* genes; (ii) individuals with both "*wilkinsoni*" COI and *Pho* genes; (iii) individuals exhibiting "*pityocampa*" COI haplotype and "*wilkinsoni*" *Pho* alleles. We could not find any individual bearing "*wilkinsoni*" COI and "*pityocampa*" Pho sequences. Additionally, no F1 hybrids, expected to have a copy from each parent for the nuclear loci, were found in our sampling.
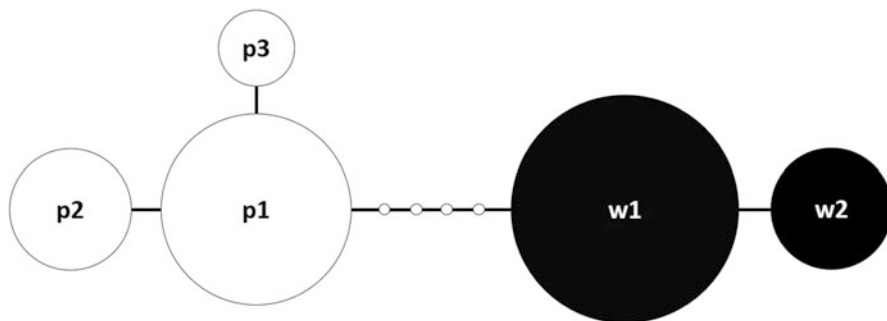
Fig. 4.6 Photolyase haplotype network for *T. pityocampa* (*white*; p1, p2, p3) and *T. wilkinsoni* (*black*; w1, w2) populations in the studied region

The cartography of the results (Fig. 4.7) evidences the existence of a parapatric distribution of the two taxa. Populations from Bulgaria (29 – see the site codes on the map), mainland Greece (28), Thassos (27) and Samothraki islands (26), and western Turkish Thrace (23–25) belonged to the *T. pityocampa* clade for both markers, while populations from Samos (8), Rhodes (7), southern (1–6) and northern (16–21) Turkey were in the *T. wilkinsoni* clade. We also found two localities, one in Tekirdağ (22) and the other in Balıkesir (15) where *T. pityocampa* and *T. wilkinsoni* individuals (i.e., identified as such by both markers) co-occurred. Moreover, introgressed individuals were found in western Turkey (9; 12–14) and the proximate islands Chios (10) and Lesbos (11). Non-introgressed individuals also occurred in each of these localities, recognized as *T. pityocampa* except for İzmir (9) where *T. wilkinsoni* occurs. This narrow region, where characteristic genes from both taxa were discovered either in the same or in different individuals, corresponds to the contact zone. The absence of F1 hybrids in such an area is noticeable, and could be linked either to the rarity of hybridization cases or to insufficient sampling.

However, introgression, characterized here as an inconsistency between mitochondrial and nuclear markers, should result from hybridization events. Cytoplasmic genes are usually exchanged more easily than nuclear genes (Ballard and Whitlock 2004). In pine processionary moth, a sex-biased dispersal has been suggested from genetic markers (Salvato et al. 2002; Simonato et al. 2007; see above, 3.2.3). As better dispersers, males could reach the edge of the distribution range more easily than conspecific females and mate interspecifically. Since mitochondrial DNA is maternally inherited, such a biased dispersal behaviour could favour mitochondrial introgression.

Introgressed individuals always had *T. pityocampa* mitochondrial haplotypes and *T. wilkinsoni* nuclear alleles. Even if more data are needed to confirm these results, this asymmetrical introgression suggests that interspecific matings occurred mainly between *T. pityocampa* females and *T. wilkinsoni* males. Such asymmetric introgression is quite frequent after range expansion, and is modulated according to disturbance, spatio-temporal demographic processes, and intraspecific gene flow (Petit and Excoffier 2009; Excoffier et al. 2009; Currat et al. 2008). Even if the phylogeography of *T. pityocampa* could suggest an eastward expansion (with
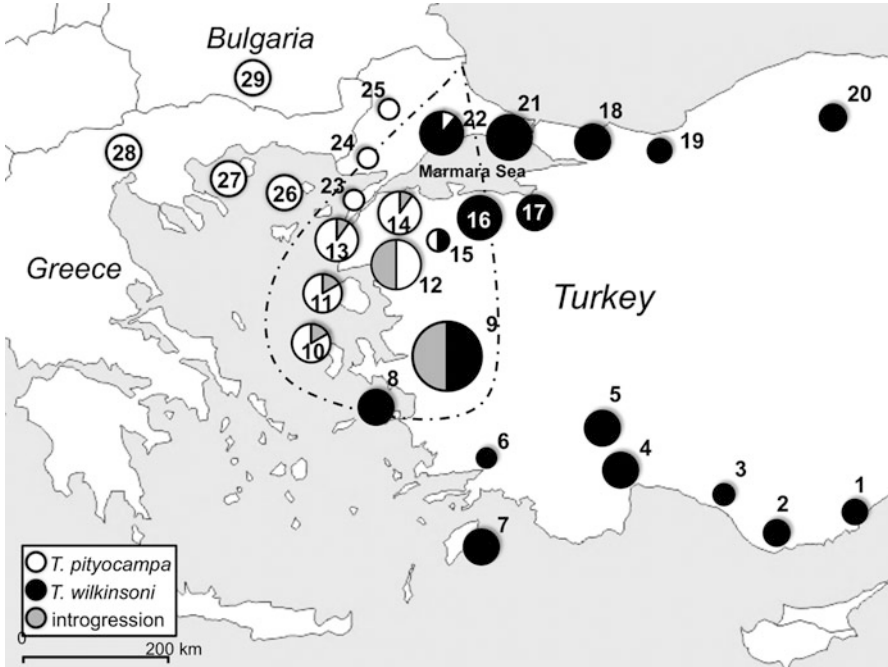
Fig. 4.7 Distribution of sampled pine processionary moth, identified as *T. pityocampa* (*white*), as *T. wilkinsoni* (*black*) and as introgressed samples (*grey*) based on sequence data. *Dotted line* shows the approximate position of the contact zone. The numbers refer to locality codes as cited in the text

reduced genetic diversity), i.e. toward the contact zone, data are less conclusive for *T. wilkinsoni*. Other biological considerations such as Haldane's rule, i.e. less viability of heterogametic hybrids (Haldane 1922; Schilthuizen et al. 2011), presence of sterilizing *Wolbachia* endosymbionts (Turelli et al. 1992; Werren 1997), or differential phenology of sexes and/or species could also be implicated.

In order to describe the present hybrid zone more precisely, more molecular markers and a larger sampling are required. A higher number of markers will make it possible to reveal mosaic patterns of introgression. ITS-1 and microsatellite primers are available for both species (Rousselet et al. 2004; Santos et al. 2007; Burban et al. 2012) and could add valuable insight to the current information. Furthermore, next generation sequencing is promising as it will allow to gather much more genomic data and to bring a precise view of genetic introgression (McCormack et al. 2013; Twyford and Ennos 2012). On the other hand, laboratory hybridization studies as well as spatially characterized bionomic surveys are needed (Bull 1991). The combination of such genetic, experimental and ecological approaches is necessary both to decipher the history of the contact zone and try to predict its future (Buggs 2007). Similar approaches should be also developed in North Africa, where different mitochondrial clades and sub-clades co-occur.

4 Range Expansion in the Pine Processionary Moth: A Population Genetics Approach

Jérôme Rousselet, Mauro Simonato, Paola Salvato, Jean-Pierre Rossi, Emmanuelle Magnoux, Alain Roques, Andrea Battisti, and Carole Kerdelhué

4.1 Range Expansion of the Pine Processionary Moth and Expected Genetic Footprints

In response to the recent climate warming, range expansion of the pine processionary moth to upper latitudes or elevations has been reported in several European countries since the beginning of the 1990s (Benigni and Battisti 1999; Démolin et al. 1996; Hellrigl 1995; Hódar et al. 2003; see Chap. 3). The present-day northern edge of the distribution area extends approximately from the Atlantic Ocean to the Black Sea (Chap. 3) and is mainly controlled by both winter and summer extreme temperatures (Huchon and Démolin 1970; see Chap. 5). The distributional change observed in the last two decades is consequently and primarily due to increased winter temperatures favouring larval survival (Battisti et al. 2005; Buffo et al. 2007; Hoch et al. 2009; Robinet et al. 2007; Chap. 3).

For a large part of Europe, the northward expansion is limited by strong continental climate or mountain chains, although an elevational shift has been observed. In the Italian Alps, the range boundary has thus shifted upwards of 110–230 m over the period 1975–2004 (Battisti et al. 2005). The plains of western France with oceanic climate have offered and still offer the pine processionary moth the widest possibilities of spatial expansion. This insect species has gained about 95,000 km^2 in France, as its range increased from 265,000 km^2 in 1979 (see C.T.G.R.E.F.-I.N.R.A. 1980) to 360,000 km^2 in 2011 (Bouhot-Delduc 2005; Robinet et al. 2010, 2014; Chap. 3).

The current range of the pine processionary moth in France has likely resulted from three major processes of northward expansion since the Last Glacial Maximum: (i) the post-glacial recolonization during the Holocene warming; (ii) the colonization of the climatically suitable areas that have been afforested during the nineteenth and twentieth centuries, especially in north-western France; (iii) the present-day climate-driven expansion observed since the beginning of the 1990s (Chap. 3). Given that pine hosts are largely available in northern France, and that winter temperatures are now favorable in the north of the expansion front, the speed of the on-going spatial expansion is henceforth mainly governed by the dispersal abilities of the moths.

Different dispersal processes can occur during range expansion and the colonization wave front can expand at different speeds according to the proportion of long-distance events (Klein et al. 2006; Kot et al. 1996; Weinberger 1982; Chap. 5). In a homogeneous environment, diffusive expansion, modeled with a thin-tailed dispersal kernel, is expected to lead to a colonization wave of constant speed (Aronson and Weinberger 1975; Kolmogorov et al. 1937), whereas stratified dispersal with

long-distance events, modeled with a fat-tailed kernel, is expected to lead to a wave of increasing speed (Garnier 2011; Kot et al. 1996). These different forms of dispersal lead to different genetic structures after spatial expansion (e.g. Bialozyt et al. 2006; Excoffier et al. 2009; Ibrahim et al. 1996; Klein et al. 2006). The rare long-distance dispersers can establish very isolated satellite populations with strong founder effects. This stratified dispersal is generally expected to generate a high spatial differentiation (Klein et al. 2006). In case of very low frequency of long-distance dispersal events, a series of strong bottlenecks or an embolism effect can produce large patches of homozygosity in the newly colonized areas (Bialozyt et al. 2006; Hewitt 1999). Conversely, in the case of diffusive processes, only the individuals in the front of colonization found the new populations. This could imply a more progressive loss of diversity during the range expansion, leading to a weaker differentiation and an isolation by distance pattern (Klein et al. 2006).

In this subchapter, we review the population genetics and phylogeographical data available for the pine processionary moth in Western Europe that allowed to detect the genetic imprints of range expansion and to propose hypotheses about the dispersal patterns that occurred at different time scales. In particular, we will show how the combined analysis of a low mutation rate mitochondrial marker and of polymorphic nuclear loci (AFLP and microsatellites) can shed light about the origins of the currently expanding populations and the on-going processes involved in latitudinal and elevational expansions.

4.2 Past Northward Expansion: Genetic Imprints of a Diffusive Dispersal

In France, the present-day northern edge appears more or less continuous from the Atlantic Ocean to the Alps (Robinet et al. 2007, 2010). The genetic data obtained with a mitochondrial marker (Rousselet et al. 2010) associated to previous field records (Démolin et al. 1996; Chap. 3) revealed that it corresponds to the recent junction of two lineages originating from different glacial refugia and coming into secondary contact in the north of the Massif Central mountains (Fig. 4.8). Details about the phylogeographical pattern in Western Europe and the distribution of each lineage are given in the previous subchapter. While the origin of the eastern lineage, distributed from eastern France to Greece, is still uncertain, the populations distributed from western France to eastern Spain (Spanish-French lineage) likely originated from a glacial refugium located in the eastern Iberian Chain. This lineage is roughly distributed along the Greenwich Meridian and is clearly south-north structured (Fig. 4.8). Its important contribution to the northward post-glacial recolonization opened the possibility to search for genetic signs of range expansion.
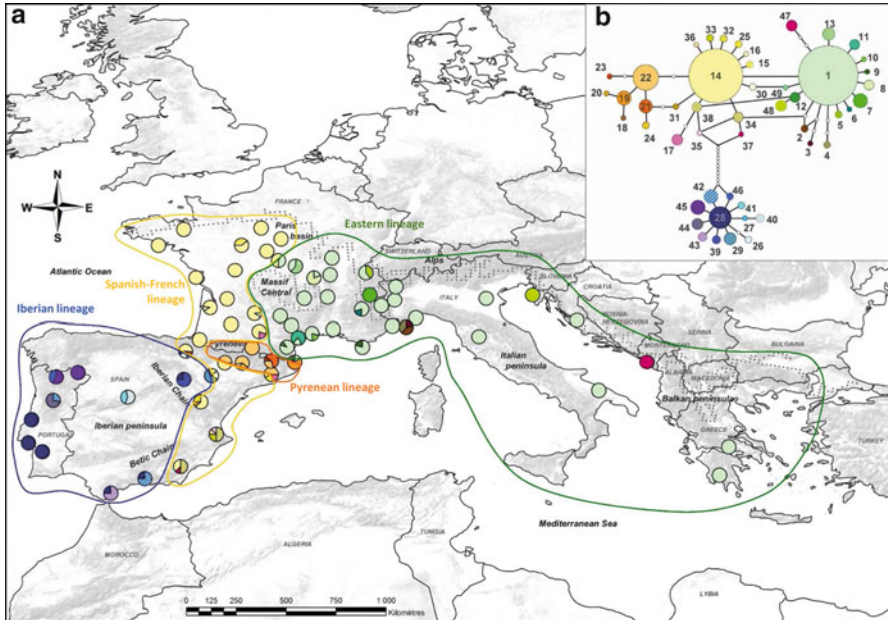
Fig. 4.8 Geographic distribution (**a**) and haplotype network (**b**) of the cytochrome c oxidase subunit I (COI) mitotypes, showing the area occupied by the four main maternal lineages identified in Europe. *Dotted line* indicates the present-day northern and elevational edges (Modified from Rousselet et al. 2010)

4.2.1 A 'Northern Purity, Southern Richness' Pattern Within the Spanish-French Lineage

The Spanish-French lineage exhibits a 'northern purity, southern richness' pattern as defined by Hewitt (1999). This population genetic structure is a typical genetic footprint of northward post-glacial expansion in cold-sensitive species. The higher genetic diversity and divergence in the southern populations ('southern richness') is attributed to their prolonged demographic stability over the Quaternary climatic oscillations, as they occur in regions where environmental conditions were suitable even during the glacial maxima. The 'northern purity' is commonly attributed to a loss of genetic variation due to serial bottlenecking associated with pioneer-like dispersal and rapid northward expansion during interglacial periods (Canestrelli et al. 2006; Bialozyt et al. 2006; Hewitt 1999). The pattern of 'refugia within refugia' can be an alternative scenario, and is well supported for the Iberian peninsula (Canestrelli et al. 2006; Gómez and Lunt 2006). Multiple, strongly isolated and differentiated refugia could have existed during the ice ages, but they did not all necessarily contribute to the northward post-glacial recolonization. Only a small subset of the overall diversity having occurred in the refugial areas could have been at the origin of the northern populations.

In the case of the pine processionary moth, over the Iberian peninsula, several differentiated refugial areas with different contributions to the northward post-glacial recolonization of Europe were identified. Rousselet et al. (2010) suggested that the different richness in host-plant species might explain these differences of contribution to the present-day range. For example, two mountain pine species, *P. nigra* and *P. sylvestris*, were probably the main continuously available hosts in the northernmost Iberian refugium located in the Pyrenees, while a Mediterranean pine species, *P. pinaster*, was also probably present and abundant in the eastern Iberian Chain (Carrión et al. 2000; De-Lucas et al. 2009). Consequently, the refugial populations of the Pyrenean lineage could have been trapped elevationally by tracking mountain pines and did not contribute to the northward expansion. While these north-eastern Iberian populations only contributed to the upward expansion, the mid-eastern Iberian populations located a little more southerly could have expanded latitudinally by tracking thermophilic lowland pine species. The expansion pathway from eastern Spain to south-western France corresponds to one of the migration routes suggested for *P. pinaster* (Salvador et al. 2000). This would be consistent with the moth following the migration route of one of its hosts, and with a 'northern purity' having resulted from a geographically restricted source of post-glacial recolonization.

However, host-plant relationships might also account for the pronounced loss of genetic diversity observed in the western French populations with the mitochondrial marker used by Rousselet et al. (2010). This oligophagous insect mostly attacks the mountain pine species and, to a lesser extent, the Mediterranean pine species (Buxton 1983; Hódar et al. 2002; Huchon and Démolin 1970; Masutti and Battisti 1990; Montoya 1981). The present-day populations of the Betic Chain in south-eastern Spain exhibited a very low larval survival on *P. pinaster* while first instar larvae were able to develop on *P. nigra* and *P. sylvestris* (Hódar et al. 2002). According to these authors, egg-laying on maritime pine can be considered (in this region) as an "oviposition mistake". The pattern of attacks reported by Montoya (1981) in the eastern Iberian Chain (i.e. mid-eastern mountainous refugial areas) is consistent with these experimental data obtained from the Betic Chain (i.e. south-eastern mountainous refugial areas). Conversely, the French populations seem to exhibit a higher plasticity in host plant use (Huchon and Démolin 1970). In south-western French lowlands, *P. pinaster* is the only native pine species and is moreover considered as a good host (Démolin 1969). In these areas likely colonized during the Holocene warming, this Mediterranean pine was available only along the Atlantic coast until recent afforestation. A host plant shift, associated to a strong bottleneck, might have been at the origin of the maternal lineage that has latitudinally expanded on Mediterranean hosts from a mountainous Iberian refuge to a lowland coastal area. A similar scenario might also explain the low mitochondrial genetic diversity of the populations feeding on *P. pinaster* in the Portuguese lowlands (see Santos et al. 2007; Rousselet et al. 2010).

In conclusion, the variability of the mitochondrial marker was sufficient to suggest a recolonization pathway between the glacial refugia in Spain and the post-glacial areas in France, but not to discriminate between different scenarios

leading to a 'northern purity' pattern. According to these scenarios, the history of the maternal lineages could be consistent with different dispersal patterns and called for the use of polymorphic nuclear markers.

4.2.2 Genetic Signs of Diffusive Dispersal Within the French Populations

The genetic structure of French populations was further studied at a finer scale using highly polymorphic markers. About 550 individuals from 20 sampling sites covering native post-glacial, afforested and recently colonized areas (Fig. 4.9) were genotyped using five microsatellite markers specifically developed (Rousselet et al. 2004). Populations from the three mitochondrial lineages occurring in France (Fig. 4.8) were sampled, with a special effort for the Spanish-French and the eastern lineages. At these scales of space and time, only population-centred analyses were conducted using the data set from Kerdelhué et al. (2006) and additional data from Robinet et al. (2012).

Genetic differentiation between sampling sites, considered as subpopulations, was analysed using F-statistics (Wright 1951). The pairwise estimates of the fixation index among subpopulations θ_{ST} (Weir and Cockerham 1984) were plotted against geographic distances. The significance of the relationship between genetic and geographic distances ($\theta_{ST}/(1 - \theta_{ST})$ against kilometers) was tested using a Mantel test (for more details see Kerdelhué et al. 2006). Genetic diversity within each subpopulation was assessed by calculating the mean number of alleles per locus.

Contrary to Kerdelhué et al. (2006), who studied fewer populations, a statistically significant and positive relationship was found with Euclidian geographic distances, but the relationship was much stronger when geographic distances between sampling sites were calculated as bypassing the Massif Central ($R^2 = 0.72$ against $R^2 = 0.37$). This nonetheless corroborates the main conclusions drawn by Kerdelhué et al. (2006) and means that (i) these nuclear and biparental markers confirmed the east/west separation observed with the maternally inherited marker; (ii) populations that are geographically closer are genetically more similar than populations that are further apart. This so-called 'isolation by distance' (IBD) pattern indicates a limited dispersal across space (Wright 1943), as suggested by Démolin (1969). Figure 4.9 indicates the main corridors of gene flows inferred from microsatellite data among the French populations. From north-western to north-eastern France, the pine processionary moth populations are thus gradually differentiated along these two axes. Nevertheless, the populations from the southern Paris basin and from the Saone valley located at each end of these corridors are strongly differentiated (pairwise θ_{ST} are about 30 %), despite the occurrence of a recent contact zone at the north of the Massif Central.

Both in eastern and western France, a linear decrease of the mean number of alleles per locus from the southern to the northern sampling sites seems to be observed (approximately from 8 alleles in the Aquitaine basin to 5 along the western part of the northern edge and from 9 in the Southeast basin to 5 in the eastern part of the northern edge; the highest value, namely 11 alleles, was observed
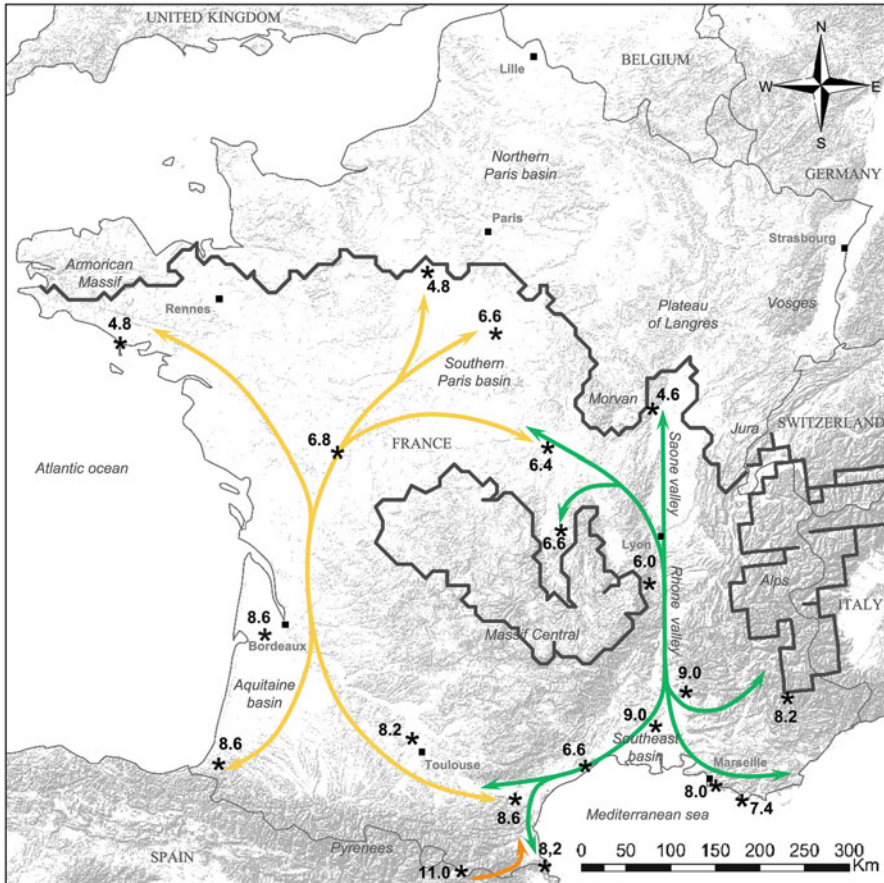
Fig. 4.9 Corridors of nuclear gene flow among the French populations of the pine processionary moth inferred from the analysis of five microsatellite markers. Note that the expansion corridors identified with these markers match the two main mitochondrial lineages shown in Fig. 4.8. *Black stars* indicate the location of the 20 sampling sites, and the values shown beside correspond to the mean number of alleles per locus. Between 25 and 30 individuals were analysed per locality

in an eastern Pyrenean population). This relationship needs to be confirmed with a higher sampling effort and more detailed analyses, but it could result from a gradual loss of genetic diversity during the past range expansion.

In conclusion, both nuclear and mitochondrial markers indicate two major corridors of northward expansion with a recent contact zone in the southern part of the Paris basin. The population genetic structure appeared to be characterized by a gradual loss of genetic diversity and an isolation by distance pattern from the southern glacial refugia to the northern areas colonized once the environmental conditions became suitable. These genetic signs are consistent with a range expansion resulting from a diffusive dispersal, i.e. only short distance individual movements. This result appeared to validate the use of a reaction-diffusion model for modelling the recent expansion of the pine processionary moth (Robinet 2006; Robinet et al. 2014; Chap. 5).

## 4.3   Current Expansion: A Combination of Active and Passive Dispersal

4.3.1   Pioneer Colonies Beyond the Wave Front and Human-Mediated Long Distance Jumps

During the 2000s, several satellite populations were detected far beyond the northern edge of the main range (Robinet et al. 2012; Chaps. 3 and 5). The first pioneer colonies discovered near Paris were located from 30 to 55 km away from the colonizing front (Robinet et al. 2012). This brings up questions about the active flight capabilities *vs.* passive dispersion of the moth in the expansion areas. Such an apparent stratified dispersal with rare long distance dispersal events was in contradiction with the genetic imprints of diffusive dispersal observed previously at large spatial and temporal scales. The flight abilities of the females measured on a flight mill suggested that these long distance jumps were due to accidental transportation rather than to active flight (Robinet et al. 2012; Chap. 2). The recent establishment of a pioneer colony found close to Strasbourg, i.e. 190 km away from the main range, was clearly incompatible with an active dispersal. Genetic markers were thus used to assess whether the five pioneer colonies occurring near Paris could originate from the expanding front located in north-western France, or likely came from further away.

Twenty individuals from the pioneer colonies were sequenced for the same mitochondrial fragment used by Rousselet et al. (2010) in order to assign them to one of the four maternal lineages described in Europe. To locate the population source more accurately, 705 individuals from 47 sampling sites over Western Europe were genotyped for five microsatellite markers and statistically assigned to their potential source among seven reference groups defined on the basis of population genetic structure (see Robinet et al. 2012).

The individuals from two of the five pioneer colonies (namely Eragny and Aubergenville) exhibited a mitochondrial haplotype belonging to the eastern European lineage rather than the Spanish-French lineage, although this latter was closer. Microsatellite markers showed consistent results (Fig. 4.10). Most of the individuals sampled in these two colonies were clearly assigned to Italy and south-eastern France, or to Italy and north-eastern France. Concerning the three other pioneer colonies (Bailly-Romainvilliers, Nanterre and Saint-Maur-des-Fossés), the haplotypes found belonged to the Spanish-French lineage. Microsatellite markers revealed that most of the individuals from Bailly-Romainvilliers were assigned to the Iberian Peninsula and to south-western France. Finally, only two colonies (Nanterre and Saint-Maur-des-Fossés) were assigned to the nearest and northern-most potential source, namely north-western France.

In conclusion, three of the five studied pioneer colonies probably originated from populations located at least 260 km apart. The precise origin of these three colonies still remains unclear because of large unsampled areas and the limited number of microsatellite markers used. Nevertheless, the result is clearly inconsistent with active dispersal abilities and provides evidence of human-mediated long
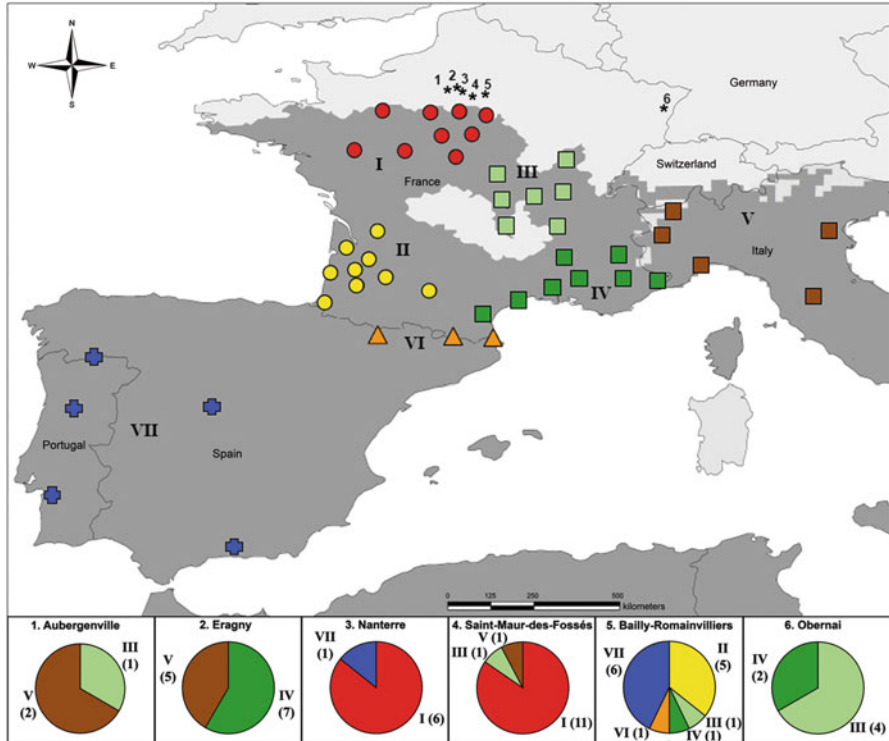
Fig. 4.10 Results of assignment tests for the six studied pioneer colonies. *Pie charts* indicate the proportion of individuals from each colony (1–6) assigned at first rank to each reference population (I–VII) using Geneclass 2.0. *Coloured shapes* on the map show the location of all the sampling sites of each reference population (using the same colour code as pie-charts). The *stars* numbered from 1 to 6 indicate the location of the pioneer colonies. Areas occupied by the moth are indicated in *dark grey* (Modified from Robinet et al. 2012)

distance jumps. To date, no egg parasitoids were detected in the pioneer colonies while some larval and pupal parasitoids were found (Robinet et al. 2012). This could indicate that the pupal stage, which is not easily detectable in the soil, was accidentally introduced into urban and suburban areas with the transplantation of large trees moved with a large amount of soil.

Concerning the two other colonies, which seem to originate from the nearby expanding front, it is difficult to definitely conclude about their colonization pathways. Noteworthy, they are both exposed to the northeastward dominant winds during the flight period in summer. Thus, although these satellite populations are likely still demographically isolated, recurrent male gene flows between them and the main range might have occurred for several generations.

4.3.2 Upward Expansion in the Alps: Impact of Gender Biased Dispersal on the Genetic Diversity of the Leading Edge

Field observations (Démolin 1969), laboratory experiments (Chap. 2) and genetic data (Salvato et al. 2002, 2005) indicate that a male-biased dispersal does occur in the pine processionary moth. Salvato et al. (2002) investigated population genetic structure and gene flow using biparentally (nuclear AFLP) and maternally (mitochondrial DNA) inherited markers. Seven populations in northern and central Italy, including three recently established sites in the expansion zone, were found to be highly spatially structured. Estimates of the absolute number of migrants per generation (Nm) were calculated assuming equilibrium between migration and drift. The twice higher value obtained with the nuclear markers including male-contributed alleles (0.47 *vs.* 0.25) suggested that males disperse more than females. Although these estimates rely on assumptions that can be violated in natural populations, especially in expanding populations, long-distance colonization by adult females appeared to be a rare event. Conversely, males seemed to give a small but constant contribution to the genetic diversity of populations on a relatively wide range. Similar results were obtained for the sister species *T. wilkinsoni* (Simonato et al. 2007; see above, 3.2.3). Dispersing males thus probably reduce the genetic loss associated with bottlenecks that could occur in expanded and/or isolated populations. This assumption was strongly supported by complementary data obtained from the expanding population located in the Western Alps.

Salvato et al. (2005) further compared the genetic diversity of pheromone-trapped males and of larvae sampled in the same stands (hereafter "resident larvae") for the same seven populations. Distribution of mtDNA frequencies revealed substantial homogeneity between trapped males and resident larvae within the six populations located in the core range of the species, including four old native forests and two 50 years old artificial stands that probably were recently colonized. Conversely, in the isolated population of Aosta Ruines Verrès located at the upper edge of the distribution area, the mitotype diversity of trapped males was higher than that of resident larvae. One mitotype found in trapped males was present in other populations but not in the resident larval population of Aosta Ruines Verrès. Consequently, this mitotype was likely transported by migrant males originating from distant and differentiated populations, but was not transmitted because maternally-inherited. Pairwise genetic distances of nuclear data (AFLP) confirmed the same differentiation patterns between adults and larvae. However, due to the dominant nature of AFLP markers, it is not known whether these pheromone-trapped males, especially the putative migrants, successfully bred and contributed to the next generation.

These results, in contrast with those obtained for the six populations located in the core range of the species, can be explained by a number of factors, such as the proximity of moth populations with different mitotypes and an innate tendency of the moths to expand beyond the range's edge, possibly supported by the ongoing increase of the minimum winter temperature. Moreover the outcome of this study underlines the potential for a significant gene flow among populations, driven by male dispersal, existing at least near the edge of the present area of distribution.

4.3.3 Conclusion

The study of the population genetic structure at large spatial and temporal scales in France and in Italy confirmed that the short-lived adults of the pine processionary moth exhibit a limited active dispersal as suggested by Démolin (1969). Genetic differentiation measured by F_{ST} using five microsatellite markers rapidly increases with geographic distance. This isolation by distance pattern and the gradual loss of genetic diversity along the main axis of the past northward expansion is consistent with a preferential exchange of migrants between adjacent demes and a diffusive dispersal. Thereby, only the individuals located at the expanding edge contribute to the advance of the expanding front, and this is expected to lead to a colonization wave of constant speed in a homogeneous environment. However, it will be necessary to investigate the colonization process at a fine scale in the current expansion areas in order to define the patterns of contemporary range expansion in various types of human landscapes (see also Chap. 5).

The contemporary northward expansion appeared to result from a combination of active and passive dispersals. In northern France, especially in urban areas, wide territories have already become climatically suitable for the pine processionary moth and offer abundant host-tree resources thanks to numerous artificial plantations (Robinet et al. 2014). The spread of the populations of the pine processionary moth into these territories appears now mostly limited by the active female dispersal abilities. Accidental introductions can allow to colonize climatically suitable territories beyond the wave front thus accelerating the spread of the insects. It will be crucial to confirm the putative pathway associated to large trees trade in order to avoid accidental transportations.

Males appear to disperse more than females and could contribute to genetic diversity of populations on a relatively wide range, reducing the risk of inbreeding and genetic loss associated with bottlenecks occurring in isolated populations. The identified male-driven neutral gene flow suggests that rapid exchanges of adaptive genetic diversity could occur between genetically differentiated lineages that recently came into secondary contact, and that may also be ecologically differentiated.

4.4 Ongoing Work and Perspectives

4.4.1 The Northward Expansion Area of the Paris Basin: A Melting Pot of Differentiated Lineages

In order to study the genetic imprints of the contemporary range expansion, a sampling grid covering about 60,000 km^2 was designed in the southern part of the Paris basin where the moth has colonized wide territories during the last 20 years (Fig. 4.11a). For this study, additional microsatellite markers were developed (Burban et al. 2012) in order to allow fine-scale and individual-centred analyses. First, 15 individuals collected every 64 km along the grid were analysed using mtDNA, AFLP and microsatellite markers. This study area corresponds to the

main current expansion area of the French-Spanish lineage. Its south-eastern part also corresponds to the expected contact zone with the eastern lineage (see Figs. 4.8 and 4.11), allowing to study the pattern of admixture between them.

Surprisingly, the mtDNA marker allowed to identify three rather than two major genetic groups within this expansion area (Fig. 4.11). While the two expected maternal lineages actually co-occurred at this spatial scale (Fig. 4.11b), another
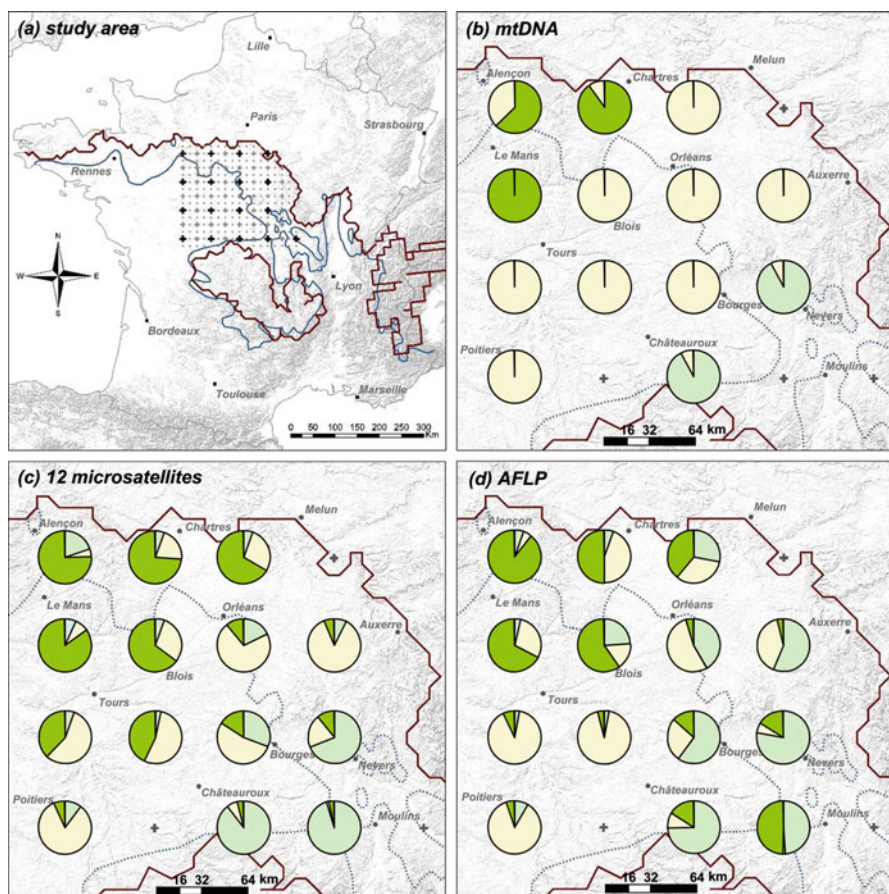

Fig. 4.11 Distribution of the three major genetic groups identified in the expanding area of the southern Paris basin using mtDNA, AFLP and microsatellite markers (**a**) location of the sampling grid (*dark crosses* represent sampling sites of the 64 km × 64 km grid, grey crosses the 32 km × 32 km grid, and *grey dots* the 16 km × 16 km grid) (**b**) each *pie chart* indicates the proportion of individuals bearing a mitotype from the French-Spanish mitogroup (*light yellow*) and from the eastern mitogroup (*dark green* for mitotype 5 and *light green* for the other ones) (**c, d**) distribution of the three genetic clusters inferred using Structure 2.3 for the microsatellite (12 loci) and the AFLP data sets respectively. The *red lines* indicate the edges of the distribution area in the winter 2005–2006, the *blue lines* indicate the maximum advance in 1979 according to J.F. Abgrall (CEMAGREF)

mitotype belonging to the eastern lineage (namely mitotype 5, Fig. 4.8) was also found in the north-western part of the grid. To date, this mitotype was found only in the northern French Alps (Rousselet et al. 2010) and its occurrence in the Paris basin likely resulted from an accidental introduction. The north-western corner of our sampling grid was indeed located near an old pioneer colony, reported in 1980 in the vicinity of Alençon (C.T.G.R.E.F.-I.N.R.A. 1980; Abgrall 2001), and now embedded within the present-day northern edge (Fig. 4.11).

The nuclear AFLP and microsatellite markers confirmed the existence of three well-differentiated genetic clusters but also revealed a larger admixture area than that identified by the mtDNA marker, which revealed narrow contact zones (Fig. 4.11b–d). They further suggested that the individuals occurring along the main axis of expansion showed signs of introgression between all the lineages that occurred in parapatry in this area before the climate-driven expansion (Fig. 4.11). This result highlights again the role of the male-driven gene flow at the leading edge.

In order to address the issue of the dispersal pattern in a recent expansion area, microsatellite markers were then used at a finer scale by analysing samples of five individuals collected every 32 km in the same study area. We used the kinship coefficient (F_{ij}) proposed by Loiselle et al. (1995) as a means of measuring the relatedness between each pair of individuals. The plot of the changes of F_{ij} for increasing distance is called the correlogram and reveals the spatial structure of the genetic data (Vekemans and Hardy 2004) (Fig. 4.12).
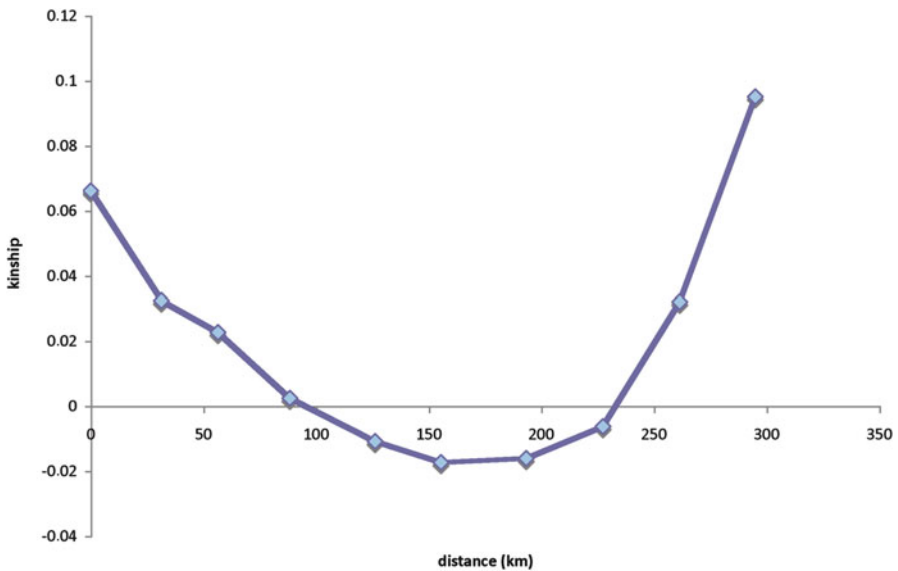
Fig. 4.12 Correlogram showing the large scale spatial variability of microsatellite data. The average of pairwise kinship values between individuals is plotted against the average of pairwise Euclidian geographic distances between them for ten classes of geographic distance

Up to a distance of 150 km, the mean kinship between individuals gradually decreased as geographic distance between them increased. This is consistent with an isolation by distance pattern, and thus with the diffusive dispersal previously found at a larger scale. Above a distance of 200 km, kinship increased as geographic distance increased, but this pattern must be interpreted with caution because of the low number of pairwise comparisons between each corner of the grid. Nevertheless, genetic relatedness between some distant individuals located in the corners revealed by the mean kinship could reflect the complex spatial distribution of the genetic clusters over the grid and the introgression patterns resulting not only from short-distance spread but also from contingent long-distance human-mediated jumps.

4.4.2 "Landscape Genetics" of the Pine Processionary Moth in the Paris Basin: Expansion in a Heterogeneous Environment

The southern part of the Paris basin where the moth recently expanded consists of different land uses, including forests with native broad-leaved trees and exotic conifers, meadow areas, wide open-fields, valleys and wetland areas, and large urbanized territories. The sampling grid in the study area presented above was also designed to cover the different landscapes of this strongly human-affected environment. Supplementary samples consisting of five individuals collected every 16 km will be genotyped for the same microsatellite markers in order to identify landscape features able to account for genetic discontinuities at a fine scale.

We especially aim to assess if the trees outside forests (TOF) can play a role in the dispersion of this species during range expansion, by creating suitable corridors. Considering only the distribution of the host tree resources, the different habitats of the pine processionary moth can be grouped into two main categories: forest lands with numerous and large artificial pine stands and non-forest lands with small stands and scattered ornamental pines. The Beauce region is a vast open-field area recently colonized that did not slow the northward expansion of the moth whereas isolated and scattered pines have been the only available resource (Chap. 5; Rossi et al. 2013). This indicates that TOF can be the sole medium of the spread. Previous field observations (C.-B. Malphettes, 2000, personal communication) suggested that moth expansion could have been accelerated in areas where host trees are scarce whereas large patches of resource like Orléans forest and Sologne could have retained philopatric females and acted as a barrier to dispersal. This also means that some landscapes mainly consisting of TOF might act as filters selecting the better dispersers contrary to more dense and continuous native forests. If true, a part of the spatial genetic structure is expected to be correlated with identifiable landscape features such as host tree abundance or fragmentation.

4.4.3 Concluding Perspectives

The use of neutral genetic markers showed that the population genetic structure in the current expansion areas can result from a combination of active short-distance

dispersal and human-mediated long-distance jumps. Moreover, a male-driven gene flow between the wave front and some of the distant satellite populations is very likely. These results question the existence of flows of adaptive genetic diversity occurring at the expanding edge, especially on possible phenological adaptations that might be experienced in the expanding populations (e.g. more frequent prolonged diapause at the edge – Géri 1980 – or phenological shift following admixture between ecologically differentiated lineages). Such a modification of the species climatic envelope would affect the further potential of colonization. Genomic resources are currently being developed for the pine processionary moth, and will soon allow the development of genome-scans approaches. These methodologies, based on high-throughput sequencing, will give access to a tremendous number of neutral genetic markers that can be useful for fine-scale landscape genetics studies, and will at the same time allow to detect the genomic regions under selection, and thus potentially involved in adaptive traits.

5 Global and Local Genetic Structure of the Northern Pine Processionary Moth, *Thaumetopoea pinivora*

Anna Cassel-Lundhagen, Andrea Battisti, Cecilia Ronnås, and Stig Larsson

5.1 Introduction

As opposed to the pine processionary moth *T. pityocampa*, *T. pinivora* is not considered as a major forest pest. It mainly occurs in local populations in mountainous areas in southern Europe and along the coasts of the Baltic Sea in northern Europe. However, like the pine processionary moth, *T. pinivora* causes nuisance to humans, and was recently the focus of attention of the general public because of a major outbreak in a touristic area on the island of Gotland in south-eastern Sweden. A need to gain knowledge about its biology, and particularly its dispersal behaviour, became apparent in order to better understand its abundance and distribution.

As described in the previous subchapters, genetic data can provide information about a species dispersal behaviour. Colonization events tend to result in geographic structuring of genetic diversity so that the dispersal pathways can be revealed (Hewitt 1999; Taberlet et al. 1998). However, in highly mobile species traces from a colonization event (as manifested in haplotypes shared by the founder and founding population) may be blurred by subsequent gene flow from other areas (Ibrahim et al. 1996). In the case of low or no gene flow subsequent to colonization, low genetic diversity is expected to persist in the new populations (Ortego et al. 2011; Whitlock et al. 2003), especially if the population remains small in size (Vrijenhoek 1994). Because dispersal is expected to be most frequent between neighboring locations, and gradually cease as the distance increases, the level of

genetic differentiation is expected to increase with geographic distance. However, the correlation between geographic and genetic distance is expected to change over time depending on the rate of dispersal between populations relative to the amount of genetic drift acting within them (Epperson 2003). Over time, new mutations will also accumulate, causing further genetic structuring. Thus, the combined genetic patterns found within and between local populations can be used to reveal the species historic and contemporary dispersal behaviour.

5.2   The Phylogenetic Position of Thaumetopoea pinivora in Relation to Other Summer Processionary Moths

The northern pine processionary moth *Thaumetopoea pinivora* (Treitschke) (Lepidoptera, Notodontidae) belongs to a well-defined clade in the phylogeny of the genus (see beginning of this chapter) that includes the so-called summer processionary moths associated with coniferous host tree species. These species are characterized by larval development across spring and summer, which makes them biologically different from the winter processionary moths. The molecular phylogeny fully justifies such a difference and clearly separates the two clades (Simonato et al. 2013), although the species share the same coniferous host plants.

Four species are included in the clade of the summer processionary moths. *Thaumetopoea pinivora* is the only one associated with *Pinus* spp. whereas the other three species feed on *Cedrus* spp. in various parts of the Mediterranean basin. *Thaumetopoea bonjeani*, which is the most closely related species to *T. pinivora* (although clearly separated based on a large number of mitochondrial and nuclear genes, see the phylogeny subchapter), is monophagous on *Cedrus atlantica* in the mountains of Algeria and Morocco. The other branch of the clade includes *T. libanotica*, which is associated with *Cedrus libani* in the mountains of Lebanon and Syria, and the recently described *T. ispartaensis* from the same host plant is located in the Taurus mountains in southern Turkey.

As far as it is known from natural history (Chap. 2), the four species share the same developmental pattern, pointing at a common origin. However, the geographic data that are currently available do not allow us to locate the most likely geographic area of origin. All four species occupy mountain forests that are well isolated in the Mediterranean basin; thus, we can only speculate that they may be relics of a species that occurred in a former more extended distribution of *Cedrus* forests back in the Tertiary (Qiao et al. 2007).

Thaumetopoea pinivora is the only species that does not feed on *Cedrus* as it has switched to *Pinus*. This could explain why it has been able to expand from the Iberian Peninsula and colonize part of Europe after the last Quaternary glaciation (see below). The lack of *T. pinivora* (or other summer processionary moths associated with *Pinus*) in other parts of the Mediterranean basin where mountain forests of pines are abundant, similar to those of the Iberian Peninsula, supports the notion that the

switch probably originated from a species feeding on *Cedrus. Thaumetopoea bonjeani* appears to be the most likely candidate as it has a distribution that is geographically close to *T. pinivora* in the Mediterranean area, and because these two species show the shortest genetic distance in the molecular phylogeny. This hypothesis is further strengthened as the two species share the same sexual phenomone (Frérot and Démolin 1993; Frérot et al. 1990), a number of morphological traits (Démolin 1988; Démolin et al. 1994; El Yousfi 1989; Gachi 2004), as well as the species that parasitize their eggs (Schmidt et al. 1997).

A thorough phylogeographic analysis of the clade of summer processionary moths could shed more light on the relationships among the species, especially if other taxa were found in target areas such as the high elevation coniferous forests in the Mediterranean basin. Because these species are much less conspicuous than the winter processionary moths it may be that their occurrence has so far been overlooked.

5.3 Glacial and Postglacial History

Thaumetopoea pinivora has a peculiar global distribution, occurring at high-elevation locations in southern Europe while primarily residing close to sea level in northern Europe. In addition, the distribution is highly scattered and local populations tend to appear more or less isolated (Chap. 3). The main host plant for *T. pinivora*, Scots pine (*Pinus sylvestris* L.), is widespread across Europe (Hultén and Fries 1986). Data obtained from pollen, macrofossil, and phylogeographic analyses of mitochondrial DNA variation across Europe suggest that *P. sylvestris* survived the last glaciation in at least four different glacial refugia: Spain, southern Italy, Asia Minor, and central Europe (Cheddadi et al. 2006; Naydenov et al. 2007; Sinclair et al. 1999), and possibly in cryptic refugia at northern latitudes (Kullman 2002; Larsen et al. 1987). There is evidence that *P. sylvestris* colonized central and northern Europe more than 8,000 years ago, and that the northern pine populations originate from refugia in Italy or central Europe (Cheddadi et al. 2006; Kullman 2008). If *T. pinivora* followed the spread of its host after the last glaciation, i.e., the colonization pathways of *P. sylvestris* mirror the establishment of *T. pinivora,* then we should expect to find significant genetic divergence between the southern and northern European populations of *T. pinivora*. If, on the other hand, all extant populations of *T. pinivora* share the same origin, then we should expect lower levels of genetic divergence across sites (Table 4.2).

The exact habitat requirements are poorly known, and thus the causes of the fragmented distribution are largely unresolved. When knowledge about habitat requirements is limited, as for *T. pinivora,* or when information about the former distribution of the habitat is unavailable, it can be difficult to determine whether geographically separated populations are the result of recent colonization events or if such populations have existed for a long time. A fragmented distribution could also indicate that the populations are remnants of a more widespread distribution

Table 4.2 Suggested large-scale genetic patterns after an expansion event, and subsequent dispersal strategies, of *Thaumetopoea pinivora* depending on time since expansion, the prevailing colonization model and the species dispersal capacity

		Recent events			Ancient events		
Colonisation model	Dispersal capacity	Founder effects	IBD pattern	Unique alleles in colonized locations	Founder effects	IBD pattern	Unique alleles in the colo- nized location
Long- distance few	High	+	−	−	−	−	−
	Low	+	+	−	−	+	+
Stepping stone	High	−	+	−	−	+	−
	Low	+	+	−	−	−	+

(+) indicates that the pattern is found and (−) that it is not

Founder effect = reduced levels of genetic variation as a consequence of few individuals (few genotypes) colonizing a new location; IBD pattern (Isolation By Distance pattern) = a correlation between geographic distance and genetic distance between pairs of populations. Such pattern occurs if dispersal rate is negatively correlated to distance; Unique alleles = alleles that are found only within a specific population or region

(Habel and Assmann 2010). Finally, such a distribution could also occur if the species has a narrow habitat requirement but is capable of long-distance dispersal, enabling the species to track rare suitable habitat patches (Schroeder et al. 2007).

In order to reveal the mechanism behind the current distribution we evaluated the glacial and postglacial history of *T. pinivora* by using genetic data from both nuclear microsatellite markers and a fragment of the cytochrome oxidase I (COI) gene in the mitochondrial (mt) DNA. A combined analysis of the fast-evolving microsatellites (reflecting more recent processes) and the non-recombining mitochondria (mirroring more ancient events) allowed us to evaluate the species' establishment and dispersal pattern across a wide temporal and spatial range (Table 4.2).

We found that *T. pinivora* exhibits very little mitochondrial diversity across its whole range (Cassel-Lundhagen et al. 2013). Only eight sites within the 633 bp long fragment of the mitochondrial COI gene were polymorphic, and 90 % (116 out of 129) of the analysed individuals, found across the whole range, shared the same haplotype. Thus, the data suggest that the populations have not been present in their current locations long enough for a significant number of mutations to emerge. Data also indicated that *T. pinivora* does not exist as a large and continuous population because such a population should have accumulated genetic diversity over time (Bromilow and Sperling 2011). Because the number of mutations is determined by the length of time that a population has existed, we conclude that all of the analysed populations appear to have a recent history. This was also verified by the micro-satellite data which exhibit few unique alleles despite a wide geographic range.

The lack of mtDNA variation across all sites further suggests that the glacial refugium of *T. pinivora* is not associated with any of the studied populations. In general, populations that are older than the last glaciation are typically charac-terized by high haplotype diversity and possibly deep divergence (Hewitt 1996).

The mtDNA structure of *T. pinivora* stands, for example, in contrast to that of the bark beetle *Tomicus piniperda*, another cold-tolerant species that is also associated with *Pinus sylvestris*. In a study covering the European range of *Tomicus piniperda* a high number of unique haplotypes and accumulated mutations were found in geographically restricted regions (Horn et al. 2009). Horn et al. (2009) also suggested that refugia may occur outside Europe and this could potentially apply also to *T. pinivora* as it is known that several European cold-tolerant species have a distribution that extends into Asia (Schmitt et al. 2010).

   As opposed to the mtDNA data, the *T. pinivora* microsatellite diversity was markedly higher in the Spanish populations and was gradually lowered towards the north. The low number of unique alleles in the northern populations supports our conclusion that the colonization was recent (cf. Slatkin 1985) but the differences between the populations were not as pronounced as expected if severe bottlenecks had occurred. The data rather indicate that the colonization process was gradual with most of the variation retained in the expansion front (Ibrahim et al. 1996). The lack of traceable genetic bottlenecks also point against the species having colonized northern Europe through rare long-distance dispersal events with subsequent small population sizes. Simulations of our empirical data compared with a set of suggested scenarios indicate that *T. pinivora* colonized northern Europe approximately 2,000 years ago at least at two separate occasions. Considering the current occurrence, the *T. pinivora* distribution must thereafter have diminished significantly in size leading to the currently fragmented occurrence.

5.4   Thaumetopoea pinivora *Outbreak and Local Population Genetic Structure*

Thaumetopoea pinivora was once considered a rare species in Sweden and was until recently red-listed (Gärdenfors 2000). Only a few populations were known to exist in Sweden, one at the southern tip of Gotland Island and one on the neighbour Öland Island, both in the Baltic Sea. In 2004–2006 the density of the Gotland population increased dramatically leading to an outbreak (Larsson et al. 2008). Because outbreaks can lead to resource depletion, and subsequent starvation, they can have drastic effects on insect fitness (e.g. Klomp 1966). One could therefore expect density-dependent emigration to occur into low density and/or unoccupied areas (Berryman 1987; Bowler and Benton 2005; Denno et al. 1995) and this, in turn, may result in new populations being established (Benzie and Stoddart 1992; Nair 1988). When a high-density population is situated at the edge of the species' range, as in the case of *T. pinivora* on Gotland, increased dispersal can also result in an extension of its distribution (Travis et al. 2009; Wilson et al. 2010).

   Due to the known high dispersiveness of *T. pinivora* males, and the fact that the food source is readily available throughout Gotland Island, we expected the outbreak to expand the distribution as the competition for resources within the

outbreak area increased. To examine if this was actually the case we genotyped recently discovered populations at increasing distances from the high-density area. As neither of these populations had been previously known, and as they were relatively easy to observe due to the aggregating behavior of the larvae and their health impact on humans (Battisti et al. 2011), we assumed them to be newly established from the ongoing outbreak. Using 12 polymorphic microsatellite markers we tested for presence of founder events, if there were reduced allelic richness due to rare long distance dispersal, and if there were signs of recent establishment in terms of sharing of alleles between the outbreak area and the isolated populations (Ronnås et al. 2011).

Contrary to our expectations, three aspects of the results suggest that none of the isolated populations was recently established. First, the distant populations exhibited similar levels of genetic variation to the high-density one and locally unique alleles were found in the isolated sites (Table 4.2). Second, there were no signs of founder events or genetic bottlenecks. Finally, there was significant genetic structuring in the populations located further away from the high-density population. Thus, the outbreak appears not to have resulted in spread into the surrounding areas, despite the high population density (Ronnås et al. 2011). On the contrary, *T. pinivora* appears to have a fragmented local distribution on Gotland Island, possibly the remnant of a former more widespread population similar to what seems to be the case at the larger, continental scale.

5.4.1 Being Rare and Being Common, and the Genetic Paradox

As opposed to *T. pityocampa*, *T. pinivora* appears typically to reside in relatively isolated populations. From a population genetics view, we expected that this should result in small local effective population sizes with pronounced effects of genetic drift. Therefore, it would be expected that the overall levels of genetic variation were lower on a global scale for *T. pinivora* than for *T. pityocampa* and that the level of differentiation was more pronounced. We did not, however, find this pattern. Although microsatellite data cannot be easily compared among species, it is striking that the levels of heterozygosity are comparable between the two species (Santos et al. 2011b). There is also relatively good agreement in COI haplotype variability within locations. In each genotyped location of *T. pityocampa*, in general, only 1–3 haplotypes were found (Kerdelhué et al. 2009; Rousselet et al. 2010). This was also the case for *T. pinivora* (Cassel-Lundhagen et al. 2013). On a global scale, on the other hand, the pattern was considerably different. In *T. pityocampa*, haplotypes were found in geographically well-defined clusters while all populations across the whole range of *T. pinivora* shared the same main haplotype. The considerably different genetic structure between the two species is likely due to the differences in their current distribution. If a species colonizes a new area, then the same haplotype will spread across all populations. If an area, on the other hand, is already inhabited with a population carrying a

certain haplotype, then the spread of an immigrant haplotype is less likely. Thus, the difference in genetic structure may be due to variation in time since establishment. Whether the difference is also due to variation in dispersal behaviour remains to be elucidated.

5.4.2 Conclusions from Genetic Data in Light of *Thaumetopoea pinivora* Distribution

The microsatellite data of *T. pinivora* from Gotland Island revealed some interesting patterns when compared with data from sites across Europe. All microsatellite alleles, except two unique alleles on Gotland, were found across the whole distribution range. Thus, most alleles were found also in Spain, Russia, or Germany. The combined global distribution pattern and genetic structure of *T. pinivora* is puzzling. While it is, as far as we know, a poor disperser, it also has a very scattered distribution. Furthermore, there is an ongoing outbreak on Gotland but this has not yet resulted in any range expansion, or documented new colonizations. We do know that its colonization history in northern Europe is relatively recent. The combined data from genetic studies (Cassel-Lundhagen et al. 2013) rather suggest that *T. pinivora* may be considered as a relict species (Habel and Assmann 2010) where its total distribution is retracting rather than expanding. The habitat requirements of *T. pinivora* appear to be relatively narrow, and populations have not been found in forests managed for wood production. Even though it is not possible to reconstruct the availability of suitable habitats in the past, it is very likely that the intensification of agriculture and forestry during historical time has resulted in a decline in the occurrence of the sparse, slow-growing pine stands that *T. pinivora* prefers.

Glacial relict species are species that had their most restricted distribution during the cold maxima and have expanded since, while interglacial relicts have rather had their more widespread distribution during cold periods with subsequent reductions in interglacial periods. The interpretations of our genetic data do not really support any of these extremes but rather suggest that *T. pinivora* has both expanded and retracted during the interglacial. Thus, it appears that a combination of suitable climate *and* appropriate vegetation was not in place until several thousand years after the ice retreated from northern Europe (Cassel-Lundhagen et al. 2013).

The finding of a relatively high diversity extending across all of Europe, despite the fact that *T. pinivora* appears very locally, suggests that it has had a "burst" of stepping-stone expansion lasting for a limited period, followed by habitat fragmentation and loss of populations. Thus, the combined data suggest that *T. pinivora* can be considered a cold-tolerant relict species residing in habitats appearing irregularly in time and space, with situation-dependent dispersal rather than being widespread and highly dispersive, as could be expected if the species was in general overlooked rather than truly rare.

6 Allochronic Differentiation and Ecological Divergence in a Phenologically Shifted Population of the Pine Processionary Moth in Portugal

Manuela Branco, Helena Santos, Susana Rocha, Maria-Rosa Paiva, Eduardo Mateus, Christian Burban, and Carole Kerdelhué

6.1   Introduction

Speciation is the evolutionary process by which new species arise. The initial reproductive isolation between lineages may occur due to physical separation, such as by a geographical barrier or as a result of habitat fragmentation, in which case speciation is said to occur in allopatry. Speciation can also arise in consequence of ecological specialization and assortative mating within a given geographical region. Most cases of so-called "sympatric speciation" have been studied among either cichlid fishes or phytophagous insects, in which populations primarily diverge through habitat or host-plant specialization (Dres and Mallet 2002; Fan et al. 2012; Kornfield and Smith 2000). A different model of sympatric speciation called "allochronic speciation" was proposed in the 1960s (Alexander and Bigelow 1960). In this particular case, isolation is due to a temporal shift of the reproductive period without geographical separation or habitat change.

In this subchapter, we will review a plausible case of incipient allochronic differentiation in the pine processionary moth in Portugal and provide new evidence about its origin and divergence patterns. We first present a synthesis of the conclusions obtained by the use of molecular markers, we then introduce the results concerning the ecological differentiation observed for various life-history traits, and we finally point out some perspectives and clues for future research.

Thaumetopoea pityocampa is ubiquitous in Portugal, due to a wide distribution of pine trees and mild climatic conditions (see Chaps. 2 and 3). It typically develops with a 1-year biological cycle, with some variation according to latitudinal and altitudinal gradients as observed in other regions. Sexual reproduction occurs between August and September, and larval development unfolds in the fall and early winter. Processions take place in January or February. In August 1997, a huge outbreak of last instar larvae (fifth instar) was observed in "Mata Nacional de Leiria" (Paiva, personal observation), a maritime pine forest located ca. 120 km north of Lisbon. This situation was unexpected, because fifth instar usually occur in December. Field monitoring and observations set up during the following years showed that two different types of processionary moths occurred in Leiria pinewood: some of the individuals develop following the classical life cycle described above, while others reproduce as early as May, then develop as larvae in the summer months and experience the diapause procession in mid-September (Pimentel et al. 2006, see Chap. 2). Based on the timing of larval development,

the individuals developing with the classical cycle were called Winter Population (WP) while the individuals with a shifted life cycle were supposed to belong to a divergent Summer Population (SP). Many evolutionary questions rapidly arose from these first observations, and research was undertaken in the fields of phylogeography, population genetics and ecology to bring the first answers. We specifically addressed the following points: (1) Does the SP belong to the same species as the sympatric WP? (2) If so, does it really form a differentiated population? (3) What is the most likely origin of the SP? (4) Is it possible to estimate the age of the SP, and to quantify potential gene flow with the WP? (5) Can we identify key ecological traits that differ between the SP and the WP?

6.2 Evolutionary Scenarios Based on Molecular Data

In order to determine whether the SP actually belongs to the same species as the WP, and should thus be considered a *T. pityocampa* population with a shifted phenology, rather than a cryptic species showing a different life cycle, Santos et al. (2007) first sequenced a part of the mitochondrial COI gene and the nuclear ITS1 marker for a few individuals sampled in Leiria pinewood from the SP and from the WP, as well as in 10 surrounding localities in Portugal, Spain and France. Interestingly, the major COI haplotype and the ITS1 alleles found in the SP were also found in the WP occurring in Leiria, as well as in the other Portuguese populations, and in central and southern Spain. Only one rare mitochondrial haplotype was found exclusively in the SP, being however closely related to the main haplotype found in Leiria pinewood. Results thus demonstrated that the SP did not belong to a cryptic species, and should instead be considered a population of *T. pityocampa*, with a peculiar phenology. Moreover, the genetic data showed that the SP was phylogenetically close to other Iberian populations, evidencing a regional origin for both the mitochondrial and the nuclear markers. The very low nucleotidic divergence observed between the SP and other local populations, further suggested that the phenological shift occurred relatively recently, but did not allow to precisely estimate the time of the divergence. The markers used were not sufficiently informative to identify the closest related sister group in the Iberian Peninsula.

We thus further sequenced the whole mitochondrial COI gene (ca. 1,500 pb), in order to obtain more polymorphic, informative sites and to eventually point to the origin of the SP, at least for the maternal lineage. We sequenced 8 individuals from the SP, 8 from the sympatric WP, and 3 individuals in 12 localities in Portugal, Spain and France. We obtained 21 haplotypes, and the resulting most parsimonious network showed two haplogroups, that corresponded to the Iberian and the European sub-clades previously identified using shorter mitochondrial gene fragments (Kerdelhué et al. 2009; Rousselet et al. 2010; subchapters 3 and 4 above). The geographical distribution of the COI haplotypes and the network are shown in Fig. 4.13. These new data brought significant information concerning the evolution of the SP, as they showed that the main haplotype found in this peculiar population
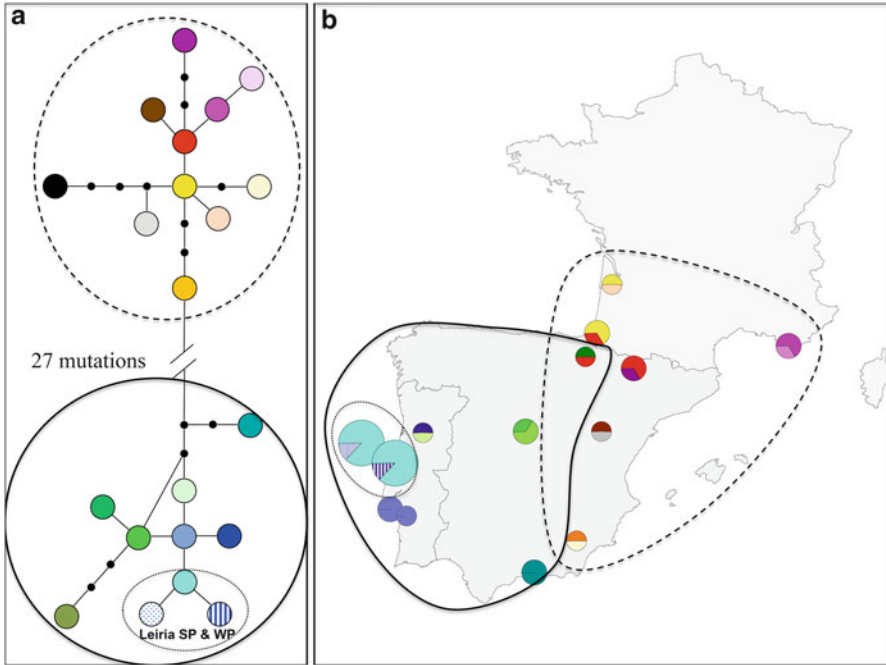
Fig. 4.13 Most parsimonious network of haplotypes (**a**) and geographical distribution of the COI haplotypes in the 14 sampled populations (**b**). Haplotype frequencies and sampling sizes are represented by the area of the *circles* on the map. In the network, each *line* between *circles* corresponds to a mutational step and each small *black circle* to a missing intermediate haplotype

was found only in individuals of the sympatric Leiria WP, and differed by only one or two mutations from the other haplotypes found in Portugal. This strongly suggests that the SP diverged locally, from an ancestral *T. pityocampa* population having a typical "winter" cycle and probably already occurring in Leiria pinewood. According to this hypothesis, the foundation of the SP would have arisen from a sudden phenological shift that rapidly led to a strong limitation of the gene flow with the individuals of the ancestral population.

The next objective was to characterize the genetic differentiation between the SP and the sympatric WP, and to determine if gene flow could still occur in spite of the observed shift in the reproductive period. Santos et al. (2007, 2011b) have monitored the activity of the adults in the field, through male catching with pheromone traps set up in Leiria pinewood from April to October of each year. Results clearly showed the occurrence of two non-overlapping peaks of male emergences, one from early May to mid-June, and another one from mid-July to September, with little inter-annual variations. A period of 1–4 weeks without male catches was observed each year, in early and mid-July. The first peak coincided with the expected reproductive period of the SP, while the second peak corresponded to the period of adult emergence known in Portugal for the WP. Given that the adults

of *T. pityocampa* live for 1–3 days only, the flight pattern observed should preclude gene flow between the two populations.

To test the hypothesis that a strong genetic differentiation between the SP and the WP would occur, and to check if the genetic composition of both groups was stable in time, Santos et al. (2011b) further applied a population genetics approach using six microsatellite loci, and genotyped larvae and males trapped over different seasons and years. Females sampled as pupae in the field, that later emerged in the laboratory, were also studied. Very high F_{ST} estimates of differentiation between the SP and the WP were found, reaching 0.18–0.24 in different years. Such values were much higher than the F_{ST} measured between populations of pine processionary moth sampled several hundreds of kilometres apart, as the F_{ST} between populations from Portugal and southern Spain did not exceed 0.15. Individual assignment tests were also performed using a Bayesian framework (Structure software, Pritchard et al. 2000). This approach produced interesting information, as it showed that: (1) The vast majority of the sampled individuals were classified in two clear-cut genetic clusters corresponding to the two phenological classes (SP *vs.* WP individuals); (2) All males trapped in the spring were unambiguously assigned to the SP, showing that the adults emerging in May and June actually gave rise to the next generation of the SP; (3) Most of the males caught in the summer were well assigned to the WP cluster, while few individuals were well assigned to the SP cluster. As the results presented in Santos et al. (2011b) were based on six microsatellites only, including two markers showing high levels of null alleles, we took advantage of recent technological developments (Burban et al. 2012) to genotype the same samples, as well as individuals sampled the following year, using 10 other microsatellite loci and excluding the two problematic markers. Results confirmed the conclusions found in Santos et al. (2011b), with enhanced assignment thresholds (Fig. 4.14).

In conclusion, the population genetics approach confirmed a strong pattern of genetic differentiation between the two sympatric SP and WP, which suggests that gene flow is extremely reduced, in spite of a shared geographical range and continued use of a common host plant. The primary process that probably acted to hamper the genetic exchange between the two populations is a major shift in the reproductive phenology. It is thus likely that the two "phenological populations" that occur in
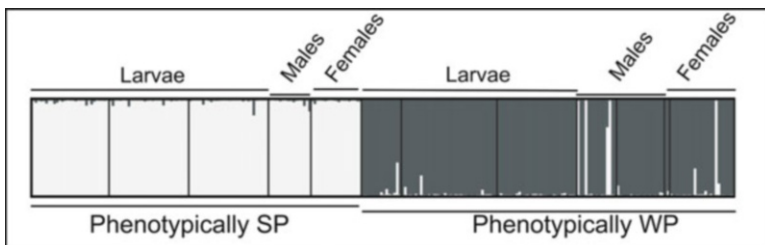
Fig. 4.14 Graphical representation of the individual assignment proportions (estimated membership coefficient for each individual) obtained by running the Structure software with 2 hypothetical clusters and 264 individuals genotyped with 14 loci (larvae, males and females sampled over several years and "phenologically" a priori identified as SP or WP)

Leiria pinewood are under allochronic differentiation and may constitute a unique case of incipient allochronic speciation in a phytophagous insect. Yet, we showed that some "phenologically WP males" actually belong to the SP genetic cluster (light-grey individuals occurring in the dark-grey cluster, see Fig. 4.14).

This result suggests that some individuals originating from the SP cluster can episodically emerge as adults within the period of WP reproduction, after a longer pupal diapause. Such individuals can be seen as SP larvae "reverting" to the WP phenology as adults. Such a mechanism could allow for some gene flow to be maintained, even though the peaks of reproductive activity of the SP and WP are non-overlapping. Analyses are still under way to estimate the levels of gene flow between the two populations, and to test whether recurrent gene flow might have occurred throughout the evolutive track of the SP. Attempts to estimate the time of the SP foundation from a putative WP local ancestor have so far remained inconclusive.

6.3 Evidence for Significant Ecological Differentiation of the Summer Population

Understanding the ecological differentiation occurring during a speciation process is of major relevance in evolutionary biology. Whatever the mechanism involved (local adaptation, drift, plasticity, among others), ecological divergence of key-traits in the separated lineages can even reinforce the ongoing speciation via specialization, or counter-selection of hybrids (Rundle and Nosil 2005). As the molecular data led to the hypothesis of a relatively recent evolution of the SP from a local WP ancestor, we took advantage of this unique model to test whether ecological differentiation could be found in the SP, as compared to the local WP. We focused on key ecological traits involved in reproduction, such as pheromone characterization, fecundity and egg parasitism, as well as on physiological capacities to survive high temperatures, as SP larvae face very hot summer conditions considered lethal for the earlier larval stages (Huchon and Démolin 1970). This research is particularly relevant within the context of global warming, as it also allows for testing the capacity of a species to rapidly adapt to new environmental conditions.

6.3.1 Comparative Study of Reproductive Traits in the SP and the WP

To test whether gene flow between the SP and the WP could be further limited by divergent chemical recognition signals, we compared the female sex pheromone emitted by both populations. The volatile emissions of virgin SP females were collected in the laboratory by SPME (Solid Phase Micro Extraction) and the characterization of the volatiles was performed by gas chromatography and mass spectrometry (Paiva et al. 2011b). Results showed that the pheromone composition
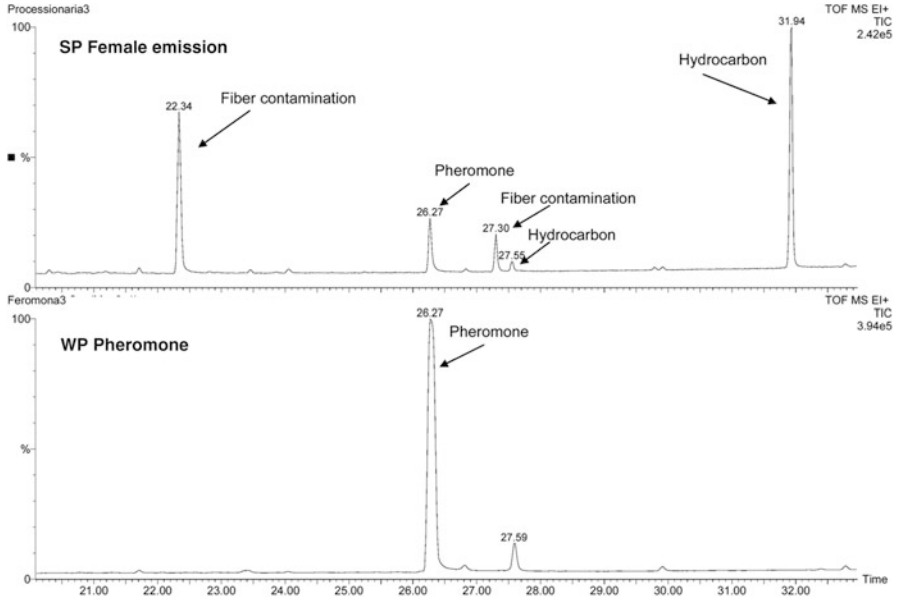
Fig. 4.15 Chromatograms obtained by Solid Phase Micro Extraction (SPME) for the volatile emissions of SP females and for the standard, commercially available, sex pheromone of the WP (Laboratories of FCT, UNL, Portugal, 2010)

of the two populations of pine processionary moth did not differ. Furthermore, no additional compounds could be identified in the volatile emissions of the SP (Fig. 4.15). This work thus indicates that the mechanisms of olfactory communication have not diverged regarding sexual attraction, so that cross mating between the SP and WP probably remains possible.

Pimentel et al. (2012) as well as Santos et al. (2013) further studied the post mating traits involved in reproduction and larval development. The egg batches of pine processionary moth populations have been observed to follow a general latitudinal trend, as they contain numerous small eggs in areas where they are exposed to cold winters and fewer, larger eggs under milder environmental conditions (Pimentel et al. 2010). It was hypothesized that this trend should result from different selective pressures occurring in these two types of environments: pine processionary moth survival to harsh winters is enhanced by the size of the colony (i.e., the number of larvae in a single nest), which favours high fecundity rather than embryo size.

On the contrary, when the selective pressure due to low temperatures is relaxed, selection tends to favour embryo size and survival. A trade-off between the number and the size of the eggs produced by each female would then explain the observed general pattern. As the SP larval development occurs in the spring and summer, it was expected that the selective pressure on colony size would no longer be in operation, and that a lower number of larger eggs would be produced by comparison with the WP. Moreover, due to the shift in phenology, it was also expected that

Fig. 4.16 Phenotypic differences of the scale cover of the egg batches observed for *T. pityocampa* SP (*left*) and WP (*right*), in Leiria pinewood (Modified from Santos et al. 2013)

the SP would escape from the pressure of specialist egg parasitoids. Results confirmed our hypotheses and showed that the SP lays fewer and larger eggs, per egg batch, than the sympatric WP. As final larval dry weight as well as adult size did not differ significantly between populations, total growth was higher in the WP than in the SP. Embryo mortality due to parasitoids and colony extinction before the third larval instar, were shown to be higher in the WP than in the SP (Pimentel et al. 2012). Preliminary results suggest that the SP would be mainly attacked by generalist egg parasitoids, and that the morphology of the scales covering the eggs would be less efficient in protecting the embryo from parasitism, due to a looser scale cover, as a result from differences in the shape of the scales (Fig. 4.16).

6.3.2 Larval Susceptibility to Extreme High Temperatures

Following the phenological shift, "the mutant" Leiria SP became suddenly exposed to new climatic conditions. In particular, larval development shifted from winter to summer, so that SP larvae are subjected to daily temperatures on average 6.6 °C higher than those of the WP. Research was thus conducted to determine whether SP larvae would be less susceptible to extreme high temperatures than WP larvae. Different mechanisms allow insects to overcome unfavourable temperatures, namely physiological and biochemical adaptations (Angilletta et al. 2002; Denlinger and Yocum 1998), behavioural changes such as micro-habitat selection (Breuer et al. 1989), or aggregation (Jones and Oldroyd 2006). To test this hypothesis, two types of parameters were studied by conducting the following experiments: (1) Temperature thresholds: L1 and L2 larvae were reared under controlled conditions at high temperatures, to evaluate the upper thresholds of survival; (2) Larval behaviour: direct observations of larvae placed in potted pine trees, during extreme hot summer days.

For the first experiment, the rearing procedures are given in Santos et al. (2011a). Each treatment consisted of three complete 24 h temperature cycles mimicking three consecutive hot days. In each cycle, the "night" was set 10 °C below the tested maximal temperature (MT), the temperature was then increased
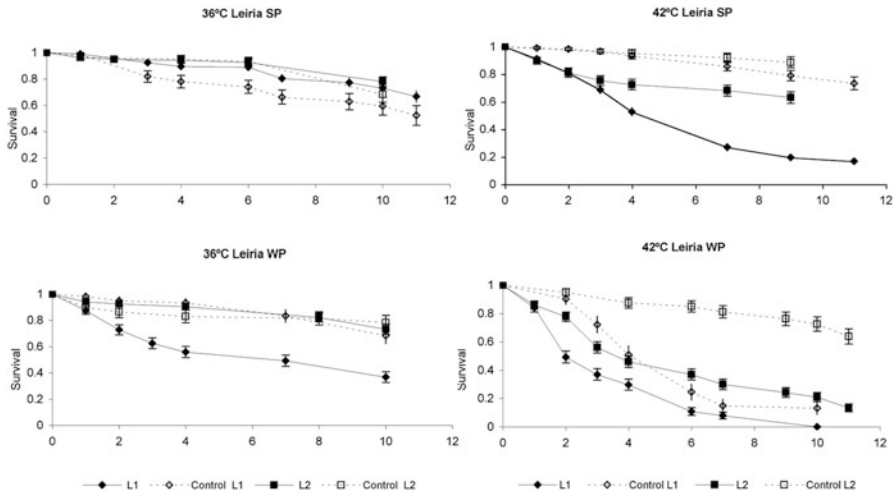
Fig. 4.17 Survival of first instar (L1) and second instar (L2) larvae during heat treatment (3 days) and post-treatment period (7–8 days), for maximal temperatures MT of 36 and 42 °C (From Santos et al. 2011a)

within 6 h to reach the desired MT, which was kept for the next 4 h, and progressively decreased to the night temperature over the following 6 h. After a trial was over, the surviving larvae were placed at room temperature during 1 week. During the whole process, larvae were fed with maritime pine branches planted in Oasis® floral foams. Survival in the tested sets of larvae and in the controls (kept at room temperature) was registered daily. The tested MT were 36, 38, 40 and 42 °C for both populations. Results unambiguously showed that survival was higher in the SP than in the WP, and always higher for L2 than for L1 larvae. Whereas a temperature negative effect upon L1 WP was already observed at 36 °C, a negative effect was only observed for L1 SP at 42 °C (Fig. 4.17). An upper threshold of survival for SP young larvae about 6 °C above that of the WP is thus assumed. At 36 °C there was even a positive effect of temperature on L1 SP survival, which might be related to a release from pathogens (Blanford and Thomas 2000). A high mortality was observed in one of the control groups of L1 WP (Fig. 4.17, low right corner), but the mortality in the heat treated group was still significantly higher. Mortality, estimated by Cox regression model, was for overall heat treatments 108 % higher for Leiria WP in comparison with Leiria SP. Pooling both populations, mortality increased by 24 % for each additional °C, from 36 to 42 °C, and decreased by 53 % from the first to the second larval instar.

We moreover observed behavioural reactions that could contribute to improve larval tolerance to high temperatures and dryness. During the hottest summer days, SP larval behaviour was assessed every hour, and responses categorized using four parameters, namely cohesion, activity, exposure and microhabitat selection, each one with two or three categories, to allow for qualitative analyses. The larvae tended to keep a gregarious behaviour, even at outdoor temperatures of up to 42 °C. Whenever

temperatures raised, the larvae showed a significant tendency to abandon the needles and move to shaded areas, either near branching points or to the ground. Similar observations could not be conducted for the WP, as larvae do not occur in summer in this population. Nevertheless, we observed a quite similar behaviour in the field in south-western France particularly during hot days, in October 2009, in a phenologically winter population. Considering the rearing conditions described above, we could also demonstrate for Leiria WP a significant positive effect of the presence of Oasis® floral foam in the rearing boxes in survival at 42 °C, by comparing survival under the conditions tested above, with situations were pine branches were not planted in a floral foam. Conversely, the presence of the floral foam had no significant effect on the controls reared at room temperatures. For logistic reasons, a similar experiment was not conducted for the SP. This result was explained by a tendency of the larvae to aggregate in the vicinity of this humid micro-habitat whenever conditions were unfavourable.

The results obtained thus suggest that the thermal tolerance presently exhibited by SP larvae was quickly acquired, considering the WP as its plausible ancestor. Still, the mechanisms underlying these observations need to be investigated. Within population variability regarding tolerance to high temperatures, is a trait that could rapidly evolve under strong evolutionary constraints (Thuillier et al. 2005). It can be hypothesized that physiological adaptations should be involved, such as divergences in the up regulation heat-shock and stress-related genes, as observed in other insects (Mahadav et al. 2009).

Models forecasting the dynamics of insect species under changing scenarios generally use parameters extrapolated from one, or a few locations only. Models usually underestimate, or even ignore geographic variations of adaptive traits. The results obtained for this peculiar population of pine processionary moth experiencing a phenological change suggest that traits involved in adaptation to the current global warming could evolve rapidly, enabling some species and populations to remain *in situ*, while coping with the resulting environmental changes. The behavioural responses that we observed further enhance the capacity of this species to circumvent unfavourable conditions.

6.4   Perspectives for Future Research

Research is in progress, aiming at a better understanding of the origin of the SP and the evolutionary, ecological and applied consequences of the existence of this population with a shifted phenology. Concerning the molecular approaches, we will develop new programs both on neutral markers and on loci potentially involved in adaptation. Single-nucleotide polymorphisms (SNPs), that can be found all over the genome, will be developed in the near future to complete the microsatellite data that we have so far generated. Gathering information over numerous SNPs will allow research to shift from population genetics to population genomics, and to improve the analysis of various evolutionary scenarios regarding the foundation of the SP. Moreover, this "genome-wide scan" approach will enable the use of new

algorithms to identify selected loci, and ultimately to identify the regions of the genome potentially involved in the evolution of the biological cycles and adaptation to new environmental conditions. We could also specifically target candidate genes known to be involved in the phenology of other insect species, and develop SNP genotyping designs focused on this set of genes.

The presence of the SP in the coastal recreational area of Leiria pinewood raises particular concern, since the most urticating larval stages (L4 and L5) develop in the summer months, thus increasing the risk of skin allergies, gastric and respiratory troubles among other health problems, to humans and animals. Since its discovery, the geographical range of the SP has been observed to expand mainly southwards, presently reaching the touristic region of Nazaré, while further expansion can be expected. We are thus planning the development of a species distribution modelling approach, in order to characterize the regions to which the SP could adapt. This approach should contribute to delineate strategies leading to slow its expansion and/or protect susceptible areas. Result dissemination will constitute a priority. In particular residents and visitors to the area should be informed on how to prevent accidents and local authorities involved.

7   General Conclusions

The overview of the main results obtained in phylogeny, phylogeography and population genetics for the genus *Thaumetopoea*, and in particular for *T. pityocampa*, *T. wilkinsoni* and *T. pinivora*, shows that such approaches have brought valuable information in the past 15 years about both the past and contemporary evolutions of populations in front of climate and environmental changes. The field of molecular genetics and phylogenetics is now in front of the genomic and post-genomic era. Genomic resources, based both on genome and transcriptome (i.e., expressed gene sets) sequencing will be available in the near future and will allow to set up new designs to gain precise information both for the neutral history of populations and species (population genomics and phylogenomics) and for the study of specific genes involved in adaptive traits. These perspectives will undoubtedly bring valuable information to understand the fate of populations and species faced to rapid global changes.

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