

# Evolution of a genetic polymorphism with winter warming illustrates how the thymes they are a-changin'

J. D. Thompson<sup>\*,1</sup>, A. Charpentier<sup>£</sup>, G. Bouguet<sup>£,§</sup>, F. Charmasson<sup>\*</sup>, S. Roset<sup>£</sup>, B. Buatois<sup>\*</sup>, P. Vernet<sup>#</sup> & P.-H. Gouyon<sup>¶</sup>

<sup>\*</sup> UMR 5175 Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, 1919 route de Mende, 34293 Montpellier cedex 5, France <sup>£</sup> UMR 5175 Centre d'Ecologie Fonctionnelle et Evolutive, Université de Montpellier 2, Place Eugene Bataillon, 34398 Montpellier, France <sup>§</sup> Flore en Thym SAS - 5 rue de la Lucques, Sydel Pays Coeur d'Hérault, 34725 Saint André de Sangonis <sup>#</sup> Laboratoire de Génétique et Evolution des Populations Végétales, UMR 8198, CNRS - Université des Sciences et Technologies de Lille 1, 59655 Villeneuve d'Ascq cedex, France <sup>¶</sup> Muséum National d'Histoire Naturelle, 12 rue Buffon, CP 39, 75005 Paris, France

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**Many species show changes in distribution and phenotypic trait variation in response to climatic warming. Evidence of genetically based trait responses to climate change is however less common. Here, we detected evolutionary variation in the landscape-scale distribution of a genetically based chemical polymorphism in Mediterranean wild thyme (*Thymus vulgaris*) in association with modified extreme winter freezing events. By comparing current data on morph distribution with that observed in the early 1970s we detected a significant increase in the proportion of morphs that are sensitive to winter freezing. This increase in frequency was observed in 17 of the 24 populations where, since the 1970's, annual extreme winter freezing temperatures have risen above the thresholds that cause mortality of freezing-sensitive morphs. Our results provide an original example of rapid ongoing evolutionary change associated with relaxed selection (less extreme freezing events) on a local landscape scale. In species whose distribution and genetic variability are shaped by strong selection gradients there may be little time lag associated with their ecological and evolutionary response to long-term environmental change.**

adapation | climate | evolution

## Introduction

Ongoing changes in regional climates are pushing many species to shift their distribution towards higher latitudes and altitudes (1, 2, 3, 4, 5, 6, 7). Such changes in species distribution, with an expansion in previously hostile areas and contraction in areas becoming less favorable, can occur rapidly both in plants and animals (2, 3, 5, 6). As a result, major changes in community composition due to differential migration rates may occur (8). Indeed, habitat fragmentation may prevent many species from showing such a distributional response to climate change. As a result, only those species that can respond by phenotypic plasticity or genetically based local adaption will persist (9). In animal and plant species, phenotypic plasticity of phenological traits can allow individuals to adjust to climate change (1, 10, 11). In addition to changes in distribution and plasticity, an evolutionary response to climate change may occur if species evolve a genetically based adaptation to climate change (12, 13). It is important to distinguish this genetic response from a plastic response of individuals if we are to fully understand the evolutionary potential of species to evolve with climate change (14). Adaptive trait variation in relation to climate change has been shown in the classic study of *Drosophila* (15, 16) and in experimental and natural populations of a small number of plant species (17, 18, 19, 20). However, in some cases the evolutionary response to climate change may be slow due to genetic constraints (21) causing a time lag between the environmental change and an observed evolutionary response. Understanding how species track climate change by genetically based adaptive trait variation and which traits facilitate the evolution of such adaption is important; such issues determine which

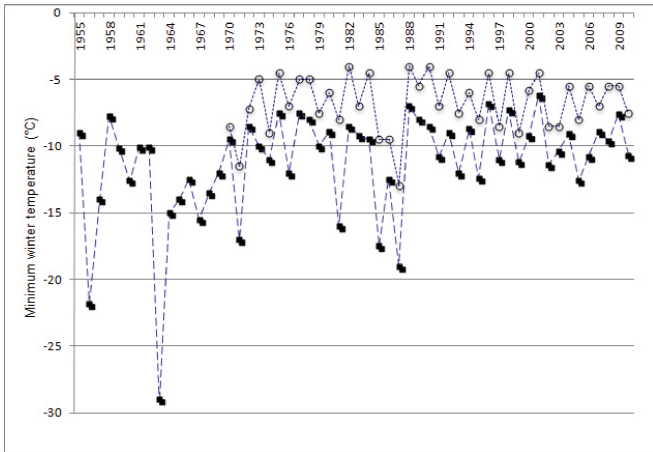
species may persist locally and which may shift their distribution (22, 23).

In this study we test for an evolutionary response of a genetic polymorphism in essential oil composition in Mediterranean wild thyme, *Thymus vulgaris*, to reduced selection associated with a warming of extreme winter freezing events over a sharp spatial climatic gradient. Our study was done in and around the Saint Martin-de-Londres basin (43°48'N, 03°46'E) which covers an area of ~80 km<sup>2</sup> and whose southern limits are ~20 km north of Montpellier in the Mediterranean climate region of southern France. The center of the basin (lowest altitude: 145m) is surrounded by calcareous hills, ranging from 300 to 658m. The study area has a Mediterranean climate with summer drought but also severe winter freezing temperatures within the basin as a result of a dramatic temperature inversion (Fig. 1). In this area, there are six different chemotypes which are the expression of a genetically controlled polymorphism in *T. vulgaris* (24). Two phenolic chemotypes (carvacrol and thymol) are largely dominant on the slopes outside of the basin on stony soils above 250m elevation and four non-phenolic chemotypes (linalool, thuyanol-4, alpha-terpineol and geraniol) occur within the basin below 200m elevation on deeper more humid soils (25, 26, 27), where they experience the winter temperature inversion. There is thus a sharp cline in chemotype frequency over only 3-5 km that goes from 100% of either phenolic or non-phenolic chemotypes to 100% of the other form, with a narrow transitional zone (Fig. 2). In short, non-phenolic chemotypes show marked adaptation to habitats which in the past have frequently experienced extreme freezing temperatures in early winter whereas phenolic chemotypes are sensitive to intense early-winter freezing and occur in habitats where extreme summer drought can exclude non-phenolic chemotypes (28, 29).

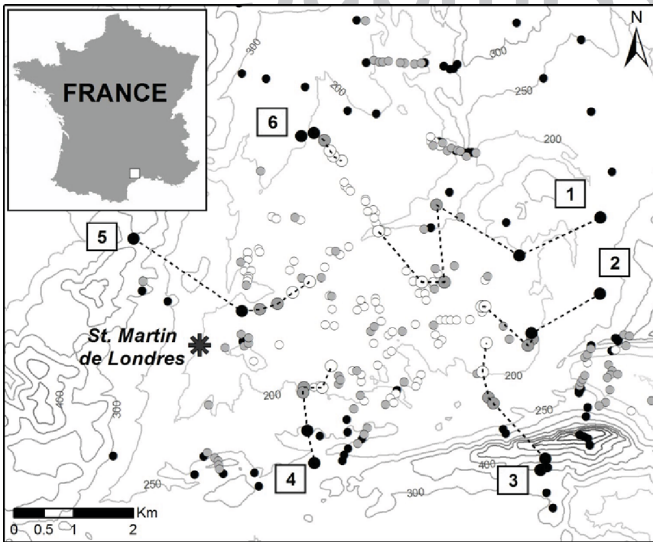
In the part of the study area where non-phenolic chemotypes dominated populations in the early 1970s, extreme winter temperatures have become less severe (Fig. 1) with a significant increase in temperature of extreme freezing events ( $r = 0.36$ ,  $n = 56$ ,  $p < 0.01$ ). In the 20 years prior to and during the initial study, winter temperatures fell below levels (-15°C in December) that would exclude phenolic chemotypes from sites dominated

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**Fig. 1.** . Coldest annual temperature from 1955 to 2010 at the weather station of Saint Martin-de-Londres (filled squares) which occurs in the zone dominated by freezing-tolerant non-phenolic chemotypes, and from 1970 to 2010 at the CEFE-CNRS experimental gardens on the northern periphery of Montpellier (open circles) where natural thyme populations are dominated by freezing-sensitive phenolic chemotypes.



**Fig. 2.** . Spatial distribution and chemical composition of sampled thyme populations in the early 1970's and the 36 populations that were re-sampled in the present study along six transects. The chemical composition of re-sampled populations indicated on the map is that which was observed in the initial study. Black circles - phenolic populations, open circles - non-phenolic populations, grey circles - mixed populations. On each transect the six populations are connected by a dashed line and are represented by a slightly larger circle than populations that were not re-sampled in the present study.

by non-phenolic chemotypes (29) on five occasions. In the 16 years following the initial study, three such events were recorded. In the last 20 years no such extreme events have been recorded in the zone dominated by non-phenolic chemotypes. The last extreme freezing event that could impact the composition of thyme populations occurred 25 years ago.

Here, we test the hypothesis that phenolic chemotypes (thymol and carvacrol) now occur in sites where they were previously absent or have increased their frequency in transitional sites due to a relaxation of selection normally associated with extreme early-winter freezing temperatures. To do so, we compared the chemotype composition of populations observed in

the early 1970's (26) to that in 2009-2010 for 36 populations sampled along six transects. Each transect is less than 10km long each containing six populations: with two "phenolic", "mixed" and "non-phenolic" populations (Fig. 2). To provide an indication of whether population-level changes are due to within-population adaption or migration among populations we also examine whether any increases in the abundance of phenolic chemotypes are primarily in non-phenolic or mixed populations that are spatially the closest to pre-existing phenolic populations.

**Results**

We compared the contemporary composition of each of the 36 re-sampled populations with their composition in the early 1970s by geographically pairing samples, i.e. the two samples of each population, and performing a non-parametric Wilcoxon test across the paired samples of a given population. Non-parametric tests were used due to a severely skewed distribution associated with a consistent trend towards increasing abundance of phenolic chemotypes. Indeed, we found that the mean percentage of phenolic chemotypes in a population was significantly ( $df = 35, S = 68.5, p < 0.01$ ) higher in the contemporary samples (overall value of 53.1%) than in those of the initial study (47.7%) (Table 1). The number of populations in which plants with the thymol chemotype were detected increased from 20 in the early 1970s to 30 in 2010 (the increase was from 16 to 22 populations for carvacrol, the other phenolic chemotype). Eight of the 13 exclusively non-phenolic populations in 1973 currently have a mixed composition whereas only one of the 12 populations composed of exclusively phenolic plants in 1973 was observed to contain non-phenolic plants in 2010 (Table 1). In a goodness of fit G-test these changes produced a significant total ( $G = 12.2, df = 3, p < 0.01$ ) and a significant heterogeneity among the two types of population ( $G = 4.34, df = 1, p < 0.05$ ). The changes in composition of the initial non-phenolic populations were associated with the appearance of the thymol chemotype in all eight of the populations whose composition changed and the carvacrol chemotype in three of them. For the group of 11 populations that were mixed in 1973, the mean percentage of phenolic chemotypes significantly increased (Wilcoxon test for paired samples:  $df = 10, S = 24, p < 0.05$ ) from 43.2% to 52.4% (Table 1). Nine of these populations showed an increase in the percentage of phenolic plants, primarily due to a 9% increase in the mean percentage of thymol plants (the carvacrol chemotype showed no mean percentage increase in mixed populations). In the eight populations that were mixed at both dates, five showed a 20-30% increase in percentage of phenolic plants, two showed no variation in composition, and one showed a 25% increase in non-phenolic plants.

Finally, we examined whether changes in chemotype frequency are associated with distance among populations. If migration is responsible for changes in chemotype frequency, increased frequency of phenolic chemotypes would be most likely to occur in non-phenolic or mixed populations that are spatially close to phenolic populations. For the 13 populations that were composed of only non-phenolic plants in 1973, the distance to the closest sampled population at that time ranged from 95m to 947m. For the eight populations that became mixed in 2010 the mean distance to a known phenolic population was significantly (Student  $T = 2.25, df = 11, p < 0.05$ ) smaller (mean + S.E. = 381m + 119) than the distance between the five populations that remained composed of only non-phenolic individuals and a known phenolic population (mean + S.E. = 601m + 200).

**Discussion**

Changes in the intensity and frequency of extreme climate events have fundamental significance for the response of natural organisms to climate change. Extreme climate events are well known to affect the sustainability of agricultural practices (30, 31), drive

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**Table 1. . Percentage of plants with a freezing-sensitive phenolic chemotype in 36 populations of *Thymus vulgaris* sampled in 1973 and 2010 along six transects (see Fig. 2) designed to sample initially phenolic (populations 1 and 2), non-phenolic (populations 5 and 6) and mixed (populations 3 and 4) populations.**

Population	Transect 1		Transect 2		Transect 3		Transect 4		Transect 5		Transect 6	
	1973	2010	1973	2010	1973	2010	1973	2010	1973	2010	1973	2010
1	100	100	100	100	100	100	100	100	100	100	100	90
2	100	100	100	100	100	100	100	100	100	100	100	100
3	82	100	91	100	40	67	70	44	60	80	23	24
4	19	47	78	87	16	17	26	46	14	0	0	17
5	0	0	0	0	0	7	0	6	0	14	0	3
6	0	0	0	0	0	3	0	44	0	17	0	0

dramatic shifts in distribution ranges (2, 3, 5, 6) and impose strong directional selection pressures on natural populations (17, 18, 19, 20). Here, we report a rapid and probably ongoing spatial reorganization of a genetic polymorphism that is closely associated with a warming of extreme winter freezing events on a highly localized spatial scale.

The changes we report involve a reduced intensity of freezing events and changes in frequency of freezing tolerant and non-tolerant phenotypes in natural populations of the Mediterranean aromatic plant, *Thymus vulgaris*. We observed both a significant appearance of freezing-sensitive phenolic chemotypes in sites where they were historically absent and an increase in their frequency in previously mixed populations. Such changes have occurred in 17 of the 24 populations where they could potentially occur. For more than 20 years the freezing temperatures that have been shown to cause mortality of the phenolic chemotypes in early winter (28, 29) have not occurred in the study area (Fig. 1). Although our observations do not conclusively demonstrate a causal relationship, the highly sensitive nature of plants with a phenolic chemotype to the occurrence of intense early winter freezing and the reduced intensity of such extreme freezing events in the study area are indicative of an evolutionary change in response to relaxed selection. Such a decline in the frequency of extreme frosts are a repeated element of observed climate change in many areas (30), hence there may be many species currently adapting to such relaxed selection. Our study shows illustrates how this can occur on a highly localized landscape scale.

Although most of the changes in population composition we detect are fairly small, some changes involve shifts of up to ~40% increase in percentage of phenolic plants in a population and several changes are related to the appearance or complete loss of morphs from a population. Also, the time period over which the changes have occurred is of a very short duration. We sampled adult plants and thus may have minimized the chances of observing changes; it would now be interesting to sample a cohort of seedlings and small plants to see if changes are even more apparent in the most recently established plants. The difficulty here is that once thyme plants get through a very small seedling stage they rapidly develop a woody stem and can often live more than 10 years, sometimes with little extra growth in size after 3-5 years. It is impossible to age such plants and some small woody plants are likely to have the same age as much larger plants. A study of seedlings in the wild and in offspring raised from seed

collected in the field could however provide information on the ongoing nature of the observed changes.

The increased frequency of phenolic chemotypes in mixed populations and their appearance in populations where they were previously absent is primarily due to increased presence of the thymol chemotype. This chemotype has appeared in all eight of the historically exclusively non-phenolic populations that are now mixed populations. The carvacrol chemotype has appeared in only three of these populations. An important factor that may contribute to these differences concerns the relatively enhanced resistance to intense freezing temperatures in the thymol chemotype compared to the carvacrol chemotype. The later is the chemotype that most suffers from extreme early winter freezing events in the field (28). In the wild, carvacrol plants occur in habitats that are the least subject to winter freezing whereas thymol plants often occur are in colder sites which are spatially closer to those of non-phenolic plants (25, 26, 27). The changes we present have occurred on a landscape scale and there is evidence that that they may result from a combination of both local within-population adaptation and gene flow among populations.

First, the epistatic genetic control of the chemical polymorphism (24) may allow for fairly rapid local changes in chemotype frequency and within-population adaptive variation to a change in selection pressure. The chemical polymorphism is associated with a single biosynthetic chain of monoterpene production and five interacting loci. The genes for non-phenolic chemotypes precede those for the phenolic chemotypes in the epistatic chain of loci. In the presence of a dominant "G" allele (the first genetic locus in the chain) a plant will have the geraniol chemotype (the essential oil being primarily composed of the geraniol monoterpene) regardless of its allelic composition at subsequent loci. If a plant is recessive for the "G" allele, but has the dominant "A" allele at the second locus, it will produce the alpha-terpineol molecule and be that chemotype, again regardless of composition at subsequent loci. The series continues with the thuyanol-4 and then linalool (non phenolic) chemotypes to the fifth and final locus where a plant which has only recessive alleles for the four "non-phenolic" loci and a dominant "C" allele will have the carvacrol phenotype. Finally, in the absence of any dominant genes in the chain a plant will have the thymol phenotype. As a result, crosses among non-phenolic plants can produce other chemotypes including the phenolic chemotypes whereas crosses among phenolic plants only produce phenolic chemotypes. Hence, the genes for phenolic

chemotypes, particularly thymol, can be maintained in populations despite strong selection against them since their expression is masked by the presence of genes coding for non-phenolic chemotypes. Any change in selection regime could thus allow for a rapid evolution in the frequency of phenolic chemotypes, especially thymol. Hence, it is probable that at least some of the observed changes result from within-population genetic change and adaptation to relaxed selection on offspring produced by crosses among individuals heterozygous at the loci for non-phenolic chemotypes.

It is important to note here that there is no qualitative plasticity in chemotype expression: a given plant maintains its chemotype (i.e. phenotype) whatever the conditions of growth. The variations which can occur involve quantitative changes such as seasonal variation in the production of monoterpenes; it is well known that the best time to harvest thyme for its essential oil content is during the flowering season when production is maximized and that production diminishes in winter (32). In samples of different individuals of a given chemotype we have observed quantitative variation in the percentage composition of the dominant monoterpene; however, all individuals can be qualitatively assigned to same chemotype (33). This recent study showed that carvacrol plants growing in marginal environments (i.e. towards the transition zone with non-phenolic chemotypes) produce a reduced percentage of carvacrol and a higher proportion on precursor molecules in their essential oil. They do not, however, change chemotype.

Second, the fact that historical non-phenolic populations which experienced the appearance of phenolic plants are closer to known phenolic populations than those populations which did not show such changes provides evidence that gene flow has at least in part contributed to the evolutionary change in chemotype frequency. This result is coherent with our previous work on the population genetics of *T. vulgaris* done in the study area during the 1990s. In a study of the population genetic structure of natural populations on three transects, each of which spanned non-phenolic, mixed-chemotype and phenolic populations in the same manner as our current study (two of the transects involved several populations used in the present study), we found low spatial differentiation for allozymes despite strong spatial structure of the genes determining chemical phenotype (34). This study thus provided evidence of fairly high levels of gene flow among thyme populations in our study area. The fact that this study was done in the mid 1990s is indicative of a history of gene flow across the study landscape.

In *T. vulgaris*, chemotype migration can occur through the dissemination of seeds or pollen. Thyme seeds are not anemochorous and have no structure to facilitate animal dispersal and thus do not disperse more than few meters or tens of meters (35), whereas pollen is insect dispersed, potentially over much large distances. Hence, as the comparison of nuclear and chloroplast DNA genetic structure in thyme populations in our study area suggests (36), gene flow mainly occurs via pollen, whose movement is thus a likely source of gene flow for novel chemotype establishment. In our study, distances from non-phenolic populations to "source" phenolic chemotypes in mixed populations were around a few hundred of meters, a distance that is compatible with foraging distances estimated for bees (37), the most abundant contemporary pollinators of thyme (38). In addition, the study populations occur in a relatively non-fragmented garrigues landscape where there are no major barriers to pollinator movement other than small roads, oak woodland or agricultural fields. In our study system it thus appears that a combination of local adaptation and migration has contributed to the changes in the spatial organization of the secondary compound polymorphism. A detailed spatial analysis of population limits, neighboring population size and distances among populations in relation to changes

in chemotype composition is now necessary to more accurately identify the relative contributions of local adaptation and gene flow to observed changes in chemotype composition. Untangling their relative contributions to ongoing evolution of the polymorphism provides a fascinating perspective.

In thyme, chemotypes may be directly adapted to their climatic environment or alternatively to changes in other features of the biotic or abiotic environment that occur as a result of climate warming. In addition to the fact that phenolic chemotypes have been repeatedly shown to grow faster than non-phenolic chemotypes in the absence of early-winter freezing (28, 39), trichomes (glands where the oil is stocked on the outer surface of leaves) on non-phenolic adult plants are more fragile with respect to freezing temperatures than those on phenolic plants (29). The later result indicates that non-phenolic plants may be able to liberate monoterpenes outside of the plant rather than into the leaf upon freezing. Non-phenolic chemotypes may thereby escape any negative effects of freezing (see 40) by releasing their essential oil into the atmosphere following severe freezing (which phenolic chemotypes are less able to do).

However, in *T. vulgaris* as in other plants that produce secondary compounds, the chemical variation cannot be treated in isolation from the range of biotic interactions that chemotypes may have with co-occurring species. First, changes in herbivore pressure may facilitate increased abundance of phenolic chemotypes due to their overall enhanced resistance to generalist herbivores (41, 42). If such herbivores show a response to climate change, then they may facilitate the colonization of non-phenolic populations by phenolic chemotypes. In addition, *T. vulgaris* shows close interactions with co-occurring grasses (43, 44) which can mediate the nature of plant / parasite / herbivore interactions entertained by the different chemotypes. The non-additive interactions which can arise between thyme chemotypes in this multi-trophic setting make it clear that although the changes we observed in chemotype composition of natural populations are closely associated with climate warming, the precise processes mediating the response of chemotypes to climate change may lie in a highly complex combination of abiotic and biotic challenges.

Other than at one of our study sites, non-phenolic chemotypes, which are less tolerant of summer drought than phenolic chemotypes, have not colonized phenolic populations located around the basin on rocky and shallow soils more prone to extreme Mediterranean summer drought stress (27, 28). In such sites there have been ongoing episodes of strong selection against non-phenolic chemotypes, e.g. the extreme summer conditions in 2003 which caused mortality of all chemotypes, but to which the phenolic chemotypes, particularly carvacrol, showed enhanced resistance (28). However, if this were the cause of increased frequency of phenolic chemotypes in our study populations, one would have predicted a significant change primarily caused by an increase in frequency of the carvacrol chemotype, which is not the case.

Climate-induced changes in the distribution of species have been increasingly modeled and documented. We thus now benefit from a corpus of knowledge on the nature of species' responses in terms of distribution change (references 1-7), how modified distribution patterns may lead to changes in community composition (-8) and also potentially cause genetic erosion in areas of range contraction (45). Here, we provide an original illustration of a change in spatial organization of a genetic polymorphism in response to reduced extreme freezing events which illustrates how, in the absence of any visible shifts in a species range, species' responses to climate change may be guided by gene flow and local adaptation. It is often thought that constraints associated with life-history traits and genetic variability (21, 22, 23) and time lags (46, 47) may delay the evolutionary response of organisms, particularly plants, to long-term environmental change. Our

study, done over a similar time span as work reporting ecological changes in plant species distribution (3, 5), illustrates that a rapid evolutionary response to temperature modifications can occur where genetic variation is combined with a change in a previously strong selection pressure, even for a perennial woody plant.

### Material and Methods

Six transects (Fig. 2) each less than 10km long and containing six populations: with two "phenolic", "mixed" and "non-phenolic" populations were sampled across the gradient in chemotype frequency. Transects 2, 3 and 6 were sampled in April 2009, and transects 1, 4 and 5 were sampled in April 2010. Due to changes in land-use, one transect had three mixed populations. To be as objective as possible in the choice of the micro-site to resample populations once at a given site, we placed the point indication (from an original map of field surveys in the early 1970s) of each original population on a digitalized 1/25,000 scale map of the region and selected its precise latitude and longitude coordinates. With these coordinates we returned to each site with a GPS and once at the precise point in the field we collected leaves on 30 adult plants (as in the original 1970s sampling scheme) sampled

at least two meters apart within a sampling area of up to 14m by 16m.

For each plant, essential oils were extracted by putting leaves in 1mL of methanol for 24 hours. Analytical gas chromatography was carried out on a Shimadzu GCMS-QP21010 fitted with a flame ionization detector (FID) and a fused silica capillary column SLB-MS (30 x0.25 mm; 0.25 µm thickness). Helium was used as a carrier gaz. The injector was heated at 160°C. The oven temperature was programmed from 50°C to 180°C at around 25 min, and increase to 270°C for a total time of analysis around 38 min. Compounds were identified by comparison of their retention times and mass spectra with those of standards published by Mass Spectral Library (NIST, 2005) and in the literature (48). 52 possible components of the essential oil were used to identify chemotypes. The original chemical analyses and chemotype identification used gas chromatography and visual identification of peaks based on retention times.

### Acknowledgements.

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