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Population genetics in the homothallic lichen-forming ascomycete *Xanthoria parietina*

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Abstract: The genetic diversity within and among populations of *Xanthoria parietina* was studied at the subspecific level with a fingerprinting technique (RAPD-PCR) applied to sterile cultured multispore isolates, each being derived from a single apothecium. Populations from coastal, rural and urban sites from NW, SW and central France and from NE Switzerland were investigated. Between 1 and 8 microsites of a few decimetres square, each comprising 13 to 27 thalli of *X. parietina*, were analysed per population. A total of 132 isolates from epiphytic and 3 isolates from epilithic specimens were investigated. Phenotypic variation was recorded among some of the thalli in the field and among sterile cultured isolates in the laboratory. A high diversity of genotypes was observed, even among thalli growing side by side in phenotypically homogenous populations. An average of 73·5 % polymorphism was found in all samples. As shown with Principal Coordinates Analysis (PCO), most of the genetic variation (90%) resided within, not among, populations. As *X. parietina* had previously been shown with molecular and fingerprinting techniques to be homothallic, the potential genetic background of this diversity is discussed. Intense genotype rather than gene (allele) flow seems to be an important element in *X. parietina* populations.

Key words: AMOVA, fingerprinting techniques, PCO, RAPD-PCR, sterile cultured isolates

Introduction

Xanthoria parietina is one of the most common and widespread species of lichen-forming ascomycetes in Europe. Its characteristic golden yellow thalli occur on a wide range of natural and anthropogenic substrata, from shaded to fully sunlit sites on nutrient-rich or eutrophic substrata. Its area of distribution ranges from the Caucasus to westernmost Europe and from North to South Africa; it is common on the east coast of northern North America, was found in Ontario and occurs on the west coast from Baja California to the southern part of British Columbia (Lindblom 1997; Brodo *et al.* 2001, 2007; McCune 2004). *Xanthoria parietina* was introduced into Australia and New Zealand, where it grows side by side with morphologically similar native species (Galloway 1985; Rogers 1992; Eichenberger 2007). Honegger *et al.* (2004*b*) concluded from fingerprint analyses of specimens from four continents, that the Australian *X. parietina* might have been introduced from southern Europe.

Xanthoria parietina forms no vegetative symbiotic propagules but is invariably fertile, its central thalline areas being covered by apothecia. Fingerprinting techniques (RAPD-PCR) applied to single spore isolates derived from single asci (i.e., the progeny of meiosis) and to the sterile cultured vegetative mycelium of the ascocarp of *X. parietina* revealed identical fingerprints and, thus, it was concluded to be homothallic by Honegger *et al.* (2004*a*). The genetic background was elucidated in molecular analyses of the mating type loci (*MAT1-1-1* and *MAT1-2-1*) plus flanking regions of a range of *Xanthoria* species, where the homothallic *X. parietina* was shown to contain *MAT 1-2-1* in all descendants of meiosis, *MAT 1-1-1*

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having been lost (Scherrer *et al.* 2005; nomenclature according to Turgeon & Yoder 2000); thus recombination seems unlikely to occur in *X. parietina*. *Xanthoria elegans*, another homothallic species, had *MAT1-1-1* and *MAT1-2-1* in all siblings (Scherrer *et al.* 2005). Heterothallic species such as *X. calcicola*, *X. polycarpa* or *X. capensis* have either *MAT1-1-1* or *MAT1-2-1* in half of the siblings derived from one ascus and polymorphic fingerprints (2 × 4 genotypes among the 8 sporelings per ascus) resulting from successful recombination (Honegger *et al.* 2004*a*; Scherrer *et al.* 2005).

As the homothallic *X. parietina* is unlikely to recombine we wondered whether natural populations have a clonal structure. Lindblom & Ekman (2006, 2007) analysed the intraspecific genetic variation in populations of *X. parietina* samples in Norway and SW Sweden (Scania). Sampling was performed at irregular intervals over transects 30–150 m long, and epilithic and epiphytic samples were included. Part of the IGS and the complete ITS region were used as molecular markers. Genetic differences were documented within and between populations, but a high percentage (34% of IGS haplotypes, 24% of ITS haplotypes) of samples belonged to one haplotype. The authors concluded that *X. parietina* in these studies was characterized by intense gene flow within and between populations.

As shown in previous studies on *Xanthoria elegans* and *X. parietina,* a higher resolution of genetic variation among sample sets is achieved with fingerprinting techniques (e.g. RAPD-PCR markers; Murtagh *et al.* 2002; Scherrer & Honegger 2003; Honegger *et al.* 2004*b*) than with comparative sequence analysis. The present study aims to increase the resolution and expand the geographic range by analysing the genetic diversity within four populations of *X. parietina* in coastal, rural and urban areas in NW, SW and central France and NE Switzerland at the subspecific level with a fingerprinting technique (RAPD-PCR) applied to sterile cultured multispore isolates.

Materials and Methods

Collecting, isolation and sterile culturing

Collecting sites and collectors are listed in Table 1. Larger specimens were photographed and the outlines of smaller samples were painted on a transparent overlay prior to removal of single apothecia, whose site was noted on the photograph or overlay. Each multispore isolate was derived from one single apothecium per thallus. In three out of the four collecting sites, sampling was performed on two different substrata which were found at distances of a maximum of 30 m from each other. Up to eight microsites per collecting site were investigated. Within microsites (i.e. one sample set) the thalli had been growing side by side on the same stem or branch (i.e. on a small area).

Multispore isolates were obtained by placing a fully hydrated, single apothecium under an inverted Petri dish containing agarized Bolds Mineral Medium (BBM; Deason & Bold 1960) with double the amount of nitrogen. Numerous packages of ascospores were usually ejected on the agar surface within a few minutes; they germinated within less than a week. After a few weeks the germlings were transferred to an agarized, later to liquid, nutrient medium as routinely used in our laboratory (a modified malt yeast extract medium, 1/10 strength, as described by Honegger & Kutasi 1990). Voucher specimens were air dried and stored at −20°C; they will be deposited in the mycological collection of the joint herbaria of the University and ETH Zürich (Z + ZT). Vouchers of the sterile cultured isolates are cryopreserved and stored under liquid nitrogen (LN_2) in our laboratory.

DNA isolation and RAPD-PCR

Approximately 5 mg of mycelium from the liquid cultures was placed with *c.* six 1mm diameter glass beads in a 1·5 ml reaction tube, which was immersed in LN_2 for a few minutes. It was then cryogenically disrupted with a MM 301 mixer mill (Retsch GmbH, Haan, Germany) at a rate of 30 vibrations/sec for 1 min. Total genomic DNA was isolated using the GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis) following the manufacturer's protocol.

Eight decamer primers were used (Table 2), which had yielded good results in previous studies on *Xanthoria* species (Honegger *et al.* 2004*a*, *b*). PCR amplifications were carried out with 1 µl of template DNA in 25 µl reaction volumes, consisting of 19·75 µl sterile ddH_2O, ×1 reaction buffer for DyNAzyme, 0·2 mM dNTP, 0·2 µM RAPD primer and 0·5U of DyNAzyme DNA polymerase (Finnzymes, Espoo). The following cycles were run on a PTC-220 DNA Engine Dyad™ Peltier Thermal Cycler (MJ Research, Waltham, MA): initial denaturation for 3 min at 94°C, 40 cycles of 30 s at 93°C, 40 s at 37°C, 1 min 20 s at 72°C, 30 s at 93°C and a final extension of 5 min at 72°C. 15 µl of each amplification product were separated in 1·5% agarose gels in ×1 TBE buffer at 40 V for 5 h, stained with ethidium bromide and photographed under UV light. Constant reaction conditions and thermal profile during amplification were ensured (Meunier & Grimont 1993). The

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TABLE 1. *Collecting sites for* Xanthoria parietina, *collectors, numbers of microsites and isolates examined and percentage of polymorphisms detected*

Population	Collector	Collecting site	Co-ordinates	Voucher No.	Phorophyte	Number of microsites	Number of isolates	% Polymorphism
SW France: Roussillon, Dept. Pyrénées-Orientale	*R. Honegger*	Villefranche du Conflent		120	*Populus* sp.	5	14	57·33
		rural	42° 35′ 0″ N 02° 22′ 0″ E	121	*Prunus spinosa*	3	13	75·33
Central France: Bourgogne, Dept. Yonne	*R. Honegger*	Relais fleuri near Avallon		144	*Salix* sp.	3	24	76·67
		rural	47° 29′ 27″ N 03° 54′ 33″ E	145	*Cornus albus*	3	22	80·67
NW France: Bretagne, Dept. Nord-Finistère	*R. Honegger*	old port of Roscoff, Channel		164	*Tamarix gallica*	1	13	78·00
		coastal	48° 43′ 08″ N 03° 85′ 48″ W					
NE Switzerland: city of Zürich	*S. Nyati &* *R. Honegger**	University campus, Irchel		319	*Parthenocissus tricuspidatus*	5	22	69·33
		urban	47° 22′ 0″ N 08° 33′ 0″ E	320/1-/2 320/3	*Salix* sp. sandstone	2 1	24 3	77·33 38·00

*thalli of vouchers nos 319 and 320 were documented photographically and left *in situ*, only small fragments carrying mature apothecia were removed.

TABLE 2. *RAPD primers (Operon Decamer oligonucleotide primers) used in PCR in* Xanthoria parietina

No.	code	5' to 3'
1	OPA-09	GGGTAACGCC
2	OPA-10	GTGATCGCAG
3	OPB-10	CTGCTGGGAC
4	OPB-17	AGGGAACGAG
5	OPD-03	GTCGCCGTCA
6	OPD-13	GGGGTGACGA
7	OPD-16	AGGGCGTAAG
8	OPD-20	ACCCGGTCAC

reproducibility of this technique was tested in previous studies (Honegger *et al.* 2004; Honegger & Zippler 2007). High reproducibility is achieved provided that freshly isolated DNA is used and the PCR conditions are standardized. Only intense, unambiguous bands were visually scored and included in the presence-absence matrix.

Data analysis

Presence (1) or absence (0) of 150 polymorphic RAPD bands (as achieved with eight primers) were visually scored and recorded in a binary data matrix. Bands of equal fragment size were assumed to be homologous. The computer program GenAlEx 6 (Peakall & Smouse 2006) was used to calculate the statistics of genetic variation based on individual haplotypes for each population. To assess the distribution of the total genetic variation per population we calculated the percentage of polymorphic bands (P) and Nei's standard genetic distances between pairs of populations (Nei 1972, 1978). Genetic distances between pairs of isolates were calculated with the Nei coefficient (Nei 1972). Genetic distances were spatially visualized with the principal coordinates analysis (PCO) and plotted in a chart. This analysis shows the relationships between isolates and between populations, based on the first two principal coordinates of each distance matrix.

The partitioning of genetic variance within and among populations and groups of populations was assessed by an analysis of molecular variance AMOVA (Excoffier *et al.* 1992). The significance test was performed with 999 permutations. This analysis also calculates fixation indices (Φ statistics), which are indices of population subdivision analogous to F-statistics (Weir 1996).

Results

Phenotypic variation among *Xanthoria parietina* samples in the field and laboratory

In three of the four collecting sites the populations of *X. parietina* appeared

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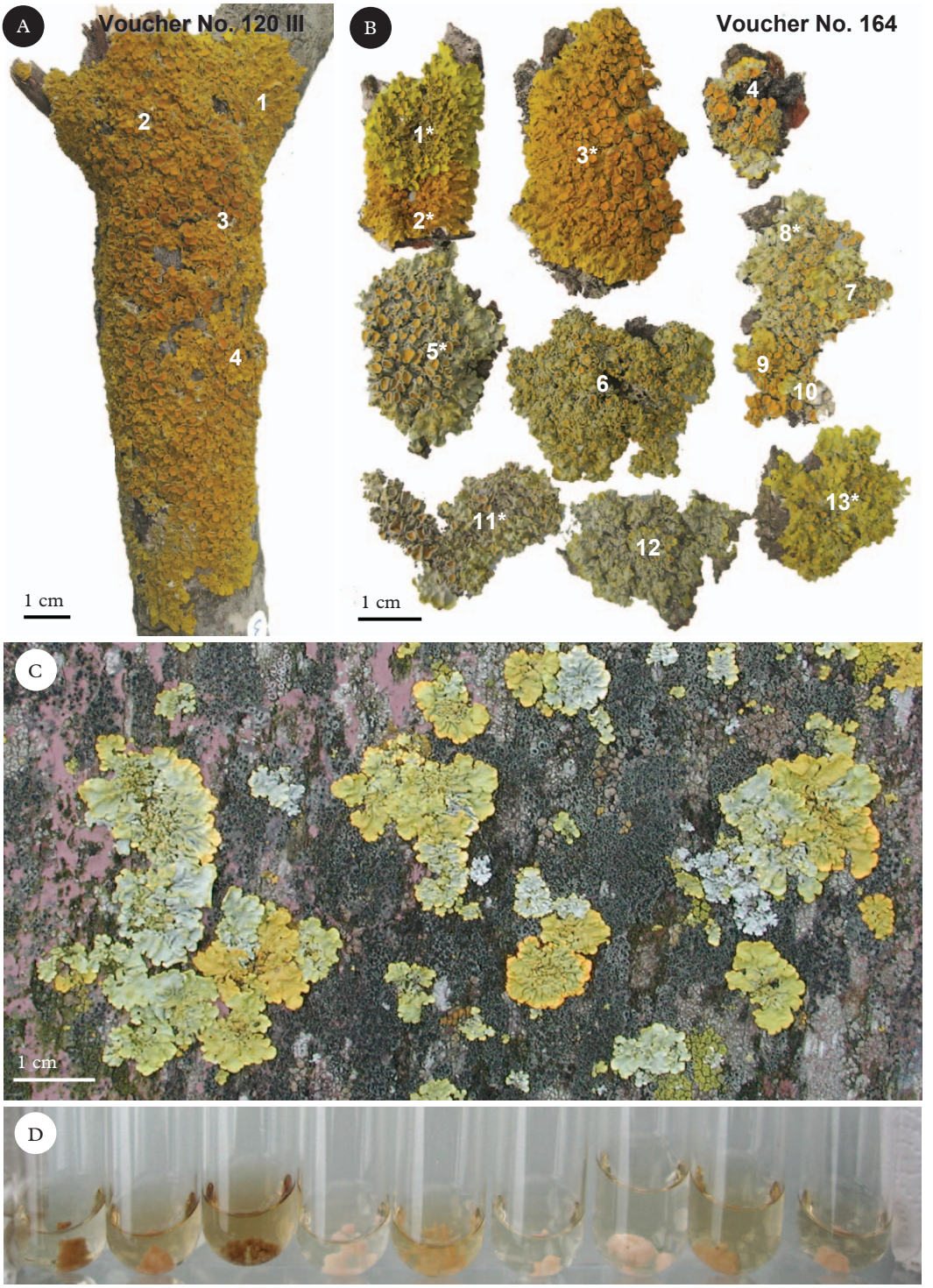

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phenotypically homogenous. The thalli revealed the characteristic coloration: intensely yellow on fully sunlit parts (Fig. 1A) and greenish grey on shaded parts, best visible in single thalli which grow around branches. Apothecial discs (i.e. the hymenial layer) and the ostioles of pycnidia always revealed an intense yellow coloration due to the deposition of crystals of the anthraquinone parietin (Søchting 1997). However, in one exceptional population from coastal Bretagne a whole spectrum of colours from greyish to orange-yellow was seen in thalli which had the same exposure and illumination, low and high anthraquinone producers growing next to each other (Fig. 1B & C). Some thalli had greyish lobes, but intensely yellow margins (Fig. 1B4, 1B11, 1C). In seven out of 13 samples of voucher 164 the lichen products were studied with thin-layer chromatography methods according to Culberson & Ammann (1979; H. Gansner, unpublished data); all revealed chemosyndrome A (parietin as the main secondary product) according to Søchting (1997), as typically found in *X. parietina*.

Even when populations were homogenous in the field, phenotypic differences were observed in sterile cultured multispore isolates (Fig. 1A & D1). Germlings of different samples from the same collecting site revealed distinctly different growth rates on non-nutrient mineral medium (Fig. 2A–C). Differences in growth rates were less prominent on nutrient media, but differences in pigmentation (probably carotenoids; these contribute to the coloration of the thallus of *X. parietina* and other *Xanthoria* spp. in nature, as shown by Czeczuga 1983) of the thallus-like fungal colonies and in the release of coloured (unidentified) secondary compounds into the culturing medium were evident (Fig. 1D).

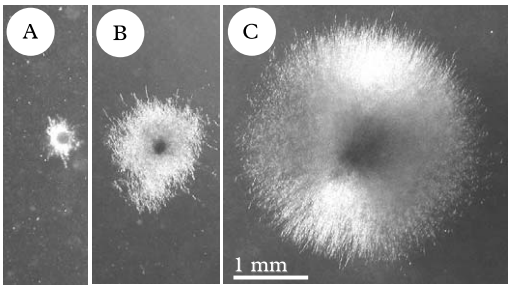

FIG. 2. Growth rates of *Xanthoria parietina* germlings from the same collecting site (University of Zurich, Irchel Park) after 5 months culturing on agarized mineral medium, same culturing conditions [date of isolation (ascospore ejection): 26 viii 2002; photographs were taken on 30 i 2003]. Each colony is a multispore isolate, as grown out of the eight ascospores contained in one ascus. All colonies from the same apothecium (multispore isolates) revealed the same growth rate and the same phenotype (see also Honegger *et al.* 2004*a*); A shows minimum, C maximum, B average growth rate as seen in most of the isolates. A, isolate 320 I bd; B, isolate 319 I f2d; C, isolate 319 IV ad. Same magnification in A–C.

Genetic diversity of *Xanthoria parietina* within and among populations

In our fingerprint analyses of epiphytic *X. parietina*, a high level of polymorphism was found in all populations, ranging from 57·3–80·7 % (13–22 isolates per site investigated; Table 1), whereas the only population from rock revealed 38% polymorphism (3 isolates tested; Table 1). The average (*P*) in the total

FIG. 1. Phenotypic plasticity in *Xanthoria parietina* in the field and laboratory (symbiotic *vs.* aposymbiotic phenotypes). A, phenotypically homogenous population with confluent thalli on a branch of poplar in SW France (voucher No. 120, microsite III); all thalli contain large amounts of parietin (see fingerprints of these four samples in Fig. 3); B, phenotypically diverse thalli on *Tamarix* shrubs in coastal Bretagne (NW France; voucher No 164) with varying amounts of parietin; this is not due to shading effects (see C) [samples with asterisks all have chemosyndrome A (according to Søchting (1997) in TLC (H. Gansner, unpublished data)], see fingerprints of some of these samples in Fig. 4; C, population of juvenile thalli developing on a fully sunlit bench 1 m from the *Tamarix* shrubs whence samples shown in B were collected, high parietin producers (intensely yellow to orange) grow next to pale, greyish thalli with very low parietin contents, the same situation is seen in voucher nos 164/5, 164/11 and 164/12 (see B), where only the apothecial discs are intensely yellow; D, sterile cultured isolates from voucher nos 120 and 121, all kept in the same culturing medium for the same period of time and revealing different coloration and secreting different amounts of secondary compounds into the medium.

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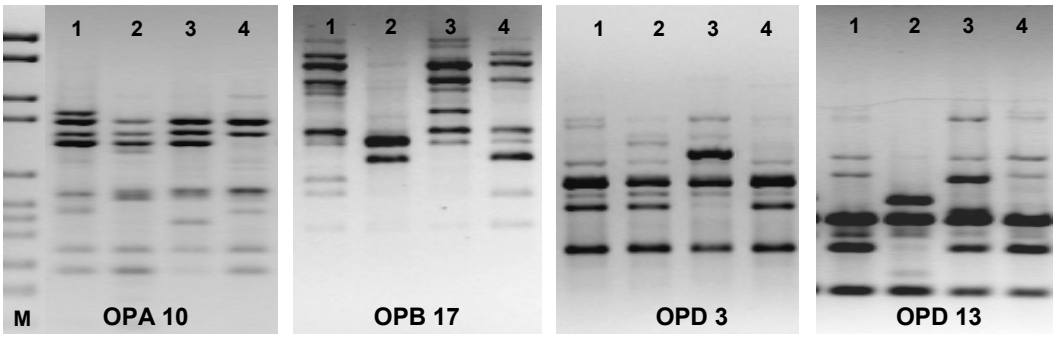

FIG. 3. RAPD-PCR (four primers) of four *Xanthoria parietina* isolates from thalli, which had been growing side by side in a phenotypically homogenous population in SW France (voucher no. 120, microsite III, see Fig. 1A). Each isolate represents a different haplotype.

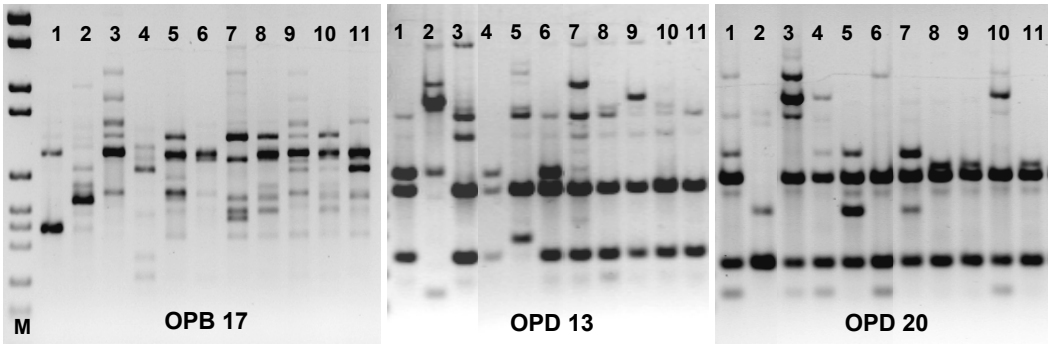

FIG. 4. RAPD-PCR (three primers) of sterile cultured multispore isolates of samples 1–11 of the phenotypically diverse population of *Xanthoria parietina* from coastal Bretagne, NW France (voucher no. 164; see Fig. 1B). Each isolate represents a different haplotype.

sample was 73·5%. It is not surprising that the phenotypically heterogenous population from coastal Bretagne (164; Fig. 1B) was genetically highly diverse, but the phenotypically homogenous population from SW France (120; Fig. 1A) reached even slightly higher values (Table 1). No genetic variation was found in isolates which derived from different apothecia on the same thallus (3 samples tested; results not shown).

The genetic distances between pairs of populations ranged between 0·029 and 0·098 (Table 3). The PCO grouped similar populations together, the phenotypically diverse population from coastal Bretagne being the most distant one (Fig. 5). The population structure was assessed by analysing the molecular variance (AMOVA). Here the populations were grouped in four regions (SW, NW and central France and Switzerland).

When the RAPD dataset was partitioned within and between populations, most of the genetic variation resided within populations (90%; Fig. 6), relative to the amount of variation among regions (3·9%) or among populations within regions (6·4%; Table 4). AMOVA produced significant ($P < 0{\cdot}001$) fixation indices. The global differentiation of populations (Φ_{ST}) amounted to 0·103, the differentiation among populations within regions (Φ_{SC}) to 0·067, and the proportion of differentiation between regions (Φ_{CT}) to 0·039.

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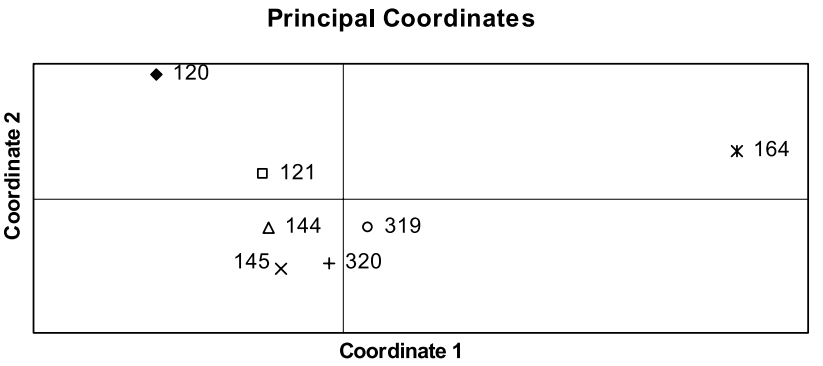

FIG. 5. Grouping of *Xanthoria parietina* populations with Principal Co-ordinates Analysis.

TABLE 3. *Pairwise Population Matrix of Nei Genetic Distance between pairs of populations of* Xanthoria parietina

120	121	144	145	164	319	320	
0·000							120
0·043	0·000						121
0·051	0·037	0·000					144
0·067	0·041	0·038	0·000				145
0·097	0·074	0·075	0·082	0·000			164
0·061	0·035	0·034	0·041	0·062	0·000		319
0·066	0·041	0·029	0·038	0·072	0·033	0·000	320

Discussion

The high genetic diversity within populations of *X. parietina*, as observed in the present study, is surprising. As a homothallic species (with *MAT1-2-1* in all progeny of meiosis, *MAT1-1-1* having been lost; Scherrer *et al.* 2005), recombination seems unlikely to occur. However, the ultimate proof for obligate homothallism, that is ascocarp and ascospore formation by a haploid mycelium derived from one single ascospore, is not yet available due to culturing problems, no sexual reproductive stages being formed in sterile cultures. Moreover a nonsense mutation, that is a stop codon at the beginning of the HMG box, was found in *MAT1-2-1* of epiphytic inland *X. parietina* (Scherrer *et al.* 2005; S. Scherrer, unpublished data); its impact on transcription and thus on gene expression remains to be explored. As an ascomycete *X. parietina* has to form a new dikaryon on the haploid mycelium prior to the formation of every fruiting body, but it is not known how this proceeds. Pycnidia as potential donors of spermatia are present and ascogonia are easy to find within and slightly below the algal layer of submarginal thallus areas (Janex-Favre & Ghaleb 1986), but no trichogynes have so far been detected with light or electron microscopy techniques (R. Honegger, unpublished data). Therefore we conclude that spermatization, a common and widespread mode of dikaryon formation in lichen-forming ascomycetes (Honegger 1984; Honegger & Scherrer 2008), is not the mode of dikaryotization in *X. parietina*.

Where does the high genetic diversity in populations of the homothallic *X. parietina* originate? In a homothallic species facultative gene flow might occur via parasexual events, for example via DNA transfer through hyphal anastomosis between different haplotypes. Such phenomena have not yet been detected among lichen-forming fungi, but cannot be excluded. In their pioneering work on mating systems in lichen-forming ascomycetes Murtagh *et al.* (2000) found uniform fingerprints among sporelings from the same apothecium, but polymorphisms among sporelings from different apothecia on the same thallus of *Ochrolechia parella* or from different thalli of *Graphis scripta*; thus they concluded the patterns were the result of facultative homothallism or opportunistic outbreeding, respectively. In *O. parella* and

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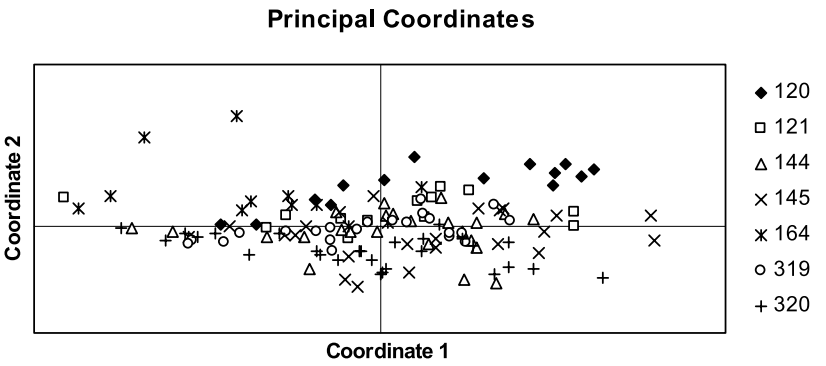

FIG. 6. Principal Coordinates Analysis of all isolates of *Xanthoria parietina*.

TABLE 4. *AMOVA, analysis of molecular variance in* Xanthoria parietina

Source of variation	df	Variance component SSD	Absolute	%	*P*
Among regions	3	187·13	0·700	4	<0·001
Among populations within regions	3	129·75	1·263	6	<0·001
Within populations	128	2264·98	17·695	90	<0·001

G. scripta the molecular basis of homothallism is unknown.

Accumulation of mutations and intense genotype rather than gene (allele) flow might be the main causes of the high genetic diversity in *X. parietina*. High genotype flow is typically found in fungal species that disperse primarily by means of vegetative propagules. Thousands of conidia are asexually produced on a single infection of powdery mildew, and hundreds of uredospores detach from a rust pustule. Both can be transported by wind over hundreds of kilometres and germinate immediately upon landing on a suitable host (for a review see McDonald & Linde 2002). The heterothallic rice blast fungus *Magnaporthe grisea*, a main fungal pathogen on rice worldwide, lacks fruiting bodies in many regions, for example because only one mating type is present, as observed in North American and Korean populations (Viji & Uddin 2002; Tredway *et al.* 2003; Park *et al.* 2008); thus allele flow via outcrossing occurs rather infrequently, yet populations are genetically diverse. This is caused by the accumulation of mutations (Park *et al.* 2008), efficient dispersal via asexually produced propagules (conidia) and partly also due to parasexual exchange of DNA via hyphal anastomoses, as observed in sterile co-cultures in the laboratory (Zeigler *et al.* 1997).

Xanthoria parietina forms no vegetative symbiotic propagules but is invariably fertile, its central thalline areas being covered by apothecia. It was therefore assumed to disperse exclusively via ascospores, which have to re-lichenize (Ott 1987). As shown in field experiments free-living photobiont cells are more common than previously assumed (Sanders 2005). However, vegetative dispersal via thallus fragments was also documented in the field (Honegger 1996). The high regenerative capacity of *X. parietina* facilitates the establishment of new thalli from larger or smaller fragments under natural or experimental conditions (Honegger *et al.* 1996). Upon being glued to adequate substrata, thallus fragments of *X. parietina* grow very well within short periods of time,

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that is 3 mm in 10 months (Armstrong 1993; Honegger 1993, 1995, 1996, 1998). Short distance dispersal of minute fragments by grazing invertebrates might be more common than previously assumed. The fecal pellets of the ever present lichenivorous mites (acari) were shown to contain viable ascospores of *X. parietina* and viable cells of its unicellular green algal photobiont, *Trebouxia arboricola* (Meier *et al.* 2002). The frequency of vegetative dispersal in *X. parietina* is not comparable to dispersal via asexually produced propagules of non-lichenized fungi (e.g. conidia, uredospores, etc.), but precise data are missing. Wind dispersal over long distances is documented for vegetative symbiotic propagules (soredia) of lichens (Marshall 1996; Tormo *et al.* 2001; Muñoz *et al.* 2004; Seaward 2008). Vertebrates, especially birds, are active and passive vectors of lichen fragments and propagules (Seaward 2008). The importance of resident and migratory birds as passive transporters of either ascospores, tiny thallus fragments of *X. parietina*, of lichenivorous mites on *X. parietina* and/or their fecal pellets over short or long distances remains to be explored, but has certainly been underestimated. *Xanthoria parietina* grows abundantly at sites where birds land and rest (Honegger *et al.* 1996) and nutrient input via droppings facilitate the growth of this nitrophilous species at the resting places. However, passive genotype transfer from adjacent populations might provide the starter material for establishing new populations and later for increasing the genetic diversity of existing ones. Theoretically only one viable photobiont cell and one or a few cells of the fungal partner (cells of the haustorial complex or germ tubes of ascospores), as found on bark substrata (Bubrick *et al.* 1984; Sanders 2005), might be sufficient to start a new thallus.

Taking homothallism and the different options for vegetative (symbiotic or aposymbiotic) dispersal into account, a clonal structure was expected at microsites, that is among vicinal thalli, which might have derived from one 'founder thallus' in phenotypically homogenous populations of *X. parietina*, as investigated in the present study. The Swiss population in the urban collecting site was expected to reveal a lower level of genetic variation because Irchel Park around the University campus was built and young trees planted 16 years before we did our sampling. In contrast, the populations in SW and central France included old trees at rural sites. However, no major differences were evident, probably because all sites are embedded within the main area of distribution of *X. parietina* and genotype flow proceeded in a similar manner throughout. A lower level of genetic variation might be found outside the main area of distribution, for example at sites where *X. parietina* was anthropogenically introduced (Australia, New Zealand).

Although the very common and widespread *Xanthoria parietina* is one of the best investigated lichens worldwide, many aspects of its fascinating biology remain unexplored.

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