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Ascospore size declines with elevation in two tropical parmelioid lichens

Didier Masson[1*] & Nicolas Magain[2]

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Abstract. Spore size and shape are biometric parameters frequently used in lichen taxonomy, especially in species characterization. However, the influence of environmental factors on the intraspecific variability of these characters remains very little investigated in lichenology. The elevational variation in spore length, width, volume and shape (length/width ratio) of two species of the genus *Hypotrachyna* (*H*. aff. *damaziana* et *H. altorum*) occurring on Réunion Island (Indian Ocean) were studied. Spore length, width and volume significantly decrease with elevation in *H*. aff. *damaziana*, and spore width and volume also significantly decrease with elevation in *H. altorum*. There is no relation between spore shape and elevation in either of the two species. A significant correlation was further observed between the intra-individual variability in spore size of *H.* aff. *damaziana* and elevation. For this species, inter-individual variability in spore volume is also correlated with mean annual temperature and mean annual precipitation of the sampling locations, and spore width and length are correlated with mean annual temperature.

Key words: *Ascomycota*, *Parmeliaceae*, *Hypotrachyna*, Réunion Island, spore morphometry, intraspecific variation

Introduction

Ascospore size provides a relevant diagnostic character frequently used for taxonomic purposes in lichenized fungi, including species delimitation (e.g., Poelt 1973; Löfgren & Tibell 1979; Clerc 1984; Martínez & Burgaz 1998; Doré et al. 2006; Argüello et al. 2007; Truong et al. 2009; Núñez-Zapata et al. 2011). However, the influence of environmental factors on spore size within a species remains largely unexplored in lichenology (Hawksworth 1973). In a review on environmental modification and lichen taxonomy, Weber (1977) wrote: 'Differences in ascospores are accorded paramount importance in lichen systematics today, but at the species level one should perhaps be asking what effects the environment might have. Spore size, numbers and perhaps even the extent of septation suggest themselves as characters on which further studies should be carried out, but scant attention has been paid to these aspects in recent years'. Four decades later, the state of affairs remains unchanged.

Hypotrachyna aff. *damaziana* and *H. altorum* are two fairly common foliose lichens on Réunion (van den Boom et al. 2011; Masson 2012), a rather young tropical volcanic island in the Mascarene Archipelago, with the highest peak in the Indian Ocean (Piton des Neiges, 3070 m). These corticolous lichen-forming *Ascomycota* reproduce sexually, and their thalli generally produce apothecia with simple, hyaline mature spores. *Hypotrachyna* aff. *damaziana* is a taxon with a well-defined phenotype on Réunion Island (Masson unpubl. data). It is morphologically and chemically similar to *H. damaziana* from tropical America and Kenya (Hale 1976; Krog & Swinscow 1979; Eliasaro & Adler 2000; Flakus et al. 2012; Nash et al. 2016). Molecular data from thirteen specimens from Réunion (Masson & Sérusiaux unpubl. data) and one Kenyan specimen (Kirika et al. 2019) show that the two populations are most probably conspecific. However, since the type of *H. damaziana* has been collected in Brazil, molecular studies on Neotropical material are needed to determine whether the African and American specimens belong to the same species. *Hypotrachyna* aff. *damaziana* thrives on Réunion in five main habitat types (as defined by Strasberg et al. 2005): submontane and montane forests, *Pandanus* wet thickets, *Acacia* montane forests and subalpine thickets, in an elevation range from 620 m to 2375 m a.s.l. (Masson unpubl. data). *Hypotrachyna altorum* has a much more restricted elevation range, 1485–1850 m, and is confined to montane and *Acacia* montane forests (Masson 2012). The combination of regular spore production and wide elevational range makes *H.* aff. *damaziana* particularly interesting for testing the influence of environmental

[1] 386 rue des Flamboyants, F-40600 Biscarrosse, France
[2] Evolution and Conservation Biology, InBios Research Center, Institut de Botanique B22, Université de Liège, Chemin de la vallée 4, 4000 Liège, Belgium
[*] Corresponding author e-mail: di.masson@wanadoo.fr

factors on spore size variability, whereas *H. altorum*, with its smaller elevational range but with a rather similar ecology, could provide an interesting model to compare. Elevational gradients are widely used as 'natural' experiments for testing the response of organisms to abiotic influences, although the diversity of environmental factors varying with elevation makes any interpretation rather tricky (Körner 2007).

Experimental studies have shown that ecological conditions, especially temperature and moisture, affect ascospore morphology in non-lichenized fungi (e.g., Williams 1959; Petrie 1994). Further, because of their unique physiology, lichens are highly sensitive to climatic conditions (Kershaw 1985; van Herk et al. 2002; Palmqvist et al. 2008; Gauslaa 2014). The two *Hypotrachyna* species under consideration thrive mainly on branches of trees or shrubs with loose crowns and rather light foliage, such as *Acacia koa* [= *A. heterophylla*, cf. Le Roux et al. (2014)], the main phorophyte for *H.* aff. *damaziana* (Masson unpubl. data) and the exclusive one for *H. altorum* (Masson 2012). It is reasonable to assume that in these well-lit, well-ventilated situations, these lichens are exposed to mesoclimatic variation.

The present work tests two assumptions: (i) spore size, and possibly also spore shape, of both *Hypotrachyna* species change with the elevation of the sampling localities; and (ii) spore size and/or spore shape correlate with climatic variables such as temperature and/or rainfall and/or solar radiation.

Materials and methods

Specimen sampling and characterization of localities

Fresh specimens of *H.* aff. *damaziana* and *H. altorum* were collected on Réunion by the first author between 2003 and 2017. They are preserved in the herbarium of the University of Liège (LG) and the first author's private herbarium. Specimens with at least one well-developed and not parasitized apothecium were collected, for *H.* aff. *damaziana*, in sixty localities distributed in 48 1 × 1 km cells of the UTM grid system. Thirty-two of these cells (two-thirds) were randomly selected for a statistical study. Specimens of *H. altorum* were collected in twenty-two localities distributed in 14 1 × 1 km cells, and all of these cells were included. Of the 46 cells selected for both species (32 for *H.* aff. *damaziana*, 14 for *H. altorum*), 19 contained more than one locality and/or more than one specimen. In this case, only one specimen from a single locality was randomly drawn for each of these 19 cells. Thus, each of the 46 samples studied consisted of a single specimen taken from a single locality in a 1 × 1 km cell of the UTM grid system. This procedure was chosen to avoid too close proximity between the sampling sites and thus to limit spatial autocorrelation. Data on the samples are included in Table 1 and Figure 1. Four collection sites are shared by the target species. All but three of the studied specimens were collected in July and August, during the 'cool' season (Jumaux et al. 2011).

The elevation of the forty-two localities was determined in the field using a hand-held GPS receiver (Garmin eTrex) and subsequently controlled using CartoExploreur 3 (Bayo) software. Climate data (mean annual temperature, mean annual precipitation, annual mean daily global solar radiation) for each locality were determined from the maps available in Jumaux et al. (2011). The averaging period is 1991–2010 for temperature, 1981–2010 for precipitation, and 2001–2010 for global solar radiation.

Spore measurements

Ascospores in their fully hydrated state were examined with a Zeiss PrimoStar compound light microscope after being freed from the asci in squash preparations of hand-cut sections of ascomata mounted in tap water. Only non-deformed spores with coloured cytoplasm filling the entire cell were taken into account. For each sample, 30 randomly selected spores from a single well-developed apothecium were measured using an ocular micrometre at 1000×. For one sample (*H.* aff. *damaziana* No. 15), spores from ten apothecia were studied to assess variability of spore size and shape within a single thallus. One of these ten apothecia (apothecium 'A'; Fig. 2) was randomly selected for the comparisons among localities. Thus, the measurements obtained from this apothecium characterize sample No. 15 in the rest of the study. Length and width values were recorded to the nearest 0.5 µm. The length/width ratio (Q) was used as an indicator of spore shape. The volume of each measured spore was estimated using the equation of an ellipsoid of revolution (Pentecost 1981).

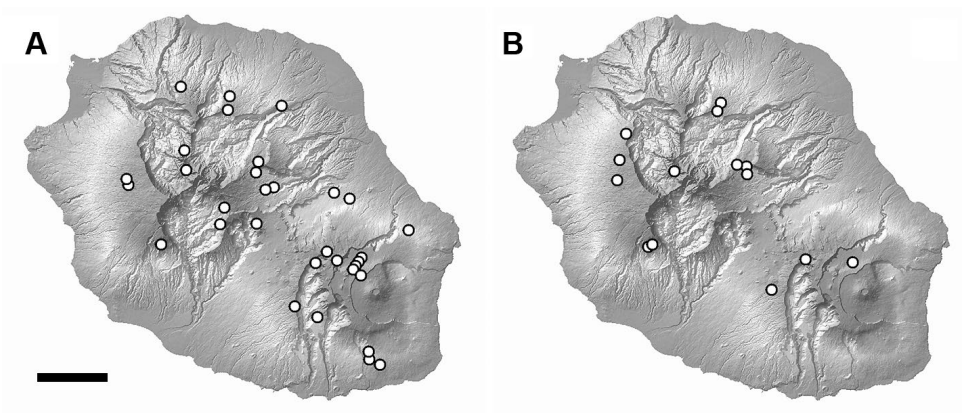

Figure 1. Sampling sites of *H.* aff. *damaziana* (A) and *H. altorum* (B) overlain on shade relief maps of Réunion Island. Scale: A, B = 10 km.

Table 1. Geographical characteristics of the sampling sites, and references of the specimens studied. Samples 1–32 pertain to *H.* aff. *damaziana*, samples 33–46 to *H. altorum*. Collection localities marked ** are shared by both species. Sample numbers in bold type indicate leeward localities; those marked § indicate specimens whose ITS has been studied.

Sample	Locality	Elevation (m)	Latitude (°)	Longitude (°)	Date	Specimen No.
1	route forestière 38	620	−21.34139	55.72041	25.08.2017	974.5128
2	Sainte-Marguerite	685	−21.11583	55.67821	28.08.2012	974.4106
3 §	l'Ancienne Nationale	775	−21.10970	55.65803	24.08.2013	974.4434
4	forêt de Dioré	825	−20.99319	55.58195	21.08.2017	974.5054
5	Rond de Basse Vallée	840	−21.33499	55.70613	16.08.2013	974.4252
6 §	les Réservoirs	880	−21.16026	55.75987	15.08.2013	974.4234
7 §	piton Ravine Basse Vallée	1070	−21.32497	55.70451	16.08.2013	974.4284
8	Takamaka	1340	−21.10227	55.56873	29 07 2005	974.1845
9 §	ravine des Calumets	1365	−21.15008	55.49438	21.08.2012	974.3937
10	plaine des Fougères bas	1400	−20.98099	55.50752	30.08.2012	974.4133
11 §	les Trois Mares	1430	−21.10729	55.55827	21.08.2015	974.4784
12	Bélouve 1	1470	−21.06770	55.55180	24.08.2012	974.4006
13	Grand Coude	1490	−21.27520	55.63487	24.08.2017	974.5120
14	Bras Sec	1505	−21.12837	55.49855	22.08.2012	974.3966
15	la Roche Écrite	1515	−20.96903	55.44046	20.08.2015	974.4755
16	plateau de la Sale	1520	−21.04753	55.44517	24.07.2005	974.1721
17	sentier des Tamarins 1	1550	−21.08315	55.54543	19.07.2005	974.1528
18	le Mapou	1580	−21.20357	55.62968	24.08.2015	974.4850
19 §	plaine des Fougères haut**	1640	−20.99986	55.50687	31.08.2012	974.4155
20	sommière la Saline	1710	−21.09057	55.35897	10.04.2003	974.0266
21	Notre-Dame de la Paix	1720	−21.26407	55.60259	17.07.2005	974.1393
22	sommière Éperon**	1735	−21.08548	55.36013	10.04.2003	974.0280
23 §	piton Tortue	1735	−21.15109	55.54555	23.08.2013	974.4413
24	plaine des Tamarins**	1760	−21.07904	55.44359	27.07.2005	974.1762
25 §	piton de Coco	1780	−21.20120	55.69633	25.08.2012	974.4035
26	plateau Goyaves**	1790	−21.17989	55.40876	19.08.2013	974.4321
27	fond de la Rivière de l'Est	1810	−21.20684	55.69185	20.07.2005	974.1596
28	Rampe Liot 2	1915	−21.21008	55.68537	20.07.2005	974.1590
29	Rampe Liot 1	2030	−21.21347	55.68500	20.07.2005	974.1604
30 §	bois Ozoux	2160	−21.19227	55.64745	22.08.2013	974.4394
31 §	Pas de Bellecombe	2300	−21.22057	55.69313	22.08.2013	974.4374
32	rempart de la Rivière de l'Est	2375	−21.20383	55.65950	22.08.2015	974.4797
33	Grand Bras Sec	1485	−20.98934	55.51027	31.08.2012	974.4161
34	plateau Citron	1535	−21.07409	55.54726	24.08.2012	974.3998
35	sentier des Tamarins 2	1550	−21.08410	55.54571	19.07.2005	974.1538
36	rivière d'Abord	1570	−21.23994	55.58475	23.07.2005	974.1678
37	sentier Oméga	1600	−21.02712	55.37857	31.07.2005	974.1877
38 §	plaine des Fougères haut**	1640	−20.99986	55.50687	31.08.2012	974.4152
39 §	Bélouve 2	1650	−21.07385	55.53042	25.08.2013	974.4488
40	sommière Éperon**	1735	−21.08548	55.36013	10.04.2003	974.0272
41	plaine des Tamarins**	1760	−21.07904	55.44359	27.07.2005	974.1763
42 §	ravine Savane	1775	−21.20372	55.69615	25.08.2012	974.4039
43	plateau Goyaves**	1790	−21.17989	55.40876	19.08.2013	974.4320
44	Dennemont	1790	−21.06087	55.36983	02.08.2005	974.1906
45	le Petit Mapou	1805	−21.19830	55.63255	24.08.2015	974.4842
46 §	Haut des Makes	1850	−21.17676	55.41276	19.08.2013	974.4323

The arithmetic mean spore length, width, Q and volume, their standard deviation and coefficient of variation (incl. Haldane correction; Haldane 1955) were calculated for each sample. Considering that the variation of spores between the different individuals of a species (intraspecific variation) is a trait of the species, whereas the spore variation within one individual (intra-individual variation) is only the attribute of that individual (Raitviir 1972; Parmasto & Parmasto 1987), statistics for the overall spore measurements of each of the two species studied are presented in the format: (M – SD) – *M* – (M + SD), where M is the arithmetical mean of the mean values of the samples (in italics) and SD its standard deviation.

Statistical analyses

Statistical analyses were performed using R (R Core Team 2018) and R packages 'car' (Fox et al. 2012), 'dplyr' (Wickham et al. 2016), 'ggpubr' (Kassambara 2017) and 'reshape2' (Wickham 2012). The normality distribution of variables was checked using quantile-quantile (Q-Q) plots

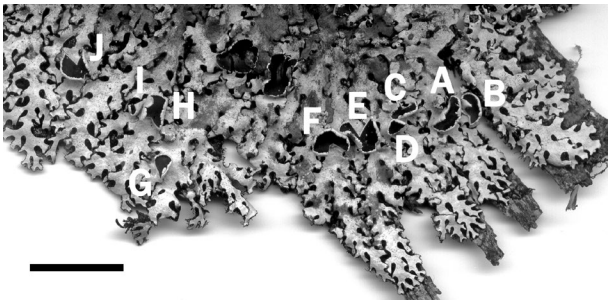

Figure 2. The ten apothecia investigated in sample No. 15 (*Hypotrachyna* aff. *damaziana*, specimen No. 974.4755). Scale bar = 1 cm.

and the Shapiro-Wilk test. Homogeneity of variance was investigated using Levene's test and by plotting residuals vs. fits. To verify whether, for each of the two species, the set of samples represents a single homogeneous population, we used the Hartigans' dip test for unimodality. One-way analysis of variance was chosen to compare the means of the spore variables between groups when the variances were homogeneous; otherwise Welch's one-way ANOVA was used. The relationships between spore traits and elevation, spore traits and climatic variables, spore traits and apothecia diameter, and between climatic variables were determined with two-tailed Spearman rank correlation tests. When analysing associations between spore traits and climatic variables, the family-wise error rate was reduced by using the sequential Bonferroni procedure (Holm 1979; Rice 1989). When significant correlations were found, multiple linear regressions were performed. Adequacy of the regressions was assessed using the gvlma (Global Validation of Linear Models Assumptions) function of the gvlma R package (Pena & Slate 2012). Comparison of fitted models was performed with ANOVA tables using the Anova function.

Results

Variation of the climatic parameters of the sampling localities

Unsurprisingly, the mean annual temperature of a locality strongly depends on its elevation (Spearman rank correlation, $r_s = -0.8201$, $P = 3.0 \times 10^{-11}$, two-tailed). However, there is no significant relationship between mean annual rainfall and elevation ($r_s = -0.1182$, $P = 0.46$, two-tailed), nor between mean annual rainfall and mean annual temperature at each locality ($r_s = -0.0343$, $P = 0.83$, two-tailed). The lack of correlation between rainfall and elevation could be partly related to local exposure to trade winds. The fourteen leeward localities sampled are distributed between 1365 m and 1850 m a.s.l., while fifteen windward localities are in the same elevational range (Table 1). Comparison of the mean annual precipitation of these twenty-nine locations depending on their wind exposure (Student's t-test, $t = 4.146$, $df = 27$, $P = 1.5 \times 10^{-4}$, one-tailed) confirms that rainfall tends to be greater in windward situations (mean value 4060 mm) than in leeward situations (mean value 2479 mm).

Annual mean daily global solar radiation of the forty-two localities is significantly correlated with elevation ($r_s = 0.3228$, $P = 0.037$, two-tailed), mean annual temperature ($r_s = -0.3893$, $P = 0.011$, two-tailed) and mean annual rainfall ($r_s = 0.6087$, $P = 1.9 \times 10^{-5}$, two-tailed).

Within-thallus spore variation in *Hypotrachyna* aff. *damaziana*

Levene's test does not support heterogeneity of variance for any of the four parameters tested (length, width, volume and Q ratio) between ten different apothecia of the same thallus (sample No. 15; Table 2). Q-Q plots suggest normality of the data, even if Shapiro-Wilk tests are significant for lengths, widths and volumes. The mean lengths of spores do not significantly differ between the ten apothecia (one-way ANOVA, $F_{9,290} = 1.185$, $P = 0.30$); the same is true for widths ($F_{9,290} = 0.425$, $P = 0.92$), volumes ($F_{9,290} = 0.295$, $P = 0.98$) and Q values ($F_{9,290} = 1.342$, $P = 0.21$). Moreover, the coefficients of variation of the mean values of the samples are low and range between 1.1% and 2.2% for the four parameters studied (1.1% for width, 1.4% for length, 2.2% for volume and Q value).

Spore variation and size of apothecia

The sampled apothecia vary in size, and therefore in age. The diameter of the hymenial disc varies from 1.2 to 6.4 mm (mean: 3.15 mm) for the samples of *H.* aff. *damaziana*, and from 1.5 to 4.1 mm (mean: 2.50 mm) for

Table 2. Variation of mean spore measurements, Q values and their standard deviation (SD) in ten apothecia of the same *H.* aff. *damaziana* thallus (sample No. 15, specimen No. 974.4755). Thirty spores were measured in each apothecium.

Apothecia	Spore width		Spore length		Spore volume		Q	
	mean (µm)	SD (µm)	mean (µm)	SD (µm)	mean (μm^3)	SD (μm^3)	mean	SD
A	9.05	0.747	13.55	0.792	586.4	108.97	1.51	0.134
B	9.10	0.792	12.93	1.073	564.1	104.70	1.43	0.187
C	9.02	1.038	13.45	0.922	584.1	157.64	1.51	0.154
D	8.95	0.844	13.27	0.878	562.4	113.62	1.49	0.152
E	9.25	0.828	13.28	1.104	602.4	130.81	1.44	0.152
F	9.10	0.759	13.27	0.598	580.2	104.05	1.47	0.127
G	9.18	0.951	13.08	0.929	587.8	142.57	1.43	0.130
H	9.10	0.845	13.32	0.886	583.8	117.62	1.47	0.154
I	9.03	0.860	13.37	1.017	580.5	132.96	1.49	0.129
J	9.27	0.848	13.10	0.865	595.6	119.66	1.42	0.143

those of *H. altorum*. For the 32 samples of *H.* aff. *damaziana*, mean spore width correlates significantly with the diameter of the hymenial disc (Spearman rank correlation, $r_s = 0.5201$, $P = 0.0023$, two-tailed); so does mean spore volume ($r_s = 0.4915$, $P = 0.0043$, two-tailed). There is no significant relationship between mean length and disc diameter ($r_s = 0.2300$, $P = 0.21$, two-tailed), nor between Q and disc diameter ($r_s = -0.2384$, $P = 0.19$, two-tailed) in this species. Regarding the 14 samples of *H. altorum*, the mean length and mean volume of the spores are significantly correlated with the diameter of the hymenial disc (length: $r_s = -0.6042$, $P = 0.0221$, two-tailed; volume: $r_s = -0.5954$, $P = 0.0247$, two-tailed). However, there is no significant correlation between disc diameter and mean width ($r_s = -0.4294$, $P = 0.13$, two-tailed) or Q ratio ($r_s = -0.1115$, $P = 0.70$, two-tailed). No significant relationship was found between the elevation of localities and the diameter of the hymenial disc in samples of *H.* aff. *damaziana* ($r_s = -0.2380$, $P = 0.19$, two-tailed) or *H. altorum* ($r_s = 0.2594$, $P = 0.37$, two-tailed).

Spore variation among localities in *Hypotrachyna* aff. *damaziana*

The overall dimensions of the ascospores measured from the 32 specimens are as follows: length 12.8–*13.51*–14.2 µm, width 8.8–*9.25*–9.7 µm, volume 532.1–*614.20*–696.3 µm^3 and Q 1.40–*1.47*–1.55. Detailed statistical parameters for each sample are presented in Table 3. It appears that spore size and spore shape vary appreciably from one sample to another. Welch's one-way analysis of variance of the data on spore size and spore shape shows that there is a very significant difference across mean length ($F_{31,329.26} = 20.519$, $P = 2.5 \times 10^{-59}$), width ($F_{31,329.25} = 10.664$, $P = 7.7 \times 10^{-34}$), volume ($F_{31,329.21} = 15.043$, $P = 3.1 \times 10^{-46}$) and Q ($F_{31,329.25} = 8.725$, $P = 1.2 \times 10^{-27}$) among the 32 samples. However, the results of the Hartigans' dip test on the data used show that there is no reason to consider the study population as heterogeneous, either for length ($P = 0.58$), width ($P = 0.97$), volume ($P = 0.62$) or Q values ($P = 0.97$). Furthermore, the comparison between the mean value of the coefficients of variation of the samples and the coefficient of variation for the mean values of the samples shows that the former ('intrathalline variation', or more exactly 'intra-apothecial variation') is greater than the latter ('interthalline variation') for each of the parameters studied (Table 4).

The average spore size of specimens varies with elevation. The mean values of width, length and volume correlate significantly with the elevation of the sampling localities: the three variables decrease as the elevation increases (Table 5). The best correlation was found with average volume (Fig. 3). The coefficients of variation (CV) for the three size parameters are also significantly correlated with elevation (Table 5). This suggests that intrathalline variability in spore size changes with elevation. The CV of length increases with elevation, while the CV of width and volume decreases. Unlike spore size variables, there is no significant relationship between the mean Q value, or the CV of Q values, and elevation (Table 5, Fig. 5). While mean spore size decreases clearly with elevation, the shape of the spores does not change.

Among the three climatic parameters selected as variables likely to be linked to interthalline variation in spore size or shape, mean annual temperature of sampling

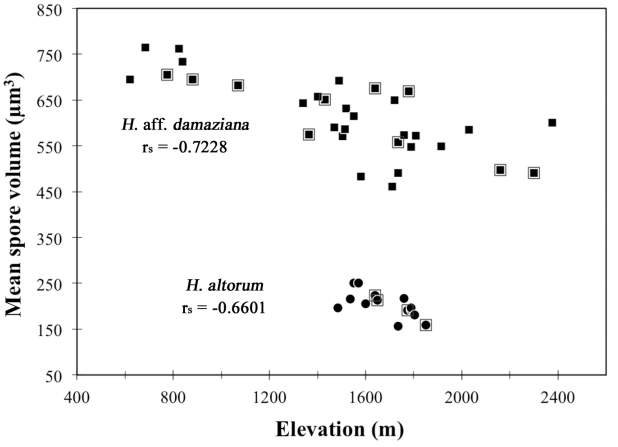

Figure 3. Scatter plot of mean spore volume versus elevation for *H.* aff. *damaziana* and *H. altorum*, and Spearman's rank correlation coefficients. Points within boxes are samples whose ITS has been studied.

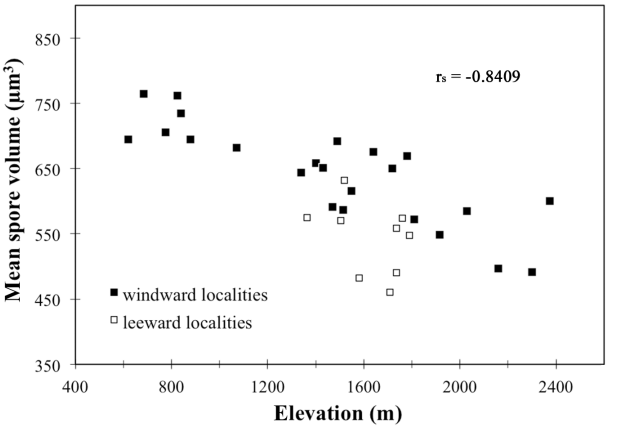

Figure 4. Scatter plot of mean spore volume versus elevation for samples of *H.* aff. *damaziana*, with windward localities separated from leeward localities. Spearman's rank correlation coefficient for windward localities only.

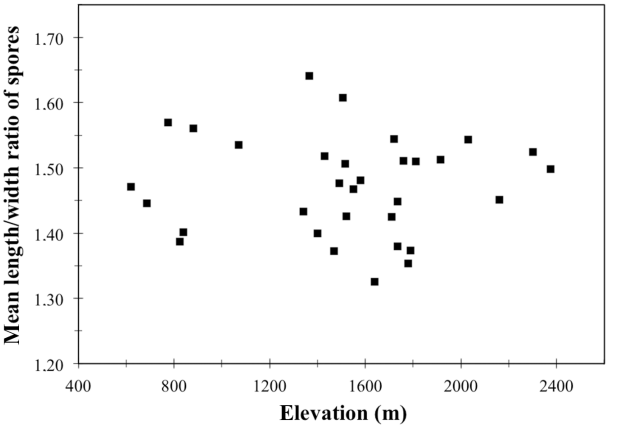

Figure 5. Scatter plot showing the absence of relation (Spearman's rank correlation, $r_s = -0.0187$) between mean spore length/width ratio (Q) and elevation among samples of *H.* aff. *damaziana*.

Table 3. Variation of mean spore measurements, Q values and their standard deviation (SD) in the 32 samples of *H.* aff. *damaziana* (samples 1–32) and the 14 samples of *H. altorum* (samples 33–46). Thirty spores were measured in each sample. Extreme values for each species are underlined for each parameter.

Sample	Spore width		Spore length		Spore volume		Q	
	mean (µm)	SD (µm)	mean (µm)	SD (µm)	mean (µm³)	SD (µm³)	mean	SD
1	9.63	1.121	14.03	0.870	694.7	186.52	1.47	0.156
2	10.00	1.114	14.32	1.118	764.2	194.83	1.45	0.165
3	9.52	1.054	14.73	0.817	705.6	153.31	1.57	0.222
4	10.13	0.982	13.97	1.074	761.6	171.56	1.39	0.142
5	9.98	0.782	13.93	0.763	734.0	131.76	1.40	0.107
6	9.47	0.718	14.70	0.887	695.0	120.00	1.56	0.137
7	9.48	0.866	14.42	0.810	682.1	118.06	1.54	0.190
8	9.48	0.748	13.53	0.809	643.6	119.94	1.43	0.113
9	8.73	0.917	14.18	0.713	574.6	131.23	1.64	0.172
10	9.63	0.840	13.40	0.865	657.9	133.64	1.40	0.139
11	9.35	0.709	14.12	0.806	651.1	109.84	1.52	0.130
12	9.35	0.789	12.77	0.785	590.8	112.78	1.37	0.115
13	9.62	0.862	14.08	0.980	692.1	165.23	1.48	0.146
14	8.77	0.740	14.02	0.924	570.4	117.25	1.61	0.131
15	9.05	0.747	13.55	0.792	586.4	108.97	1.51	0.134
16	9.47	0.776	13.40	0.968	631.5	106.33	1.43	0.175
17	9.28	0.773	13.53	1.066	615.3	112.81	1.47	0.161
18	8.53	0.601	12.58	0.920	482.7	80.37	1.48	0.145
19	9.88	0.897	13.03	0.798	675.2	144.81	1.33	0.109
20	8.52	0.650	12.07	0.691	460.7	75.14	1.43	0.137
21	9.28	0.727	14.27	1.096	650.1	132.53	1.54	0.145
22	8.63	0.556	12.48	0.725	490.3	77.91	1.45	0.106
23	9.17	0.834	12.57	0.828	558.4	113.20	1.38	0.142
24	8.99	0.688	13.51	0.900	574.0	97.74	1.51	0.151
25	9.80	0.610	13.23	0.944	669.3	107.86	1.35	0.111
26	9.13	0.798	12.45	0.824	548.0	105.38	1.37	0.155
27	8.97	0.565	13.49	0.871	572.1	75.34	1.51	0.147
28	8.85	0.645	13.32	0.914	548.9	86.97	1.51	0.154
29	8.97	0.681	13.78	1.430	585.0	111.39	1.54	0.173
30	8.67	0.634	12.53	0.991	497.0	94.10	1.45	0.134
31	8.48	0.663	12.88	0.926	491.1	101.11	1.52	0.111
32	9.15	0.789	13.60	1.170	600.5	115.71	1.50	0.196
33	6.53	0.571	8.70	0.783	196.7	41.20	1.34	0.148
34	6.78	0.503	8.87	0.615	215.6	40.25	1.31	0.107
35	7.15	0.476	9.32	0.663	250.8	40.23	1.31	0.124
36	6.98	0.500	9.77	0.785	251.1	43.99	1.41	0.143
37	6.68	0.334	8.77	0.666	205.1	22.28	1.32	0.137
38	6.88	0.520	8.92	0.720	223.5	43.26	1.30	0.116
39	6.53	0.414	9.55	0.758	213.3	24.64	1.47	0.174
40	5.82	0.464	8.73	0.626	156.3	28.68	1.50	0.125
41	6.70	0.484	9.17	0.711	217.0	38.47	1.38	0.135
42	6.58	0.417	8.40	0.770	191.1	27.86	1.28	0.156
43	6.37	0.556	8.98	0.688	192.9	41.25	1.42	0.139
44	6.62	0.387	8.50	0.881	195.8	32.12	1.29	0.140
45	6.15	0.494	9.07	0.568	181.2	33.23	1.48	0.135
46	6.10	0.481	8.08	0.588	158.3	25.73	1.33	0.148

localities is positively and significantly correlated with mean spore length, width and volume, but not with mean Q value (Table 5). There is no significant relationship between spore size or shape parameters and mean annual precipitation, except for mean spore volume, which increases with annual rainfall. No correlation between annual mean daily global solar radiation and the size or shape of spores was found.

We tested multiple linear regressions with elevation, mean annual temperature, mean precipitation, as well as diameter of apothecia disc as explanatory variables for the variation in spore width, length and volume. For all multiple linear regressions, ANOVA tests never selected a model including both elevation and mean annual temperature. This can be linked to the high correlation of these two values (see above). For spore width, a model

Table 4. Intra- and interthalline variation of spore measurements and Q values estimated from the coefficients of variation (CV) of the samples for each of the two species *H*. aff. *damaziana* (in bold) and *H. altorum* (in italics). All values are in percentages. A two-tailed Student's t-test was performed to compare the mean values of the CV of the samples between the two taxa (**: $P < 0.01$, *: $P < 0.05$, NS: not significant).

	Intrathalline variation				Interthalline variation
	min. value of CV of samples	max. value of CV of samples	mean value of CV of samples		CV of mean values of samples
Spore width	**6.3**	**11.7**	**8.5**	**	**5.0**
	5.0	*8.8*	*7.3*		*5.6*
Spore length	**5.1**	**10.5**	**6.8**	**	**5.3**
	6.3	*10.5*	*7.9*		*5.1*
Spore volume	**13.3**	**27.1**	**19.4**	*	**13.5**
	11.0	*21.6*	*17.1*		*14.2*
Q	**7.4**	**14.3**	**10.0**	NS	**5.2**
	8.3	*12.2*	*10.2*		*5.7*

Table 5. Results of two-tailed Spearman's rank correlation between *H*. aff. *damaziana* spore traits and selected environmental factors. Correlation coefficients in bold type are statistically significant at $P < 0.05$ (significance levels adjusted with the sequential Bonferroni correction for tests involving climate parameters). CV: coefficient of variation.

	Elevation	Mean annual temperature	Mean annual precipitation	Annual mean daily global solar radiation
Mean width	$\mathbf{r_s = -0.6413}$ $P = 7.7 \times 10^{-5}$	$\mathbf{r_s = 0.6981}$ $P = 8.9 \times 10^{-6}$	$r_s = 0.3186$ $P = 0.076$	$r_s = -0.3455$ $P = 0.053$
CV of width	$\mathbf{r_s = -0.5653}$ $P = 7.5 \times 10^{-4}$	$\mathbf{r_s = 0.6829}$ $P = 1.7 \times 10^{-5}$	$r_s = -0.1696$ $P = 0.35$	$r_s = -0.3864$ $P = 0.029$
Mean length	$\mathbf{r_s = -0.6268}$ $P = 1.2 \times 10^{-4}$	$\mathbf{r_s = 0.5587}$ $P = 8.9 \times 10^{-4}$	$r_s = 0.3407$ $P = 0.056$	$r_s = 0.0134$ $P = 0.94$
CV of length	$\mathbf{r_s = 0.4953}$ $P = 0.0039$	$\mathbf{r_s = -0.4284}$ $P = 0.014$	$r_s = 0.1109$ $P = 0.55$	$r_s = 0.3625$ $P = 0.042$
Mean volume	$\mathbf{r_s = -0.7228}$ $P = 3.0 \times 10^{-6}$	$\mathbf{r_s = 0.7219}$ $P = 3.1 \times 10^{-6}$	$\mathbf{r_s = 0.4020}$ $P = 0.023$	$r_s = -0.2610$ $P = 0.15$
CV of volume	$\mathbf{r_s = -0.3842}$ $P = 0.030$	$\mathbf{r_s = 0.5203}$ $P = 0.0023$	$r_s = -0.1005$ $P = 0.58$	$r_s = -0.1861$ $P = 0.31$
Mean Q	$r_s = -0.0187$ $P = 0.92$	$r_s = -0.0736$ $P = 0.69$	$r_s = 0.1504$ $P = 0.41$	$r_s = 0.3875$ $P = 0.029$
CV of Q	$r_s = -0.1041$ $P = 0.57$	$r_s = 0.1294$ $P = 0.48$	$r_s = 0.0176$ $P = 0.92$	$r_s = -0.0269$ $P = 0.88$

with mean annual temperature and apothecia size as the explanatory variables was selected. A significant regression equation was found [$F_{2,29} = 17.83$, $P = 8.9 \times 10^{-6}$, with an R-squared of 0.5206]. Spore's predicted width (in µm) is equal to 7.313 + 0.10 * mean temperature (in °C) + 0.12 * disc diameter (in mm). For spore length, a model with mean annual precipitation and elevation was selected. A significant regression equation was found [$F_{2,29} = 12.13$, $P = 1.5 \times 10^{-4}$, with an R-squared of 0.418]. Spore's predicted length (in µm) is equal to 14.248 + 0.00123 * mean precipitation (in mm) – 0.000819 * elevation (in m). For volume, a model with mean annual temperature and mean annual precipitation was selected. A significant regression equation was found [$F_{2,29} = 26.48$, $P = 2.9 \times 10^{-7}$, with an R-squared of 0.6218]. Spore's predicted volume (in µm^3) is equal to 216.824 + 21.81 * mean temperature (in °C) + 0.14 * mean precipitation (in mm). No significant regression equation was found for Q.

Average spore size increases with annual rainfall in *H.* aff. *damaziana* (Table 5), and leeward localities are, at similar elevations, generally drier than windward localities (see above). Therefore, it is to be expected that overall spore size will be smaller in the first ones, as highlighted in Figure 4. Taking separately into account samples collected in windward locations and those from leeward locations, the link between average spore volume and elevation is clearly strengthened in the case of specimens from the twenty-three windward localities (Fig. 4; $r_s = -0.8409$, $P = 5.1 \times 10^{-7}$, two-tailed), but it is not significant for those from the nine leeward localities (Fig. 4; $r_s = -0.3515$, $P > 0.10$, two-tailed).

Spore variation among localities in *Hypotrachyna altorum*

The spores of *H. altorum* are smaller than those of *H.* aff. *damaziana*, as shown by the measurements from 14 specimens: length 8.5–*8.92*–9.4 µm (Student's t-test, t = 22.443, df = 44, $P = 1.0 \times 10^{-25}$, two-tailed), width 6.2–*6.56*–6.9 µm (Student's t-test, t = 19.287, df = 44, $P = 4.3 \times 10^{-23}$, two-tailed), volume 175.1–*203.48*–231.9 µm^3 (Welch's t-test, t = 25.068, df = 42, $P < 0.0001$, two-tailed). Overall, they are also somewhat more globose (Q 1.29–*1.37*–1.44) (Student's t-test, t = 4.342, df = 44, $P = 8.2 \times 10^{-5}$, two-tailed). One-way ANOVA of the data shows that

Table 6. Results of two-tailed Spearman's rank correlation between *H. altorum* spore traits and selected environmental factors. Correlation coefficients in bold type are statistically significant at $P < 0.05$ (significance levels adjusted with the sequential Bonferroni correction for tests involving climate parameters). CV: coefficient of variation.

	Elevation	Mean annual temperature	Mean annual precipitation	Annual mean daily global solar radiation
Mean width	**$r_s = -0.6057$** $P = 0.022$	$r_s = 0.0933$ $P = 0.75$	$r_s = 0.3831$ $P = 0.18$	$r_s = 0.2508$ $P = 0.39$
CV of width	$r_s = 0.0792$ $P = 0.78$	$r_s = 0.3368$ $P = 0.24$	$r_s = -0.1157$ $P = 0.69$	$r_s = -0.0820$ $P = 0.78$
Mean length	$r_s = -0.2970$ $P = 0.30$	$r_s = 0.0921$ $P = 0.75$	$r_s = 0.2247$ $P = 0.44$	$r_s = 0.4590$ $P = 0.099$
CV of length	$r_s = 0.0220$ $P = 0.94$	$r_s = 0.1617$ $P = 0.58$	$r_s = -0.0623$ $P = 0.83$	$r_s = 0.0022$ $P = 0.99$
Mean volume	**$r_s = -0.6601$** $P = 0.010$	$r_s = 0.3098$ $P = 0.28$	$r_s = 0.3271$ $P = 0.25$	$r_s = 0.3127$ $P = 0.28$
CV of volume	$r_s = -0.0990$ $P = 0.74$	$r_s = 0.3009$ $P = 0.30$	$r_s = -0.1201$ $P = 0.68$	$r_s = -0.1973$ $P = 0.50$
Mean Q	$r_s = 0.1496$ $P = 0.61$	$r_s = 0.0135$ $P = 0.96$	$r_s = -0.3293$ $P = 0.25$	$r_s = 0.0599$ $P = 0.84$
CV of Q	$r_s = 0.2178$ $P = 0.45$	$r_s = 0.1190$ $P = 0.69$	$r_s = 0.0400$ $P = 0.89$	$r_s = 0.2240$ $P = 0.44$

there is a very significant difference across mean length ($F_{13,406} = 12.072$, $P = 2.7 \times 10^{-22}$), width ($F_{13,406} = 17.253$, $P = 1.1 \times 10^{-31}$), volume ($F_{13,406} = 19.428$, $P = 2.3 \times 10^{-35}$) and Q ($F_{13,406} = 8.943$, $P = 3.3 \times 10^{-16}$) among the 14 samples. The Hartigans' dip test shows, however, that there is no reason to consider the study population as heterogeneous, either for length ($P = 0.99$), width ($P = 0.98$), volume ($P = 0.61$) or Q values ($P = 0.82$). Intrathalline variation of spore size or shape parameters is fairly comparable to that of *H.* aff. *damaziana* (Table 4). However, the mean CV values of the samples differ significantly between the two species for length (Student's t-test, $t = 3.428$, $df = 44$, $P = 0.0013$, two-tailed), width ($t = 2.930$, $df = 44$, $P = 0.0054$, two-tailed) and volume ($t = 2.319$, $df = 44$, $P = 0.025$, two-tailed). Interthalline variation, on the other hand, is remarkably similar between the two species. As for *H.* aff. *damaziana*, the comparison between intra- and interthalline variation shows that the former is higher than the latter (Table 4).

Despite the relatively narrow elevational range of *H. altorum* (1485–1850 m), the mean width and volume values are significantly correlated with the elevation of the surveyed localities. Both decrease as elevation increases (Table 6, Fig. 3). In *H. altorum*, as in *H.* aff. *damaziana*, the shape of spores does not appear to change with elevation (Table 6). For volume and width, a regression model with elevation as the only explanatory variable was selected. Significant regression equations were found [$F_{1,12} = 8.442$, $P = 0.0132$, with an R-squared of 0.3641 for volume and $F_{1,12} = 7.387$, $P = 0.0187$, with an R-squared of 0.3295 for width, respectively]. Spore's predicted volume (in μm^3) is equal to 461.193 – 0.153 * elevation (in m) and spore's predicted width is equal to 9.708 – 0.00187 * elevation (in m), respectively. No significant regression equation was found for length and Q.

For *H. altorum*, no significant relation between the biometric parameters and the three selected climatic variables was found (Table 6).

Discussion

Intraspecific variation

The two species investigated belong to the same genus but are not closely related. According to a preliminary molecular phylogenetic analysis (Masson & Sérusiaux unpubl. data), including 14 of the 46 samples studied here (10 of *H.* aff. *damaziana* and 4 of *H. altorum*, cf. Table 1 & Fig. 3), *H.* aff. *damaziana* belongs to subgenus *Parmelinopsis* and *H. altorum* to subgenus *Hypotrachyna* (as defined by Divakar et al. 2013), subgenera that split off during the Oligocene according to Cubas et al. (2018). This phylogenetic analysis also shows that the 10 specimens attributed to *H.* aff. *damaziana* on morphological and chemical criteria are genetically very similar according to their ITS rDNA gene sequences, as are the 4 specimens attributed to *H. altorum*. This is in agreement with the 'normal' interthalline variability of the spore parameters of the two *Hypotrachyna* species (see comments on the evaluation of spore variability in Parmasto & Parmasto 1987) and with the results of the Hartigans' dip test suggesting unimodality in interthalline variability of spore features in the two taxa. All these data are consistent with the hypothesis that each of the two species represents a homogeneous population. Therefore, the interthalline variation in spore size highlighted in this work is assumed to represent only intraspecific variation within the two species. Variation due to the additional presence of one or more cryptic taxa is unlikely.

Intraspecific variation in ascospore size and shape has rarely been studied in detail in lichens, and we are not aware of any comprehensive and synthetic work on this subject comparable to that of Parmasto & Parmasto (1987) on basidiospores in the hymenomycetes. Intra-individual variation, in particular, is seldom investigated. In a pioneering work, Löfgren & Tibell (1979) studied the variation in mean length and width of ascospores between ten apothecia from a specimen of *Sphinctrina*

anglica. No coefficient of variation was calculated in that work, but according to their figure 3 it is possible to estimate variation in mean spore size between apothecia of ~3–4%. The values obtained with *H.* aff. *damaziana*, following a similar protocol, are quite comparable although somewhat lower: the four coefficients of variation calculated from the means of size and shape parameters range between 1.1% and 2.2%. No significant differences in mean spore size or shape were observed between the ten apothecia of the same *H.* aff. *damaziana* specimen. Parmasto & Parmasto (1987) came to the same conclusion in a comparison of basidiospores from different pilei having a common stem in one *Collybia* and two *Pleurotus* species. It has been shown in *Basidiomycota* that the size of spores may depend on the size of the basidioma that produces them (e.g., Hanna 1926; Parmasto & Parmasto 1987). Depending on the species, the correlation between spore size and diameter of the pileus can be positive or negative (Clémençon 1979). The same is true for the two lichenized *Ascomycota* studied: a positive significant correlation between the diameter of the hymenial disc and mean spore width and volume in *H*. aff. *damaziana*, and a negative one between disc diameter and mean spore length and volume in *H. altorum*. However, when performing multiple linear regressions with elevation, climatic parameters and diameter of the apothecia as explanatory variables, the diameter of the apothecia was only selected as a significant explanatory variable for the width of spores in *H.* aff. *damaziana*. On the other hand, models with only elevation or climatic variables were selected to predict spore length and volume in *H.* aff. *damaziana*, and spore volume and width in *H. altorum*.

The variation of size and shape between spores within an apothecium is much greater than the variation in mean spore size and shape between apothecia of the same thallus, and thus constitutes the essential component of intrathalline variation. The variability of the size and shape of spores produced by one individual is not negligible. Thus, the smallest and the largest spores, within the 90% tolerance limits of intrathalline variation, have a volume ratio of 1/1.6–2.7 in *H.* aff. *damaziana* and 1/1.5–2.5 in *H. altorum*. The size variability of spores produced by the same thallus could be caused by 'competition' between developing spores within the limited volume of each ascus. However, similar values of intrathalline variation were obtained by Parmasto & Parmasto (1987) from basidiospores, whereas the development of these is much less constrained by the available space (Halbwachs & Bässler 2015). These authors have proposed to consider this intra-individual variation as a compromise, controlled by natural selection, meeting the need for local as well as long-distance dispersal. Small wind-borne spores are dispersed over greater distances than large spores are (Norros et al. 2014).

Influence of elevation and climatic factors

As expected, mean spore size varies with elevation. In both species studied, mean length, width and volume decrease as elevation increases, with particularly significant variation for spore width and spore volume. Unlike size, the shape of spores (expressed by the Q value) does not vary significantly with elevation for the two species investigated. It is interesting to note that the intrathalline variability of the three spore size parameters of *H.* aff. *damaziana* also changes with elevation. The CV of width and volume decreases, while the CV of length increases with elevation.

Variation in mean spore volume between *H.* aff. *damaziana* specimens could be correlated with two climatic variables: mean annual rainfall and, especially, mean annual temperature of the sampling localities. Mean volume is greater in places with higher average temperature or higher rainfall. In this species, the change in temperature leads not only to a substantial change in mean spore size between specimens, but also to a significant change in intrathalline variability. No significant correlation between climatic variables and the mean spore size of *H. altorum* was found, although size decreased with elevation. It is likely that the low resolution of the available climatic data, the small number of localities investigated, and their heterogeneity with regard to their exposure to trade winds (6 windward vs. 8 leeward localities) are responsible for the lack of correlation in this species. Laboratory and outdoor cultures of various species of non-lichenized fungi have shown that the size of their spores varied significantly under the effect of various environmental factors such as temperature, humidity, light, pH, etc. (e.g., Williams 1959; Dingley 1962; Petrie 1994). These studies also make it clear that responses vary among taxa. For example, the length of spores decreases when the temperature rises from 15°C to 25°C in *Mucor dispersus* but increases in *Sordaria fimicola* (Williams 1959). The underlying mechanisms leading to these differences in variation are unfortunately not identified. The growth of a single cell such as an ascospore of *Hypotrachyna* is not directly comparable to that of the whole thallus of a lichen, but in both cases the influence of temperature seems to have the same effect. Depending in particular on their respective elevations, the thirty-two localities where specimens of *H*. aff. *damaziana* were collected have annual mean temperature varying from 11°C to 21.5°C. It has been shown that within this temperature range the growth of numerous lichen mycobionts in isolated culture increases with temperature (Thomas 1939; Henriksson 1964). Net photosynthesis, on the other hand, increases from 10°C to ~20°C in several tropical epiphytic foliose lichens (Zotz et al. 1998, 2003; Lange et al. 2004), whereas mean radial growth, measured in the field, shows a significant positive linear correlation with mean air temperature (range: ~−5°C to +15°C) in two parmelioid lichen species of the genus *Xanthoparmelia* (Benedict 1990).

Taxonomic observations

The variability in the size and shape of mature spores produced by an individual, as well as the variability among individuals of the same species, are due to environmental and genetic factors. The present study suggests an influence of elevation on spore size in two species of lichenized fungi. This influence would be exerted at both intra- and inter-individual levels in *H.* aff. *damaziana*

Table 7. Ascospore dimensions of taxa of the *H. damaziana* complex according to different authors.

Origin of material	Réunion	Kenya	Brazil		
Spore size (µm)	12.8–14.2 × 8.8–9.7	16–18 × 10–12	12–18 × 8–12	(13)16–18 × (6.5)8–12	(10)12–14 × 6.5–9
Total number of samples studied	32	1	–	–	–
Total number of spores studied	960	–	–	–	–
Source	present study	Krog & Swinscow 1979	Hale 1976	Eliasaro & Adler 2000	Jungbluth 2007

and at least at the inter-individual level in *H. altorum*. It is noteworthy that the interthalline variation, that is, the variation in mean spore dimensions or shape index (Q) between specimens of one species, remains relatively low despite the wide elevational range of *H.* aff. *damaziana* (from 620 m to 2375 m a.s.l.). This inter-individual variability is also remarkably similar between the two species *H. altorum* and *H.* aff. *damaziana* for all four investigated variables. The coefficients of variation for length, width or shape are between 5.0% and 5.7% for the two *Hypotrachyna* species. These results are slightly lower than the average values obtained by Parmasto & Parmasto (1987) from basidiospores for the same parameters (length: 6.5%, width: 6.3%, Q: 6.1%). In three parmelioid lichens of the genus *Parmelina*, interthalline variation, estimated with coefficients of variation values, ranged from 2.8% to 6.4% for length, 3.9% to 7.4% for width, and 3.4% to 10.2% for Q (our own calculations, adapted from Argüello et al. 2007 – Table 2).

Measurements of spores constitute an essential part of descriptions of fungi, whether lichenized or not (Hawksworth 1974). The general usage is to present the range of the 'normal' variation of spore measurements, often with the extremes recorded in parentheses. Most often, unfortunately, the values presented combine both intra- and inter-individual variability. The variation of spores between individuals of a species is a character of that species, and not the variation within one individual, which is a character of that particular individual. In addition, intra-individual variability in spore size and shape is not an adequate indicator of inter-individual variability (see Parmasto & Parmasto 1987 for a detailed discussion). The generally applied combination of intra- and inter-individual variation leads to a wider range of variability than that obtained only from the individual mean values of spore measurements, and it introduces considerable background noise (Raitviir 1972). The current lack of standardization in establishing ranges of variation in spore measurements usually prevents any relevant comparison between the data published by different authors. This is the case, for example, for the taxa in the *H. damaziana* complex (Table 7).

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