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Statistical comparison of morphological and anatomical characters from *verrucaria nigrescens* pers. based on the substrate along with insects attraction in the northeast zone of Iran

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Abstract
Verrucaria nigrescence Pers. has a high tendency to grow on calcareous rocks but it rarely found on rich rocks in silica of Khorasan Razavi province located in the Northeastern zone of Iran. Based on extensive studies, the relationship between favorite substrate and distribution of lichens is clear; but, there are few studies regarding the effects of suboptimal substrate and insect's behavior on morphological and anatomical characteristics. Therefore, 35 thalli of *V. nigrescence* were collected from both calcareous and siliceous rocks in the mentioned region. 33 quantitative and 19 qualitative morphological and anatomical characters were analyzed by independent t-test and Pearson chi-square test. Results showed that siliceous samples had a higher algal layer and excipulum and involucrellum width in the specimens growing on suboptimal substrates were smaller than normally developed species. A significant medulla observed below the perithecia of some siliceous samples that attracted insect's colonies. Our investigation agreed with earlier studies using the other species which shown unfavorable substrate had a prominent effect on some features; but, further work is needed to clarify the relationship between these variations and adaptive strategy of this species growing on suboptimal substrate and insect's attraction.

Keywords: adaptations, chi-square test, insect, lichen, *Verrucaria*

1. Introduction

Lichens exhibit varying degrees of specialization with regard to the surfaces they colonize, ranging from substrate generalists to strict substrate specialists (Resl *et al.* 2010) [26]. A small group of lichen colonizes over a wide range of substrates, growing on rock, soil, tree bark and wood. The large majority of lichens have narrower ranges of substrates; so, the substrate has been used as a key character led to recognition lichens (Nash *et al.* 2002 [21], Seaward 2009) [27]. These differences appear to be based largely on varying chemical requirements (Skorepa *et al.* 1979) [30]. Lichen colonization in a certain niche is also affected by the climate, precipitation and insect feeding (Smith *et al.* 2009) [33]. *Verrucaria nigrescens* Pers. seems to be more commonly reported species from different geographical regions of the country (Müller 1892 [20], Steiner 1896 [34], Szatala 1957 [36], Barkhalov 1975 [3], Seaward *et al.* 2004 [28], Sohrabi and Sipman, 2007 [31], Haji Moniri and Sipman 2009 [12], Valadbeigi 2014) [43]. Although, presence of genus *Verrucaria* is known very poorly in Iran (Seaward *et al.* 2008) [29], many species of it seems to need calcium and distributed on calcareous or limestone rocks. *V. nigrescens* also prefers limestone; but, it rarely grows in suboptimal environmental condition such as siliceous substrate which is poor in calcium carbonate (Krzewicka 2012) [16]. Another closely related taxon, *V. umbrinula* Nyl. Was found only on granite and schistose rocks (Thompson 1997) [39].

Importance of substrate in the life history of lichens is cleared ecologically (Barkman 1958 [4], Trass 1970 [42], Hale 1973 [2, 13], Tibel 1975 [40], Mayrhofer and Polet 1979 [17], Golubkova 1983 [10], Walker 1985 [46], Aptroot 1991 [1], Printzen 1995, Resl *et al.* 2010) [26]; However, there are few knowledge about the effect of substrate on the insects' attraction, growth, morphology and anatomy of lichens (Tolpysheva and Timofeeva 2008 [41], Galun 1963 [8], Malone 1977 [18], Porter 1981 [23], Tolpysheva and Timofeeva 2008) [41]. Our knowledge of the effect of optimal and suboptimal substrate on morphological and anatomical characteristics of lichens population is very limited and therefore merits additional study (Tolpysheva and Timofeeva, 2008) [41]. Accordingly, the main aim of this research was to compare morphological and anatomical characters among *V. nigrescens* Pers. specimens grow on the limestone, siliceous rocks and comparison insects' colonies around this species on both substrates.

2. Materials and methods

2.1 Collecting samples and information of studied area

For morphological and anatomical studies, three limestone sites and seven siliceous localities with Khorasan Razavi province were located in the Northeast zone of Iran that showed in Fig 1. All localities and geographical coordinates were represented in Table 1. Biogeographically, the area belongs to Irano - Turanian region and the Armenian - Iranian Province (Takhtajan 1986) [37]. Mountainous regions in the province contain both calcareous (limestone) and siliceous bedrocks (e.g. granite and quartzite, rich in silica sandstone and conglomerate) (Zomorrodian 2002) [48]. In this research, the lichen samples were collected during 2017.

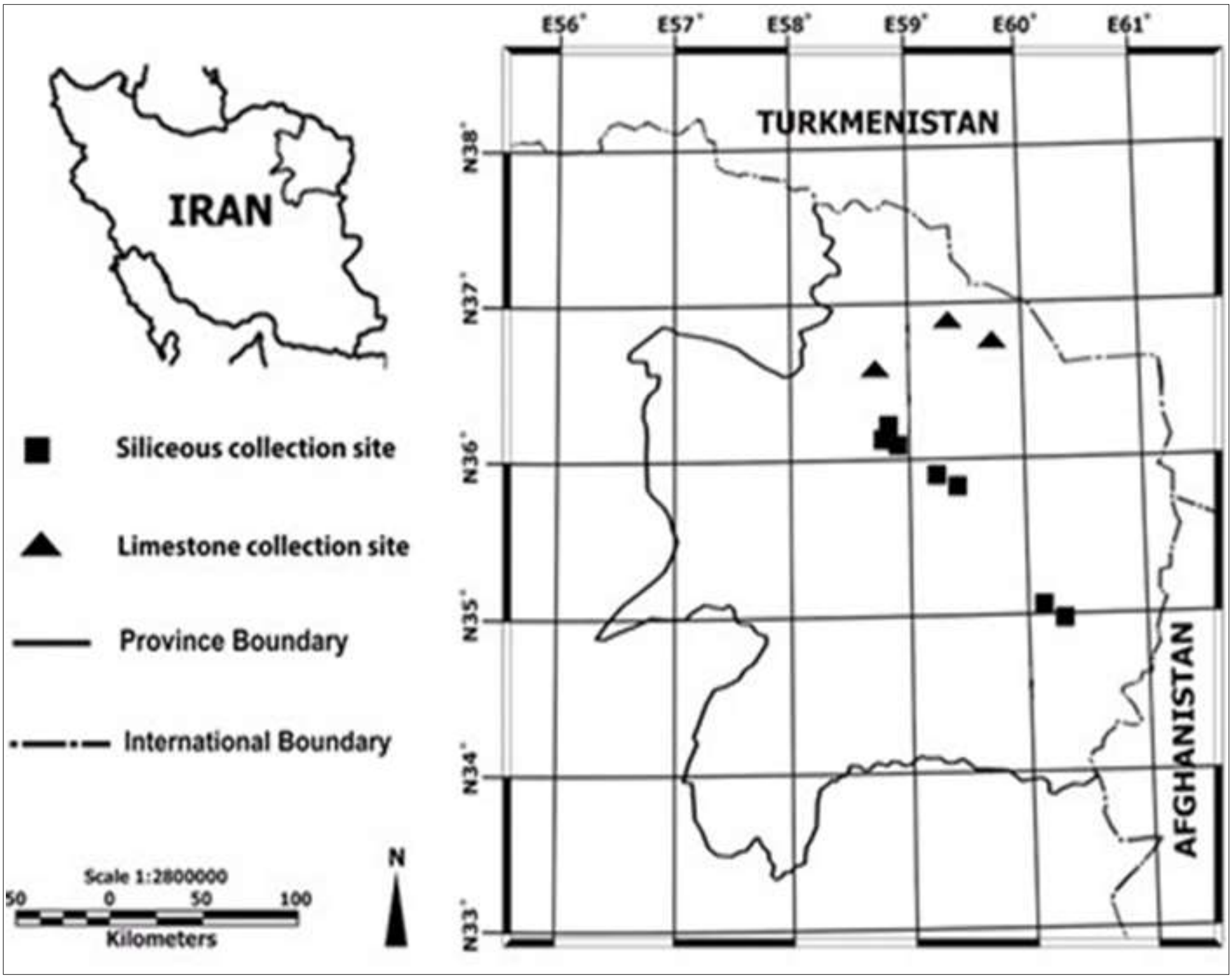

Fig 1: Limestone and siliceous sampling sites. Khorasan Razavi is located in the Northeast of Iran (35.1020° N and 59.1042° E) at the border with Turkmenistan and Afghanistan (Geological Survey of Ian, 1; 100. 000 maps).

2.2 Preparing samples

The acid test (HCL 10%) applied for all selected substrates. Limestone contains high amounts of carbonate minerals which are unstable in contact with hydrochloric acid and visible bubble of carbon dioxide released when acid begins to effervesce on a specimen (Montalvo *et al.* 2015) [19]. In perused siliceous site, *V. nigrescens* distributed very rarely and collecting samples required intensive exploring on different rocks, eventually only 9 appropriate samples were found on rich in silica sandstone, foliated quartzite and granite based on Vondrak *et al.* (2013) [45]. method. 26 proper size limestone growing thalli were selected to study morphological and anatomical characteristics (Fig 2).

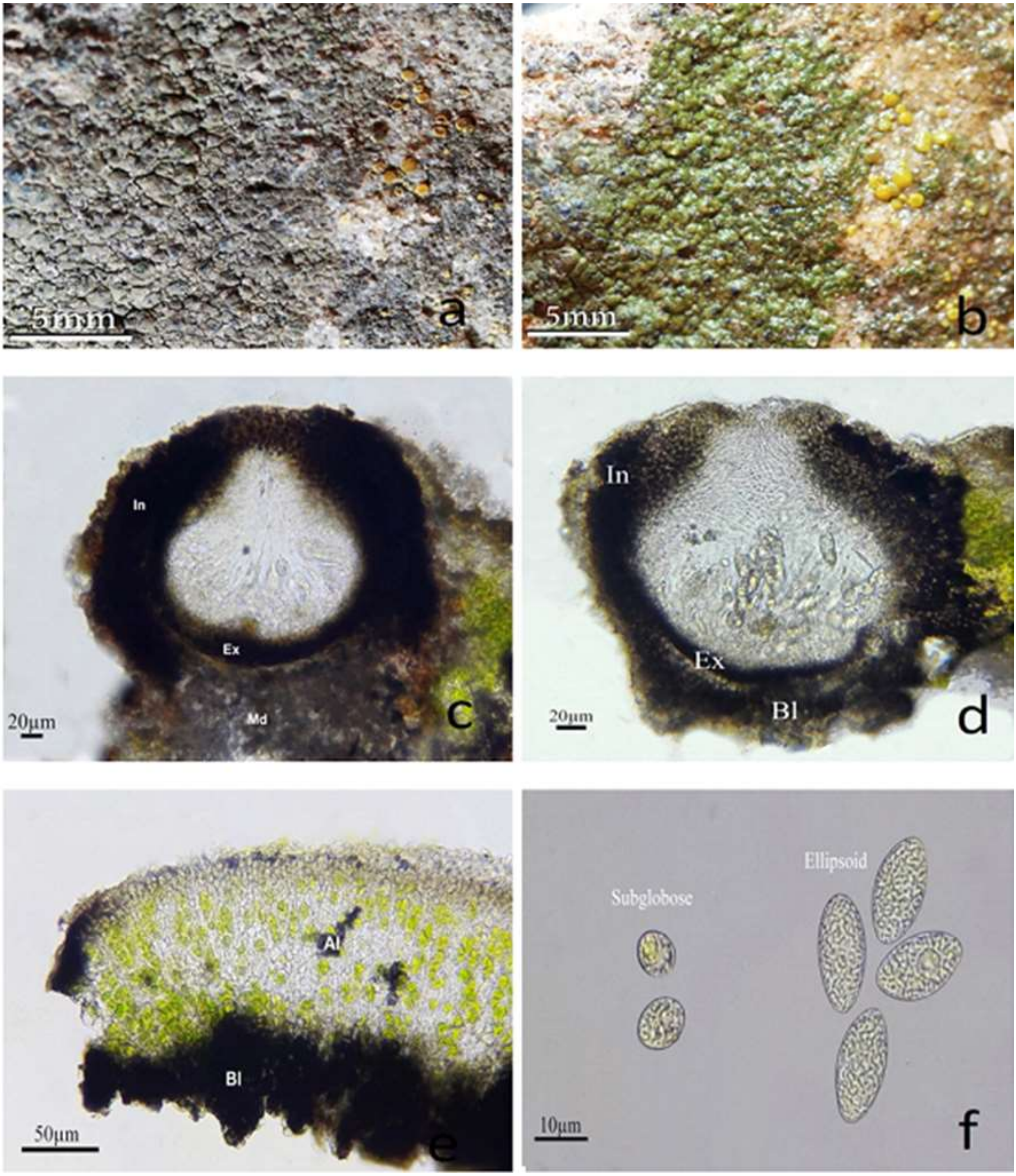

Fig 2: Morphological and anatomical structures of *Verrucaria nigrescens* Pers. a: dry areolate Thallus, b: thallus totally became green when wetting, c: perithecium rested on medulla (Md), involucrellum extending downward contiguous with exciple (Ex), d: Perithecium settled on basal black layer (Bl), involucrellum extending downward conjugating with basal layer, e: transection of thallus; basal layer (Bl) commonly filled half to ¾ of thallus thickness, in this section basal layer is thin (1/3 of thallus thikness), Al=Algal layer, f: ellipsoid and subglobose spores.

2.3 Morphological and anatomical investigations

We used Vondrak *et al.* (2013) [45]. method to measure metric characters, small to medium size individuals with less than 15 to 20 perithecia were ignored during sampling (Table 2). Morphological characters of thallus were studied by using stereomicroscope "C Stemi" (Carl Zeiss, Germany) and for the anatomical characteristics of thallus and perithecium, light microscope "Axioskope 40" (Carl Zeiss, Germany) was used. Totally, 35 specimens have been evaluated by using 52 morphological and anatomical characters**.** According to Vondrak *et al.* (2013) [45], in each sample, more than five measurements are advisable for each measurable character. Practically, it means that at least five perithecia must be destroyed on a single specimen; because, some perithecium related characters (e.g. excipulum width) that should be logically measured only once per perithecium section. In our research, areole diameter approximately ranges from 2 to 10 millimeters (2 to 8 mm in Krzewicka 2012) [16]; so, we consider that areoles had a diameter greater than 5 millimeters as 'Big Areole' and less than five as a 'Small Areoles'. Measurable characters were measured by using a calibrated eyepiece graticule and a number of measurements we used for quantitative data mentioned in Table 2.

2.4 Data analysis

Two data sets were prepared: the first data matrix was formed by 33 quantitative characters (Appendix 2). Also, the number of measurements for each quantitative character is shown in Table 4. The second data matrix formed by 19 non-metric characters (Appendix 3). Non-metric characters or qualitative variables were coded for analysis and showed in Table 3. Specimens have been evaluated by using 52 qualitative and quantitative characters (Table 4 and Table 5). For parametric and nonparametric variables, statistical evidence differences between two groups (limestone and siliceous) could be assigned by using independent t-test (Vamosi and Dickinson 2006) and chi-square test by SPSS software (Statistical Product and service solutions) version 24 (Hazra and Gogtay 2016); because, this study only examines two groups of siliceous and calcareous, the best method for analyzing of quantitative characters is in the dependent t-test. T-test is a statistical test that is widely used to compare the mean of two groups of samples that are significantly different from each other. Independent t-test examines significant values (p-value) for quantitative data in each group. This p-value is significant for both groups if the p-value is less than 0.05.

The coefficient of variation (CV) is obtained by dividing the standard deviation and mean, indicating the amount of variation in each character in each group. For qualitative characters, we used the Pearson chi-square test to determine the association between variables and substrate. In this case, only one p-value was calculated for both groups, and if this value is below 0.05, the difference between two groups is significant. Coefficient of variation (CV %) evaluated for measured variables of two studied groups to indicate variability.

3. Results

3.1 Quantitative data

Independent group t-test performed on 33 quantitative characters between siliceous and calcareous groups of *V. nigrescens* (Table 4, marked by *) and 9 characters majored and different between two studied groups based on p-value ($p<0.05$) as follows: algal layer included: algal layer minimum measured thickness (ALminT), the average thickness of the algal layer (ALAT), perithecium included: the minimum and maximum measured excipulum width (TMinEW and TMaxEW), the average width of excipulum (EAW), minimum and maximum measured involucrellum width (MinIW, MaxIW), the average width of involucrellum (IAW) and the average length of ascus (ALA). Independent t-test revealed significant differences between mean values of excipulum minimum and maximum measured width (TminEW, TmaxEW) and average width of excipulum (EAW) (Table 4); which showed the greater values in favor of limestone samples. Accordingly, carbonaceous shield-like involucrellum around perithecium had significantly greater means in all related characters for limestone specimens such as MinIW, MaxIW and IAW. It can be concluded that perithecia in siliceous *V. nigrescens* averagely had a smaller size, nevertheless, no statistically significant differences existed between means of reproductive characteristics including MinAL, MaxAL, AAL, MinAW, MaxAW, AWA, PerAS, MinALS, MaxALS, AAL, MinASW, MaxASW, AWAS and SR (Table 4).

The mean values of minimum measured algal layer thickness (ALminT) in siliceous inhabitant specimen were greater than limestone samples (60.5 and 36.63 respectively) that are attractive to insects' colonies. The ratio of the mean value for average thallus thickness (ATT) to mean value of algal layer average thickness (ALAT) was 3.9 for siliceous and 3.5 for limestone growing samples. This ratio reflects the proportion of the algal layer in the lichen thallus which was higher in siliceous samples which may be attracted to the insect colonies around the studied species.

In siliceous growing specimens, the average thickness of algal layer (ALAT) due to the coefficient of variation (Table 3), varied slightly (7.2 %) and ranged from 66.7 µm in SI1 to 82.5 µm in SI9 (Appendix 2), even in limestone living samples, CV value was less than 20 %, which indicated limited variation for this character. Generally, according to the obtained data in Table 3, limestone's V. nigrescens had thinner algal layer than siliceous samples; however, both group approximately shared similar values for related characters to thallus thickness (MinTT, MaxTT and ATT) and areole size (TSMAS, TBMAS, ASA and ABA). Higher coefficient of variation (CV) has belonged to variables associated with cortex thickness (CminT 69.5 % and 26.3 %, CmaxT 46.5 % and 27 %), (CAT 53.2 and 33.3) for limestone and siliceous samples, respectively, represents great dispersion in these variables for the studied groups. In limestone living samples, the coefficient of variation (CV) for 'ALminT' was 30.3 % which had a wide range of variability, as it ranges from 17.5 µm in LI7 sample to 57.5 in LI24 sample. CV values for all significant perithecium related characters (Marked by * in Table 4), were less than 20 % in both limestone and siliceous samples, illustrating the lower range of alteration in these variables.

Table 1: Locality, geographical position and height from sea level of sampling sites.

Samples	Locality	Geographical position	Height from sea level (m)
LI1-LI11	Kalat-e Naderi, Qarasu, on limestone beds	36°57'46.1"N, 59°40'27.5"E	1395
L12-L21	Chenaran, near Radkan village, on limestone	36°51'47.9"N, 59°04'21.1"E	1785
L22-L26	Chenaran, Akhlamad village, on limestone	36°35'44"N, 58°55'29"E	1875
SI1	Torghabeh, road to Kalat-e Ahan village, on granite	36°15'34.5"N, 59°20'00.4"E	1520
SI2-SI3	Torghabeh-Azghad village on granite	36°11'48.9"N, 59°20'48.4"E	1670
SI4	Mashahd, near Arefi viallage, on silica- pebble conglomerate	36°07'48.1"N, 59°31'06.1"E	1377
SI5	Fariman-Farhad gerd, on quarzite	35°48'20.3"N, 59°31'27.3"E,	1702

SI6-SI7	Fariman-Arreh Kamar village, on granite	35°48'20.3"N, 59°31'27.3"E	1702
SI8	Torbat-e Jam, Bezd plain, on qurzite	35°10'52.7"N, 60°23'06.5"E	1996
SI9	Torbat-e Jam, Bezd plain, on qurzite	35°10'52.7"N, 60°23'06.5"E	1996

Table 2: Number of measurements used for quantitative characters.

Characters	Number of measurements
TSMAS	20-Areole
TBMAS	20-Areole
CminT	20-Cortex from different Areoles
CmaxT	20-Cortex from different Areoles
AlminT	20-Algal layer from different Areoles
AlmaxT	20-Algal layer from different Areoles
MinTT	20-Areole
MaxTT	20-Areole
TMinES	10-Perithecium
TmaxES	10-Perithecium
MinIS	10-Perithecium
MaxIS	10-Perithecium
MinAL	20-Ascus
MaxAL	20-Ascus
MinAW	20-Ascus
MaxAW	20-Ascus
MinALS	40-Ascospore
MaxALS	40-Ascospore
MinASW	40-Ascospore
MaxASW	40-Ascospore
PerAS	10-Perithecium

Note: Some quantitative characters are averages of two or more characters; so, the number of characters in this table is less than the 33 quantitative characters were listed in this research.

Table 3: Codes and states of qualitative variables for the studied specimens of *V. nigrescence.*

No.	Variable	Abbr.	Code state		
			1	2	3
1	Substrate	SB	Limestone	Siliceous	
2	Thallus color	TC	black	Brown	
3	Wet thallus color	WTC	Whole thallus green	Not changed or little part of thallus tinged to green	
4	Areole shape	AS	Mostly Polygonal or angular	Round	
5	Areole side color	ASC	Black	Brown not detect	
6	Prothallus presence	PT	Present	Absent	
7	Cortex tissue type	CTT	Paraplechtencyma	Prosplechtenchyma	
8	Upper most cortex color	UMCC	Black-brown	other	
9	Medulla with basal black layer	MWBBL	BBL At least filled thin layer of medula	BBL absent	
10	Perithecium immersion	PeriT	Most Peri. half to 1/3 immersed in areoles	Totally immersed	
11	Perithecia number per areolae	PNA	One rarely two	More than two	
12	Perithecium position	PP	Commonly center of areoles	Edge or between areoles	
13	Involucrellum color	IC	Black-Brown	Green-brwon Mostly dimidiate or	
14	Involucrellum position	EC	Commonly extending downward conjugating whit BBL	extending downward but not conjugate ith BBL under perithecia	
15	Presence of medulla below the perithecium	MBP	Perithecia on BBL or directly on substrate	Some Prithecia on thin or thick medulla	
16	Excipulum color	EC	Blackish to brown	Green to colorless	
17	Oil drops in perithecium	ODPer	Present	Absent	
18	Vegetative propagule presence	VP	present	absent	
19	Basal black layer thickness	BBLT	thin (less than 1/3 of medulla)	1/3 to half of medulla	thick (3/4 of medulla)

Table 4: Quantitative characters used in the studied specimens of *Verrucaria nigrescens.* Means of same variables between two limestone and calcareous substrate compared by using independent group t-test (α = 0.05)

No.	variable	Abbr.	Unit	Substrate	Mean	SD	CV (%)	p-value
1	The smallest measured areole size	TSMAS	mm	limestone	0.21	0.03	14.2	0.83
				siliceous	0.2	0.03	15	0.84
2	The biggest measured areole size	TBMAS	mm	limestone	1.03	0.05	4.8	0.21
				siliceous	1	0.06	11.1	0.028
3	Average size of the small areoles	ASA	mm	limestone	0.36	0.04	11.1	0.1
				siliceous	0.34	0.03	8.8	0.06
4	Average size of the big areoles	ABA	mm	limestone	0.77	0.05	6.4	0.12
				siliceous	0.74	0.05	6.7	0.12
5	Cortex minimum measured thickness	CminT	μm	limestone	17.66	12.3	69.5	0.38
				siliceous	21.41	5.64	26.3	0.23
6	Cortex Maximum measured thickness	CmaxT	μm	limestone	30.94	15.9	51.3	0.17
				siliceous	34.44	5.98	17.3	0.13
7	Cortex average thickness	CAT	μm	limestone	24.2	12.9	53.2	0.13
				siliceous	31.62	10.5	33.3	0.1
8	Algal layer minimum measured thickness	ALminT	μm	limestone	36.63	12.9	30.3	0.00*
				siliceous	60.05	10.5	17.5	0.00*
9	Algal layer maximum thickness	AlmaxT	μm	limestone	87.54	15.9	18.5	0.07
				siliceous	98.37	12.1	12.3	0.04
10	Algal layer average thickness	ALAT	μm	limestone	68.26	11	16.1	0.05*
				siliceous	75.63	5.49	7.2	0.01*
11	The minimum measured excipulum width	TMinEW	μm	limestone	171	31.6	18.2	0.003*
				siliceous	135.2	20.9	15.4	0.001*
12	The maximum measured excipulum width	TmaxEW	μm	limestone	333.7	31.6	9.4	0.003*
				siliceous	286.1	15.8	5.5	0.001*
13	Excipulum average width	EAW	μm	limestone	254.8	23.5	9.2	0.00*
				siliceous	204.4	21.7	10.6	0.00*
14	The minimum measured involucrellum width	MinIW	μm	limestone	213	28.4	13.3	0.02*
				siliceous	189.4	18.5	9.7	0.008*
15	The maximum measured involucrellum width	MaxIW	μm	limestone	414.5	29.4	7.1	0.00*
				siliceous	359.4	43.8	12.1	0.03*
16	Involucrellum average width	IAW	μm	limestone	310.9	27.1	8.7	0.001*
				siliceous	274.4	22.5	8.1	0.001*
17	The minimum measured ascus length	MinAL	μm	limestone	49.47	5.7	11.5	0.17
				siliceous	54.08	5.22	9.6	0.09
18	The maximum measured ascus length	MaxAL	μm	limestone	82.23	9.13	11.1	0.14
				siliceous	87.02	5.02	5.7	0.06
19	average length of ascus	ALA	μm	limestone	70.16	8.96	12.7	0.4
				siliceous	72.93	6.36	8.7	0.32
20	The minimum measured ascus width	MinAW	μm	limestone	13.78	2.99	21.6	0.12
				siliceous	15.53	2.3	14.8	0.08
21	The maximum measured ascus width	MaxAW	μm	limestone	38.32	9.11	23.7	0.16
				siliceous	43.36	8.92	20.5	0.16
22	Average width of ascus	AWA	μm	limestone	24.53	4.1	16.7	0.67
				siliceous	25.13	2.1	8.3	0.57
23	Periphysoids Average size	PerAS	μm	limestone	27.66	4.31	15.5	0.08
				siliceous	24.77	4.02	16.2	0.08
24	The minimum measured spore length	MinALS	μm	limestone	16.09	1.38	8.5	0.56
				siliceous	16.4	1.36	8.2	0.57
25	The maximum measured spore length	MaxALS	μm	limestone	25.42	3.08	12.1	0.14
				siliceous	23.85	1.11	4.6	0.03
26	Average length of ascospore	AAL	μm	limestone	19.83	1.66	8.3	0.63
				siliceous	19.55	0.75	3.8	0.5
27	The minimum measured spore width	MinASW	μm	limestone	8.44	0.32	3.7	0.18
				siliceous	8.27	0.26	3.1	0.14
28	The Maximum measured spore width	MaxASW	μm	limestone	13.72	1.74	12.6	0.16
				siliceous	12.78	1.51	11.7	0.14
29	Ascospore Average width	AWAS	μm	limestone	10.11	0.61	5.9	0.71
				siliceous	10.03	0.52	5.1	0.69
30	Ratio of subglobose/ellipsoid spore	SR	%	limestone	19.03	5.1	24.3	0.94
				siliceous	18.8	5.71	22.23	0.94
31	The minimum measured thallus thickness	MinTT	μm	limestone	203.5	62.3	30.6	0.64
				siliceous	214.1	43.6	20.37	0.58
32	The maximum measured thallus thickness	MaxTT	μm	limestone	333.8	69.3	20.7	0.85
				siliceous	338.4	51.9	15.2	0.83
33	Thallus average thickness	ATT	μm	limestone	269.7	58.7	21.7	0.92
				siliceous	271.8	39.1	14.3	0.9

Independent group t-test at 5% probability ($p<0.05$), (SD) standard deviation, (CV) Coefficient of variation = SD/Means×100, (t) t-static

Table 5: Qualitative characters used in the studied specimens of *Verrucaria nigrescens* Peasron chi- square test used (α=0.05) to determined association between variables and substrate (limetone and siliceous).

No.	variable	Abbr.	Unit	Substrate	Mean	SD	CV (%)	p-value
1	Substrate	SB	Code	limestone	1	0	0	-
				siliceous	2	0	0	
2	Thallus color	TC	Code	limestone	1.53	0.5	32.6	0.2
				siliceous	1.77	0.4	24.8	
3	Wet Thallus color	WTC	Code	limestone	1.42	0.5	35.2	0.089
				siliceous	1.11	0.3	29.7	
4	Areole shape	AS	Code	limestone	1	0	0	-
				siliceous	1	0	0	
5	Areole side color	ASC	Code	limestone	1.46	0.5	34.2	0.26
				siliceous	1.88	0.3	17.5	
6	Prothallus presence	PT	Code	limestone	1.42	0.5	35.2	0.492
				siliceous	1.55	0.5	33.5	
7	Cortex Tissue Type	CTT	Code	limestone	1	0	0	-
				siliceous	1	0	0	
8	Upper most cortex color	UMCC	Code	limestone	1	0	0	-
				siliceous	1	0	0	
9	Medulla with basal black layer	MWBBL	Code	limestone	1	0	0	-
				siliceous	1	0	0	
10	Perithecium immersion	PeriT	Code	limestone	1	0	0	-
				siliceous	1	0	0	
11	Perithecia number per areolae	PNA	Code	limestone	1	0	0	-
				siliceous	1	0	0	
12	Perithecium position	PP	Code	limestone	1	0	0	-
				siliceous	1	0	0	
13	Involucrellum color	IC	Code	limestone	1	0	0	-
				siliceous	1	0	0	
14	Involucrellum position	IP	Code	limestone	1.92	0.6	32.2	0.329
				siliceous	2.88	0.3	11.4	
15	Presence of medulla beneath the perithecium	MBP	Code	limestone	1.07	0.5	42	0.012*
				siliceous	1.44	0.5	36.4	
16	Excipulum color	EC	Code	limestone	1	0	0	-
				siliceous	1	0	0	
17	Oil drops in perithecium	ODPer	Code	limestone	1	0	0	-
				siliceous	1	0	0	
18	Vegetative Propagule	VP	Code	limestone	0	0	0	0.013*
				siliceous	0.22	0.4	2	
19	Basal black layer thickness	BBLT	Code	limestone	2.15	0.7	31.1	0.35
				siliceous	1.7	0.7	38.8	

(SD) standard deviation, (CV) Coefficient of variation = SD/Means×100, (t) t-static

Table 6: Character state percentage of limestone and siliceous growing samples.

Character	Character state	Frequency	
		Limestone (%)	Siliceous rocks (%)
TC	1	46.15	22.2
	2	53.8	77.7
WTC	1	42.3	11.1
	2	57.6	88.8
ASC	1	53.8	11.1
	2	46.1	88.8
PT	1	53.8	44.4
	2	46.1	55.5
MBP	1	92.3	55.5
	2	7.6	44.4
VP	1	0	22.2
	2	100	77.7
BBLT	1	15.3	33.3
	2	50	55.5
	3	30.7	11.1

3.2 Qualitative data

Among 19 qualitative characters (Table 5), the coefficient of variation (CV) for eleven traits in both siliceous and limestone samples were 0.0 % represented, all thalli had the same state for these characters. Other eight varied traits (Table 5), where assess by Pearson's chi-square analysis to determine statistically significant differences between the two groups. Also, the frequency of character states for each varied trait mentioned in Table 6. Only the presence of medulla beneath the perithecium (MBP) and the presence of vegetative propagule showed statistically significant variation (Table 5 marked by *). As shown in Table 6, the percentage of medulla presence under perithecia is clearly greater in siliceous samples (44.4 %) and vegetative propagules only existed in some siliceous samples (22.2 %). Although, the frequency of some characters state (e.g. in TC, WTC and ASC) had different percentages. In addition, there was no significant difference between the calcareous and siliceous samples.

4. Discussion

In certain cases, we can sure reasonably that a special morphotype is substrate-controlled. If there is a continuum of morphologic change along substrate-character gradients such as hardness or mineral content, or if numerous unrelated taxa react to certain substrates, in the same way, we can usually assume that the morphotype is not genetically determined (Ahmadjian 1973) [2]. Various types of rock and rock crystal influence the thickness, form and sometimes color of tissues of endolithic crusts (Doppelbaur 1959 [6], Wetmore 1970) [47].

V. nigrescens had a high affinity for calcareous substrate and was not suitable for the insect's colonies. Despite obligate calcicolous species, it sporadically found on rich silica substrates such as sandstone and quartzite stones in the studied localities. Production of an appreciable amount of calcium oxalate is characteristic of calcicolous lichens, rather than all species growing on limestone or calcareous substrate, it was determined by differential thermal analysis, *V. nigrescens* contained no more than a trace of calcium oxalate and although this species is probably a fairly strict calcicole it may occasionally be found growing on acidic (high content of silica) rocks (Syres et al. 1967) [35].

Our study showed all limestone and siliceous growing samples had more or less variation in some quantitative or qualitative traits and attractive conditions for insect colonies around the studied species. In limestone specimen, related characters to thickness of cortex included cortex minimum measured thickness (CminT), maximum measured thickness (CmaxT) and average cortex thickness (CAT) exhibited the greatest variation. In siliceous living samples, however, cortex thickness varies in a narrower range. Moreover, the cortex thickness value was reported by Krzewicka (2012) [16]. and Breuss (Nash et al. 2007) [22], in 10 to 20 µm. In this study, cortex thickness ranged from 5 µm (LI4 sample) to 48.5 µm (LI18 sample) for limestone samples were11.5 µm (SI4 sample) to 43 µm (SI1 sample) for siliceous samples. Variations in the height of cortex could be related to different environmental conditions. One of lichen strategies for photo-protection reducing light transmittance through the upper cortex to underlying photobionts (Ertl 1951) [7]. The protective roles of cortex thickening were observed frequently in some lichens especially dessert samples which expose to high levels of radiation. Wind is one agent for the formation of a thick cortex. Cortices are thickest in populations Nibla homalea (Ach.) Rundel and Bowler (Ramalinacea) which are exposed to sandblast (Galun 1988) [9]. Apart from variations, there were no statistically significant differences between cortex characters in limestone and siliceous samples.

The mean value for thallus height (ATT) in both siliceous and calcareous samples was almost equaled; however, the algal layer thickness of siliceous samples significantly was higher than limestone growing specimens. The relation between environmental condition and lichen anatomical layer (cortex, algal layer and medulla) mentioned in some literature; Sonia et al. (2017) [32], declared lichen Physcia dubia (Hoffman) adopted to the environmental conditions on the supralittoral zone of the white sea through the variability of anatomical layers such as algal layer thickness; but, *P. caesia* (Hoffman) adapts to photosynthetic pigments level by a strong variation of the chlorophylls a/b ratio whit the stability of anatomical structures. To determine whether the increase in the algal layer thickness of *V. nigrescens* inhabited siliceous rocks is the result of more photosynthetic activity with respect to algal cell increasing or more loose density of algal cells and inclusion of fungal biont in this layer, additional factors such as photosynthetic pigments concentration in both groups suggested to be evaluated.

When comparing sexual reproductive characteristics in the studied samples, average length and width of spores and average width of excipulum and involucrellum had a slight variation in both groups (CV<10%). Even the coefficient of variation values for other perithecium related traits, was less than 23 % which indicated approximately a low level of variability for the reproduction system in each group. Independent t-test used to compare mean values of characters involved with perithecium, revealed significant differences for characters that were directly related to perithecium size (maximum, minimum and average width of excipulum and involucrellum) between limestone and siliceous samples. Even though the siliceous samples statistically had smaller perithecium, the size of the inner reproductive apparatus (Ascus, Ascospore and periphysoids) showed no significant differences between the means. Variations in sexual apparatus of *V. invenusta* Magn inhabited in the suboptimal state was reported by Pykälä and Breuss (2009) [25]; the spores of a specimen growing under unfavorable condition were narrower than in normally developed species and most perithecia of the Finnish specimens were smaller than the type specimen of *V. invensuta* from schistose rock in northern Sweden.

Krzewicka (2012) [16]. declared that V. tectorum (A. massal) Körb probably is a similar morph of *V. nigrescens* growing on suboptimal environmental conditions (substrate poor in calcium). For this reason, it occurs as non-sexual producing vegetative propagules and perithecia rarely occurred on thallus. Coppins and Aptroot (2008) [5]. also reported this taxon at the form level in *V. nigrescens*. They treated *V. tectorum* as a blastidiate morph of *V. nigrescens* based on the same color and a similar thickness of thallus (Krzewicka 2012) [16]. According to our study, unlike *V. tectorum*, asexual vegetative propagules (soredia) were very rare in our studied samples and only observed in two siliceous samples (22.2 %) and the perithecia were abundant on siliceous growing thalli. In the case of *V. tectorum*, the frequency of thin to moderate basal black layer (BBLT) was lower in the siliceous specimen; however, this trait was not significant statistically between limestone and siliceous samples.

The studied *V. nigrescens* thallus showed different reactions when wetting. In certain cases (especially black thalli), thallus markedly became shiny olive-green with moisture; but, in the other cases (mostly brown samples), reaction to wetting confined to marginal areoles or whole color stay opaque. Wetting could enhance the transparency of thallus due to the lower content of cortical pigments and the color of the green algae inside fungus shows through (Taylor 1982) [38]. The lower percentage of thalli which totally became green with moisture clearly related to the lower percentage of black thalli in siliceous samples, since these traits were not significantly different between two groups, variation in wet and dry thallus color could be correlated with environmental factors, especially the amount of sunlight transmitted to the thallus.

The presence of medulla below the Perithecium (MBP) was only qualitative significant character. Perithecia of *V. nigrescens* inhabitant limestone dominantly settled on a thin layer of the basal black layer; but, in some cases a thin or thick medulla lies beneath the perithecium. In a siliceous specimen, the presence of medulla beneath the perithecia is more common and could be visible in many transactions. The cause of the medulla existence below perithecium in the siliceous sample is unclear; however, it could probably be related to the greater activity of fungal biont to spread on hard siliceous substrate.

In conclusion, special characters of *V. nigrescens* is substrate-controlled, specimen growing under unfavorable substrate and insect colonies (low in calcium) showed the degree of variation in both vegetative (algal layer thickness) and reproductive structures (excipulum and involucrellum width). Several form and verities have been introduced for *V. nigrescens* due to thallus characteristics and presence of vegetative propagules all over areoles (Coppins and Atroop 2008) [5]; but, growth substrate was not mentioned as a distinctive factor for this taxon. However, traditional morphological characters are homoplasic and do not indicate for defined evolutionary lineage in *Verrucaria* species (Gueidan et al. 2009) [11], Molecular studies suggested to clarify the relations between limestone and siliceous growing morphotypes and their relationship with siliceous growing species such as *V. umbrinula* Nyl., *V. tectorum* and *V. fusconigrescens*.

5. References

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