Photosynthesis Research (2019) 141:245–257
https://doi.org/10.1007/s11120-019-00624-x

TECHNICAL COMMUNICATION

# Comparison of two noninvasive methods for measuring the pigment content in foliose macrolichens

**Shuai Liu[1,2] · Su Li[1,3] · Xiao-Yang Fan[1,2] · Guo-Di Yuan[1,2] · Tao Hu[1,2] · Xian-Meng Shi[1,2] · Jun-Biao Huang[1,2] · Xiao-Yan Pu[1,2] · Chuan-Sheng Wu[4]**

Received: 25 September 2018 / Accepted: 28 January 2019 / Published online: 7 February 2019


## Abstract

Chlorophyll content in lichens is routinely used as an accurate indicator of lichen vigor, interspecific differences, and the effect of site-related environmental parameters. Traditional methods of chlorophyll extraction are destructive, time-consuming, expensive, and inoperable, especially when measuring large quantities of chlorophyll. However, non-destructive methods of measurement using portable chlorophyll meters are rarely used for lichens. Considering the characteristics of lichens such as rough blade surface and absence of chlorophyll b in cyanolichens, we compared the non-destructive methods with traditional methods and evaluated their applicability in studying lichen pigment content. Two instruments, SPAD-502 and CCM-300, were used to measure the pigment content of seven foliose lichen species. These pigment readings were compared with those determined using the dimethyl sulphoxide (DMSO) extraction method. Significant correlations were observed between SPAD/CCM values and pigments (chlorophyll and total carotenoids) extracted from chlorolichens, especially species with a smooth surface. The CCM-300 was more accurate in detecting the pigment content of foliose chlorolichens. However, both instruments showed certain limitations in the determination of pigment content in cyanolichens, especially gelatinous species. For example, CCM-300 often failed to give specific values for some cyanolichen samples, and both instruments showed low measurement accuracy for cyanolichens. Based on the high correlation observed between chlorophyll meter readings and pigments extracted from chlorolichens, equations obtained in this study enabled accurate prediction of pigment content in these lichens.

**Keywords** CCM-300 · Carotenoids · Chlorophyll · Dimethyl sulphoxide (DMSO) · Foliose macrolichens · SPAD-502

## Introduction

Lichens are extremely sensitive to climate change and environmental pollution because of their physiological characteristics (Brunialti and Frati 2007; Purvis et al. 2008; Lang et al. 2009; Hauck 2010). Lichen epiphytes are also a widespread and important component of forest structure and play a vital role in maintaining the biodiversity and integrity of forest ecosystems, as well as water and nutrient cycling therein (Li et al. 2013). Chlorophyll content, chlorophyll degradation, respiration rate, and photosynthesis in lichens are important indicators of air pollution and other environmental changes (Gonzalez and Pignata 1994). Among these indicators, the degradation of chlorophyll in the symbiotic photobiont is one of the most obvious signs of damage to the sensitive lichens (Showman 1975; Beltman et al. 1980; Chettri et al. 1998). Nevertheless, chlorophyll content of lichens varies considerably within the same species because

✉ Su Li
lis@xtbg.ac.cn

1 CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming 650223, Yunnan, People's Republic of China

2 University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

3 Ailaoshan Station for Subtropical Forest Ecosystem Studies, Jingdong 676209, People's Republic of China

4 Anhui Province Key Laboratory of Embryo Development and Reproductive Regulation, Anhui Province Key Laboratory of Environmental Hormone and Reproduction, Fuyang Normal University, Fuyang 236037, Anhui, People's Republic of China

of differences among local microenvironments (especially light) and microhabitats (Renhorn et al. 1997; Gauslaa and Solhaug 2000). For example, shaded thalli of *Cladonia verticillaris* contain more chlorophyll and carotene than sun-grown thalli (Legaz et al. 1986). Epiphytic individuals of *Parmelia pastillifera* also contain significantly higher chlorophyll content than epilithic individuals, as these are generally exposed to stronger irradiance than epiphytic individuals (Tretiach and Brown 1995). Accordingly, these studies imply the need to include a large number of samples in the chlorophyll assessment of lichens in large-scale and long-term research, when considering the inherent complexity of lichen microhabitats.

The assessment of the chlorophyll content of lichens has gained importance in large-scale and long-term monitoring programs. However, these programs require an efficient, rapid, and reliable method of pigment measurement. In traditional studies, organic solvents, such as acetone, methanol, ethanol, dimethyl sulphoxide (DMSO), *N*-dimethyl formamide, and petroleum ether, were used for the extraction of chlorophyll from lichen thalli (Bruinsma 1961; Hiscox and Israelstam 1979; Moran and Porath 1980; Lichtenthaler and Wellburn 1983; Inskeep and Bloom 1985). The modified DMSO assay is more efficient and superior than other methods (Barnes et al. 1992). In this method, lichen samples are first repeatedly washed with carbonate-saturated acetone and then extracted in DMSO buffer containing polyvinyl polypyrrolidone (PVP) to minimize the conversion of chlorophyll to pheophytin. The use of DMSO during pigment extraction eliminates the need to grind plant material or centrifuge plant extracts (Shoaf and Lium 1976). Additionally, pigments extracted using DMSO can be stored in the dark for a week at 4 °C (Barnes et al. 1992) and do not need to be measured using a spectrophotometer immediately after extraction (Netto et al. 2005).

Additional methods, based on the reflectance and/or absorbance of radiation by chlorophyll, have been developed for the estimation of leaf chlorophyll content (Uddling et al. 2007). Unlike traditional methods, these methods of chlorophyll content measurement are simple, rapid, and non-destructive. One of these methods uses SPAD-502, a hand-held absorbance-based dual wavelength chlorophyll meter, developed by Minolta (SPAD models 501 and 502; Minolta Corporation, Ltd., Osaka, Japan) in the 1980s. The SPAD-502 meter records measurements instantaneously in a non-destructive manner, based on the transmittance of red (650 nm) and infrared (940 nm) radiation through a leaf sample (Netto et al. 2005). It is a simple and portable diagnostic tool and is capable of measuring the degree of greenness or relative chlorophyll concentration of leaves in a large number of experiments (Netto et al. 2005). Until 2007, the SPAD-502 meter had been used in more than 200 studies, most of which focused on agricultural products or cash crops (Uddling et al. 2007). The relationship between extracted leaf chlorophyll content and SPAD readings has been quantified (Parry et al. 2014) either as linear (Dwyer et al. 1991; Xu et al. 2000; Wang et al. 2004) or curvilinear (Markwell et al. 1995; Richardson et al. 2002; Jifon et al. 2005). However, the SPAD-502 meter has not been used for poikilohydric autotrophic organisms, such as lichens, mosses, and liverworts, probably because it requires the measuring aperture to be completely filled. Additionally, lichens have diverse growth forms, such as crustose, foliose, and fruticose, and the use of SPAD-502 is limited to certain forms. Moreover, there has been minimal interest in large-scale ecophysiological research of poikilohydric organisms to date.

Another instrument used to determine the chlorophyll content of leaves, based on reflectance and/or absorbance of radiation by chlorophyll, is the Chlorophyll Content Meter-300 (Opti-Sciences CCM-300, Hudson, NH, USA). Like SPAD-502, CCM-300 is also a lightweight hand-held device. It provides accurate readings and is easy to use. With CCM-300, chlorophyll absorbs blue fluorescence excitation light and emits a range of longer wavelengths of fluorescence (Gitelson et al. 2003). The measuring aperture does not need to be completely covered because the CCM-300 does not compare transmission through a leaf sample at two different wavelengths. The CCM-300 is capable of measuring the chlorophyll content of leaves with a small surface area (e.g., pine needles) and of other difficult to measure samples, such as fruits, mosses, lichens, and algae. Significant correlations between CCM-200 (absorption technique; the previous generation of CCM-300) and total foliar extractable chlorophyll content have been reported in many studies, most of which are focused on agricultural species, including grain crops, fruit trees, and coffee (Marquard and Tipton 1987; Schaper and Chacko 1991; Azia and Stewart 2001; Netto et al. 2005).

Despite the advantages of SPAD-502 and CCM-300, each instrument has certain shortcomings. For example, mathematical relationships between SPAD chlorophyll readings and foliar chlorophyll content vary with the plant growth stage (Chapman and Barreto 1997), season (Dwyer et al. 1995; Bullock and Anderson 1998), habitat condition (Campbell et al. 1990; Simorte et al. 2001), and genotype (Peng et al. 1993; Sibley et al. 1996). Additionally, CCM-300 shows only a linear response to chlorophyll content ranging from 41 to 675 mg $m^{-2}$. The predictive value of chlorophyll meters is also limited, when applied across species or within species, by the variability and instability of the mathematical relationship between chlorophyll meter values and foliar chlorophyll content (Uddling et al. 2007; Hawkins et al. 2009).

Although lichens have gained importance as indicators of environmental changes, the correlation between chlorophyll meter readings and extracted chlorophyll content has not yet been assessed for lichens. Consequently, it is difficult

to assess the accuracy and effectiveness of SPAD-502 and CCM-300 for the measurement of the chlorophyll content of lichens. In this study, seven epiphytic foliose lichen species, comprising different types of symbiotic algae at different growth stages, were selected from different habitats across the subtropical forests of the Ailao Mountains in southwest China. The objectives of this study were to (1) determine the empirical relationship between chlorophyll meter readings and pigment content (chlorophyll a [chl *a*], chl *b*, total chlorophyll and total carotenoids) of thalli of different lichen species and (2) determine which instrument is more accurate and, therefore, more suitable for measuring the chlorophyll content of lichens.

## Materials and methods

### Lichen sampling and treatment

Lichen samples were collected from the Ailao Mountains National Nature Reserve (23°35′–24°44′N, 100°54′–101°30′E) in southwestern China, which is home to one of the largest tracts of natural montane moist evergreen broad-leaved primary forests in China (Qiu and Xie 1998; Fig. 1a). These forests support abundant epiphytes and common macrolichen species belonging to the genera *Everniastrum, Heterodermia, Hypotrachyna, Leptogium, Lobaria, Myelochroa, Nephromopsis, Parmotrema, Ramalina, Sticta*, and *Usnea* (Li et al. 2013).

Seven epiphytic lichen species, including three chlorolichens (with green algal photobiont; *Lobaria isidiophora, Nephromopsis pallescens*, and *Sticta nylanderiana*) and four cyanolichens (with cyanobacterial photobiont; *Leptogium menziesii, Lobaria retigera, Sticta fuliginosa* and *Sticta weigelii*) (Fig. 1b–h), were collected from various habitats, including natural plots (inside, outside, and edge of forest) as well as artificial plots (fully exposed plots, and shaded shelters with high, medium, and low light conditions). Unlike chlorolichens, cyanolichens lack chl *b* and instead contain a phycobilin-containing light harvesting protein complex (Palmqvist 2000). Moreover, *L. menziesii* is a gelatinous lichen, *S. nylanderiana* has a flat surface, *S. fuliginosa* and *S. weigelii* have a highly grainy surface, *L. isidiophora, L. retigera*, and *N. pallescens* have a more pleated surface. A total of 1145 samples, including 566 samples of chlorolichens and 579 samples of cyanolichens (Table 1), were collected on rainy days or dewy mornings in November 2016 and November 2017.

### SPAD and CCM readings

All wet lichen samples were transferred to the Xujiaba Ecological Field Station laboratory (adjacent to the sampling sites) in the Ailao Mountains and cleaned to remove adhering debris, organic matter, and other unwanted species. Subsequently, lichen thalli were soaked in ultrapure water to saturation (not less than 30 min) under 600–800 μmol $m^{-2}$ $s^{-1}$ light intensity. For each sample, at least five measurements were recorded using SPAD-502 and CCM-300 portable chlorophyll meters, and average values were calculated. Damaged or diseased areas of thalli were avoided for taking measurements.

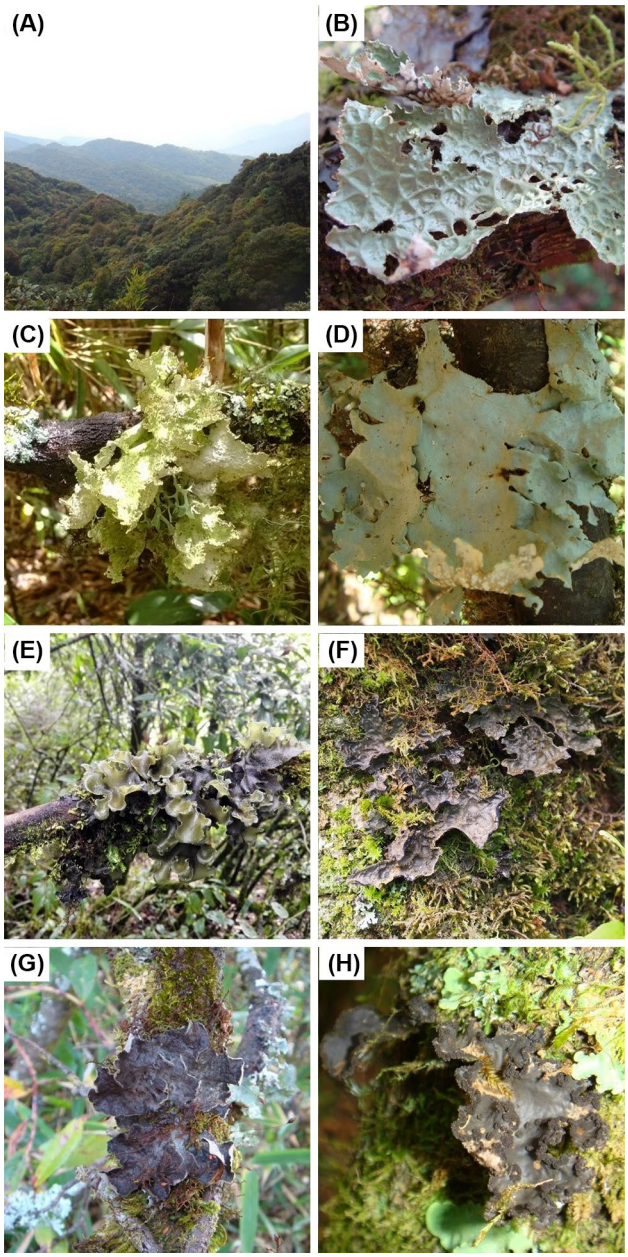


**Fig. 1** Photos of the study site and lichen species investigated in this study. **a** Subtropical forest in the Ailao Mountains; **b** *Lobaria isidiophora*; **c** *Nephromopsis pallescens*; **d** *Sticta nylanderiana*; **e** *Leptogium menziesii*; **f** *Lobaria retigera*; **g** *Sticta fuliginosa*; **h** *Sticta weigelii*

**Table 1** List of foliose lichen species investigated in this study, along with their photobionts, number of samples studied and number of samples successfully analyzed using SPAD-502 and CCM-300

| Species | Photobiont | Total number of samples | SPAD-502 Number of measurable samples (%) | CCM-300 Number of measurable samples (%) |
|---|---|---|---|---|
| *Lobaria isidiophora* | Green algae | 185 | 185 (100) | 185 (100) |
| *Nephromopsis pallescens* | Green algae | 181 | 181 (100) | 181 (100) |
| *Sticta nylanderiana* | Green algae | 190 | 190 (100) | 190 (100) |
| *Leptogium menziesii* | Cyanobacteria | 149 | 149 (100) | 116 (78) |
| *Lobaria retigera* | Cyanobacteria | 161 | 161 (100) | 148 (92) |
| *Sticta fuliginosa* | Cyanobacteria | 126 | 126 (100) | 125 (99) |
| *Sticta weigelii* | Cyanobacteria | 139 | 139 (100) | 127 (91) |

Nonetheless, CCM-300 did not record the chlorophyll content of some samples of cyanolichens for unknown reasons (Table 1). Lichen samples were then air-dried to a constant weight, which were generally less than 0.1 g, and subsequently used for chlorophyll extraction using DMSO.

### Chlorophyll extraction with DMSO

Chlorophyll was extracted from air-dried lichen thalli (20 mg) using DMSO, as described previously (Barnes et al. 1992). Unlike plants, lichens contain a large number of secondary metabolites such as lichenic acids, which degrade chlorophyll during extraction; therefore, it is necessary to wash off these secondary metabolites with relevant solutions (e.g., acetone) before pigment extraction (Barnes et al. 1992). Therefore, samples were washed six times (5 min per wash) with 3 ml of 100% $CaCO_3$-saturated acetone. The washed thalli were then placed on a filter paper and air-dried until the acetone had completely evaporated (at least 30 min). Each sample was then cut into tiny pieces and placed in an anticorrosive tube. To extract chlorophyll, 10 ml DMSO containing 2.5 mg $ml^{-1}$ PVP was added to the sample, and samples stored in the dark for 72 h to allow solvent extraction. Prior to analysis, pieces of lichen thalli were removed from the tubes, and extracts were transferred to 10-ml volumetric flasks and diluted with the same solvent.

Absorbance of the extracts was measured using a UV–visible spectrophotometer (UV-B2501; Shimadzu, Japan). The turbidity of the extracts was evaluated at 750 nm. If the absorbance at 750 nm exceeded 0.01, samples were subjected to centrifugation to remove particulate matter. Absorbance was also measured at other wavelengths including 665, 649, and 480 nm. Values obtained using the spectrophotometer were used to calculate the concentration (μg $mg^{-1}$ dry mass) of chl *a*, chl *b*, total chlorophyll (chl *a* + *b*) and total carotenoids, according to the equations described below (Wellburn 1994):

$$\text{Chl } a = 12.19A_{665} - 3.45A_{649} \quad (1)$$

$$\text{Chl } b = 21.99A_{649} - 5.32A_{665} \quad (2)$$

$$\text{Chl } a + b = \text{Chl } a + \text{Chl } b \quad (3)$$

$$\text{Total carotenoids} = (1000A_{480} - 2.14\ \text{Chl } a - 70.16\ \text{Chl } b)/220 \quad (4)$$

### Statistical analysis

The relationship between the amount of chlorophyll extracted from thalli and SPAD/CCM readings was determined by regression analysis. Cyanolichen samples without CCM readings were not used for the regression analysis between extracted chlorophyll and CCM values. After comparison with curvilinear models, simple linear regression was chosen to evaluate the relationship because it is simple, intuitive, and has a similar correlation coefficient. All graphs were drawn using Sigmaplot 12.5.

## Results

### Chlorophyll content in chlorolichens

Among chlorolichens, SPAD or CCM values showed significant linear relationships with the extracted chl *a*, chl *b*, and total chlorophyll contents (Figs. 2, 3, and 4). Both instruments showed a higher fitness for chl *a* than for chl *b* (Figs. 2, 3). Results of chl *a*, chl *b*, and total chlorophyll content indicated that CCM-300 was more suitable for measuring the chlorophyll content of chlorolichens than SPAD-502 (Figs. 2, 3, and 4) and showed a higher degree of fit for *S. nylanderiana* with a smooth surface than for *L. isidiophora* and *N. pallescens* with a coarse surface. For chl *a*, the $R^2$ value of *S. nylanderiana* ($R^2 = 0.85$) was higher than that of *L. isidiophora* ($R^2 = 0.74$) and *N. pallescens* ($R^2 = 0.64$). The results of total chlorophyll content were consistent with those of chl *a*; the $R^2$ value of total chlorophyll content of *S. nylanderiana, L. isidiophora*, and *N. pallescens* was 0.84, 0.71, and 0.67, respectively. However, results of chl *b* were different from those of chl *a* and total chlorophyll; the $R^2$ value of *S. nylanderiana* was the highest ($R^2 = 0.67$) and that of *L. isidiophora* was the lowest ($R^2 = 0.43$) (Figs. 2, 3,

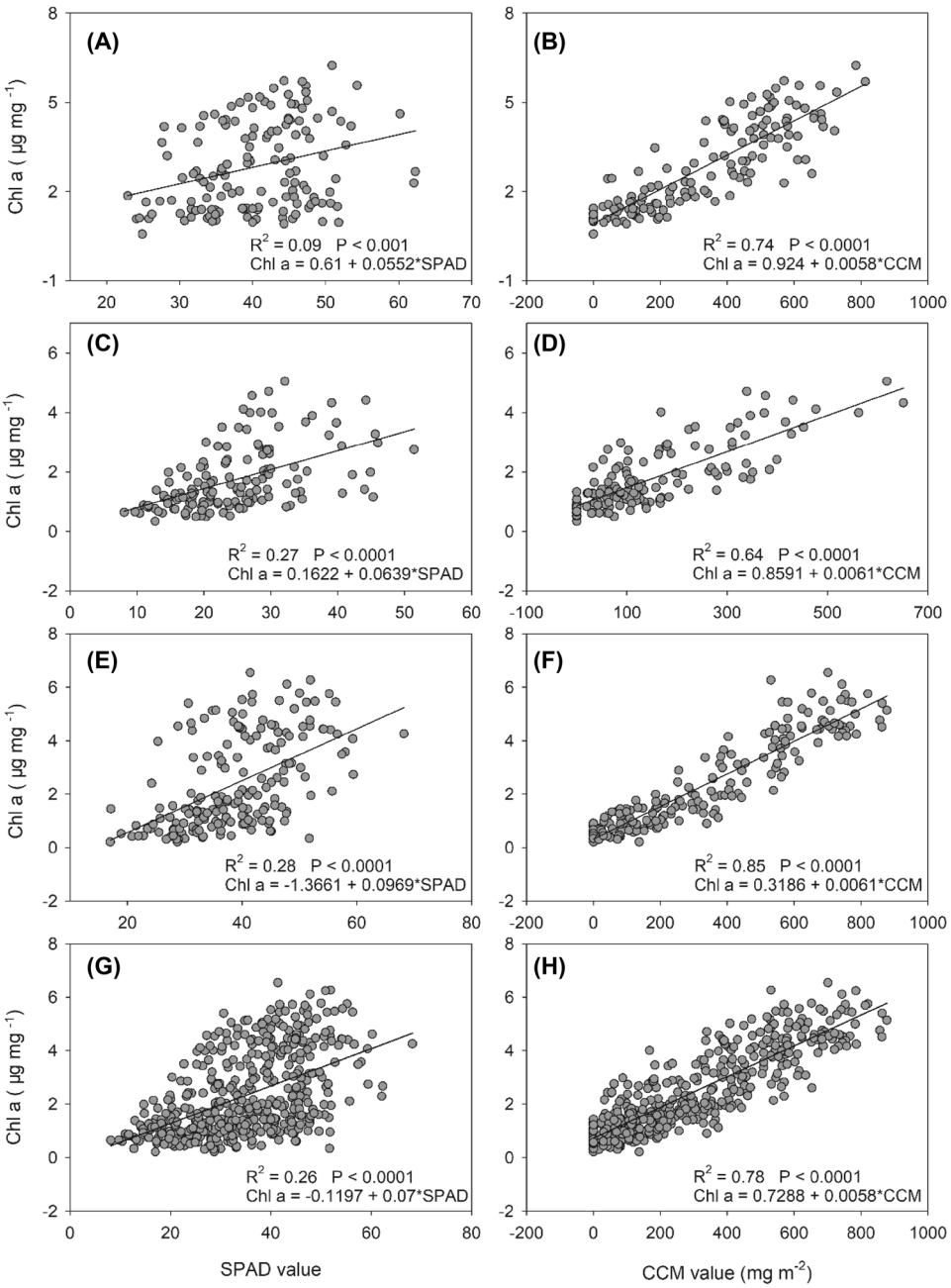


**Fig. 2** Correlation between chl *a* extracted from chlorolichens and readings of SPAD-502 (left) and CCM-300 (right). **a, b** *Lobaria isidiophora*; **c, d** *Nephromopsis pallescens*; **e, f** *Sticta nylanderiana*; **g, h** all chlorolichens

4). For all chlorolichen samples, CCM-300 showed a higher degree of fit for chl *a* (Fig. 2g, h), chl *b* (Fig. 3g, h) and total chlorophyll (Fig. 4g, h) than SPAD-502.

## Total carotenoids in chlorolichens

Significant correlations were observed between chlorophyll meter readings and total extracted carotenoids for all three chlorolichens (Fig. 5). The degree of fit using SPAD-502 was the highest for *N. pallescens* ($R^2 = 0.29$), followed by *S. nylanderiana* ($R^2 = 0.26$) and *L. isidiophora* ($R^2 = 0.05$). By contrast, the degree of fit using CCM-300 was the highest for *S. nylanderiana* ($R^2 = 0.79$), followed by *N. pallescens* ($R^2 = 0.47$) and *L. isidiophora* ($R^2 = 0.44$). Like chlorophyll, CCM-300 showed a higher degree of fit than SPAD-502 when measuring the total carotenoids of all chlorolichen samples (Fig. 5).

## Chlorophyll content in cyanolichens

Significant linear correlations were observed between SPAD/CCM values and extracted chl *a* content for all cyanolichen species, except *L. menziesii* (Fig. 6). The degree of fit was substantially below 0.3, and no significant differences were

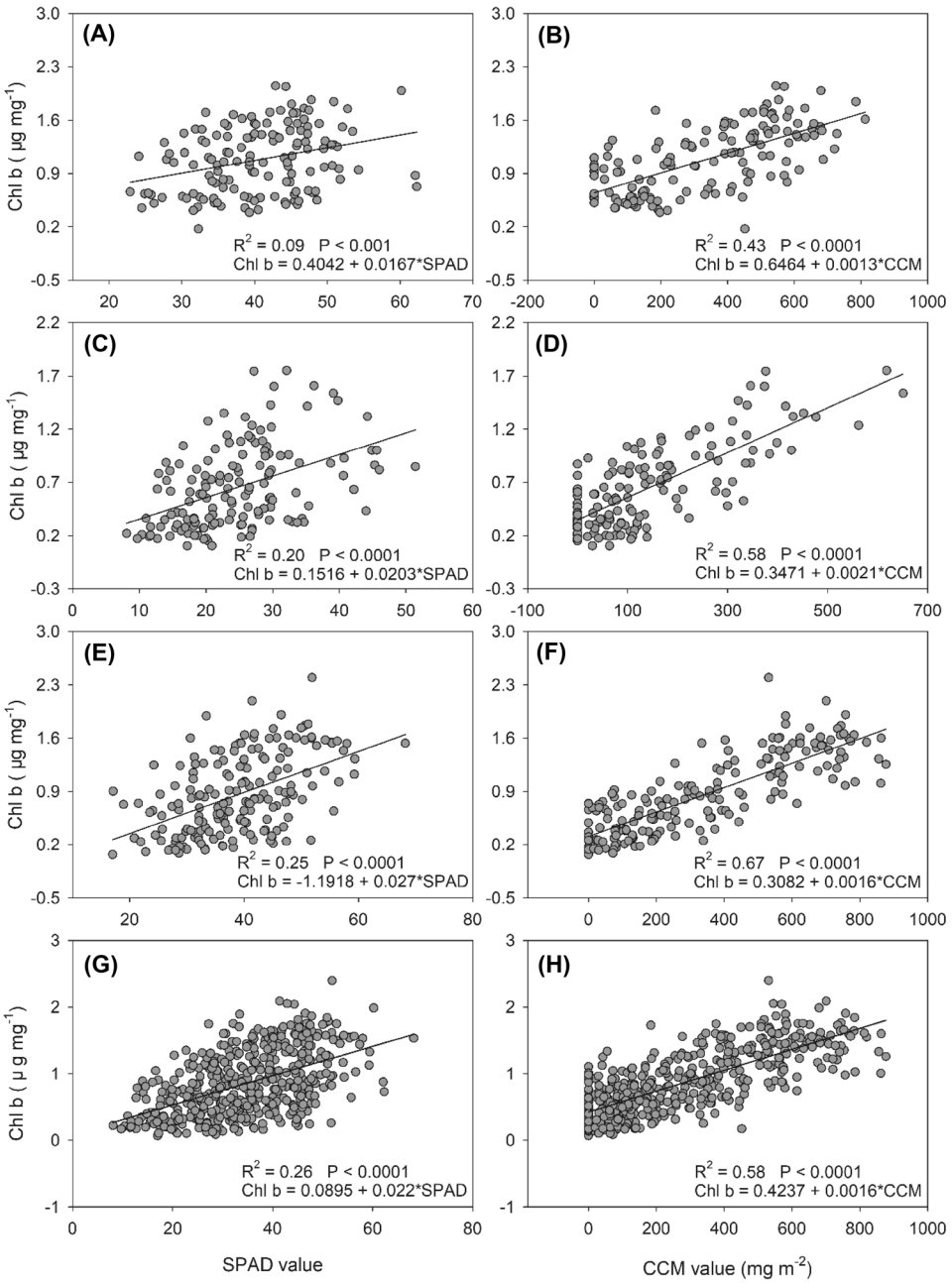


**Fig. 3** Correlation between chl *b* extracted from chlorolichens and readings of SPAD-502 (left) and CCM-300 (right). **a, b** *Lobaria isidiophora*; **c, d** *Nephromopsis pallescens*; **e, f** *Sticta nylanderiana*; **g, h** all chlorolichens

observed in correlation coefficient between the two instruments for most cyanolichens. The degree of fit for chl *a* among cyanolichens was also lower than that among chlorolichens (Figs. 2, 6).

## Total carotenoids in cyanolichens

Our results showed a significant linear correlation between SPAD-502 values and total extractable carotenoids among all cyanolichens ($R^2 = 0.11$–$0.36$), except *L. retigera* ($R^2 = 0.006$) (Fig. 7). The CCM-300 instrument was not suitable for the determination of total carotenoids in cyanolichens ($R^2 = 0.06$–$0.21$). Consistent with SPAD measurements, no significant correlation was observed between CCM-300 values and total extractable carotenoids of *L. retigera*. The extracted carotenoids showed a significant correlation with SPAD-502 values ($R^2 = 0.06$, $p < 0.001$) but not with CCM-300 values ($p = 0.55$) for all cyanolichen samples. Notably, the degree of fit for cyanolichens was substantially lower than that for chlorolichens.

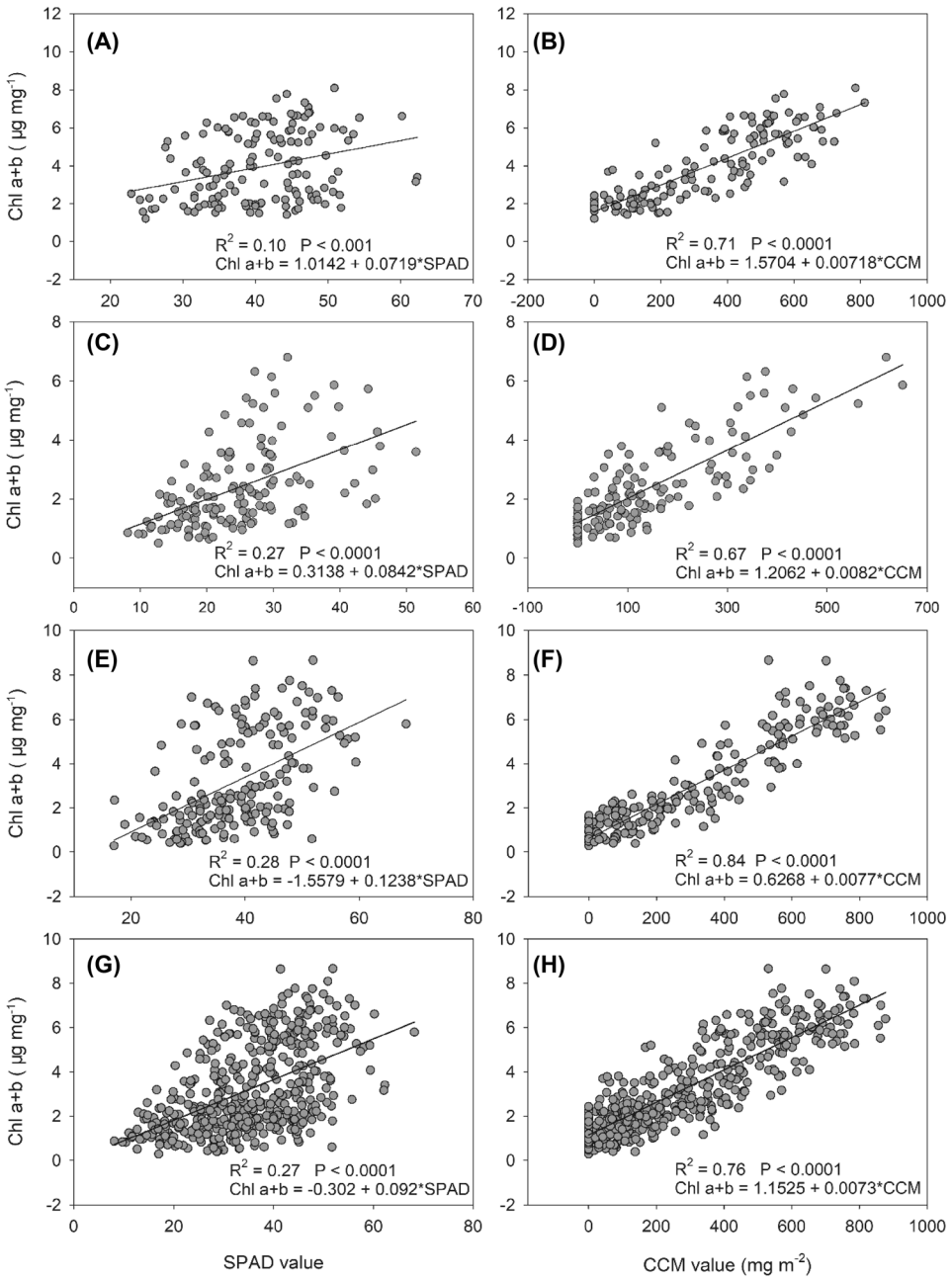


**Fig. 4** Correlation between total chlorophyll (chl *a* + *b*) extracted from chlorolichens and readings of SPAD-502 (left) and CCM-300 (right). **a, b** *Lobaria isidiophora*; **c, d** *Nephromopsis pallescens*; **e, f** *Sticta nylanderiana*; **g, h** all chlorolichens

## Discussion

The destructive method of plant chlorophyll content measurement is highly inefficient for very small sized samples and for large-scale sampling. The SPAD-502 and CCM-300 chlorophyll meters enable non-destructive quantification of physiological variables and temporal changes among threatened and endangered plant species (Hawkins et al. 2009). This is also true for lichens, which are small and highly sensitive to environmental fluctuations (Nadkarni and Solano 2002). Additionally, the growth rate of many lichen species is extremely slow (Phillips 1969). Therefore, it is reasonable to assume that the non-destructive chlorophyll meters will be an important analysis tool for lichens in large-scale and long-term field studies.

The relationship between SPAD values and extracted chlorophyll content is linear (Gratani 1992; Esposti et al. 2003; Wang et al. 2004) or nonlinear (Cartelat et al. 2005; Marenco et al. 2009; Coste et al. 2010; Cerovic et al. 2012). Similar linear (Berg and Perkins 2004) and nonlinear (Jifon et al. 2005; Gonçalves and Silva 2008; Cerovic et al. 2012) relationships have also been reported with CCM-200. Our study showed linear relationships between SPAD/CCM values and extracted chlorophyll content for all epiphytic

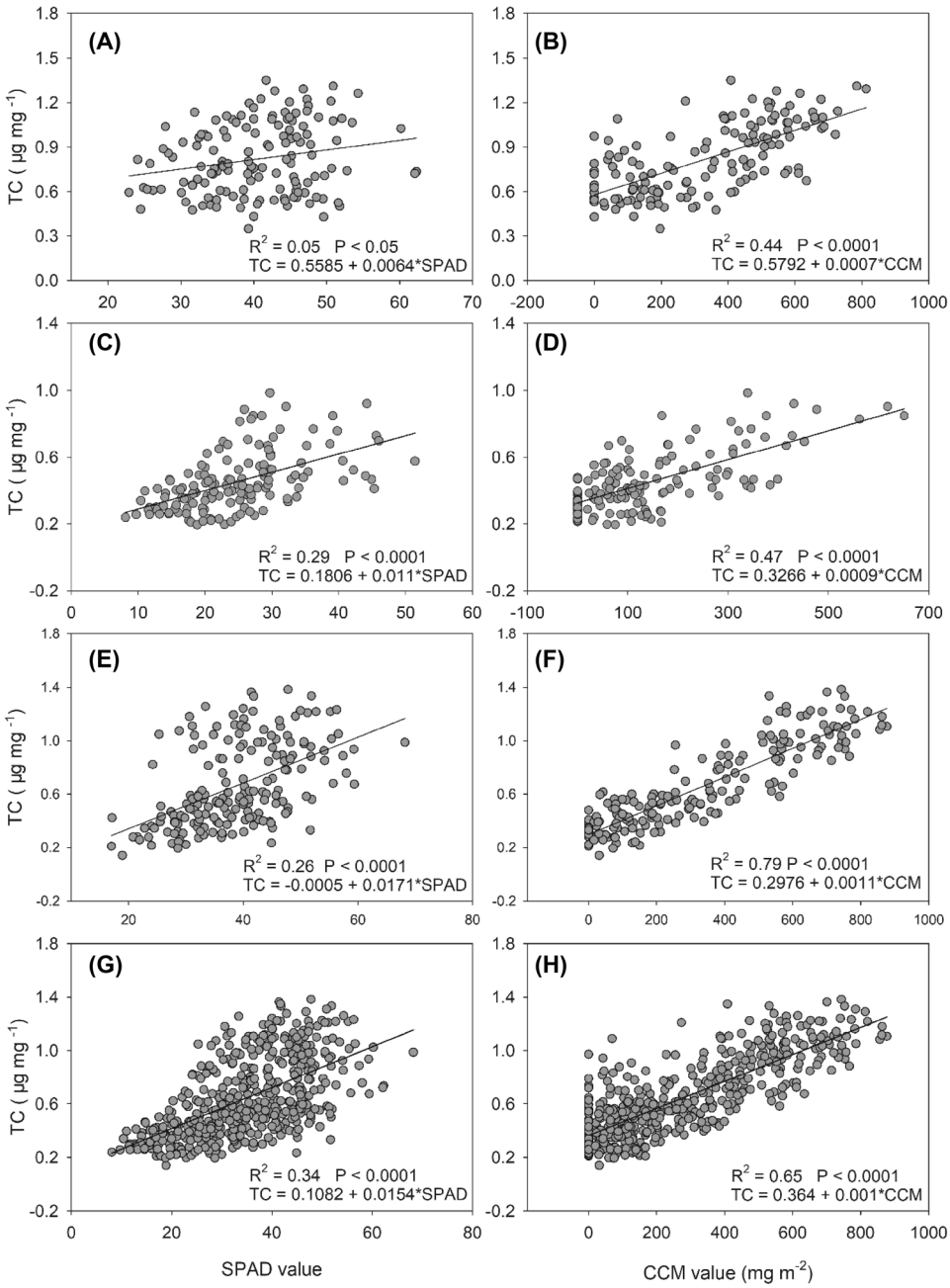


**Fig. 5** Correlation between total carotenoids (TC) extracted from chlorolichens and readings of SPAD-502 (left) and CCM-300 (right). **a, b** *Lobaria isidiophora*; **c, d** *Nephromopsis pallescens*; **e, f** *Sticta nylanderiana*; **g, h** all chlorolichens

lichen species, which is accordance with the proportional relationship between pigment concentration and absorbance, as predicted by Beer's Law (Eisenberg and Crothers 1979).

In vascular plants, the correlation between SPAD values and extracted pigment content is generally reported as ≥ 80% (Markwell et al. 1995; Uddling et al. 2007; Ling et al. 2011); similar degree of fit has been reported using CCM-200 (Richardson et al. 2002; Jifon et al. 2005; Gonçalves and Silva 2008). However, in our study, $R^2$ values for non-vascular epiphytic lichens were relatively low. The $R^2$ values between the extracted total chlorophyll content and CCM readings were higher than 60% among all chlorolichens and approximately 80% among some individual species, whereas $R^2$ values between the extracted pigment content and SPAD readings were generally 20–30%. Moreover, $R^2$ values for cyanolichens were generally below 40% using both meters, which were lower than those for chlorolichens in our study and vascular plants in previous studies (Markwell et al. 1995; Jifon et al. 2005). Additionally, no significant correlation was observed between the extracted pigment content and SPAD/CCM readings for some cyanolichens, especially the gel-like *L. menziesii*. Although CCM-300 showed a high degree of correlation between the extracted pigment content and CCM values, it was occasionally unable to measure the

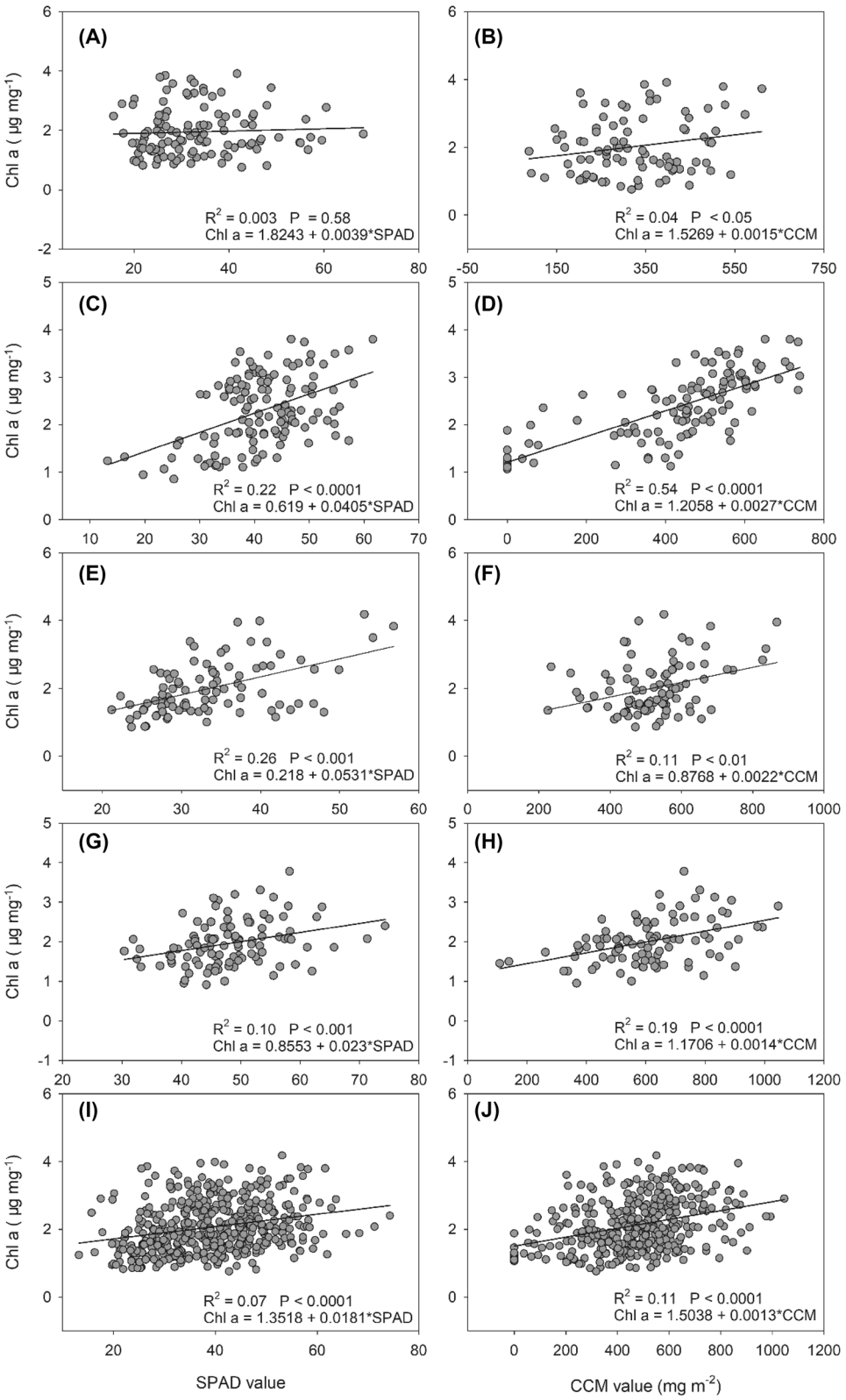


**Fig. 6** Correlation between chl *a* extracted from cyanolichens and readings of SPAD-502 (left) and CCM-300 (right). **a, b** *Leptogium menziesii*; **c, d** *Lobaria retigera*; **e, f** *Sticta fuliginosa*; **g, h** *Sticta weigelii;* **i, j** all cyanolichens

chlorophyll content of some cyanolichen samples. By contrast, SPAD-502 was able to measure all samples, albeit with a lower degree of fitness.

Several alternative explanations are possible for these results. First, the lower degree of correlation between extracted pigment content and CCM/SPAD values was

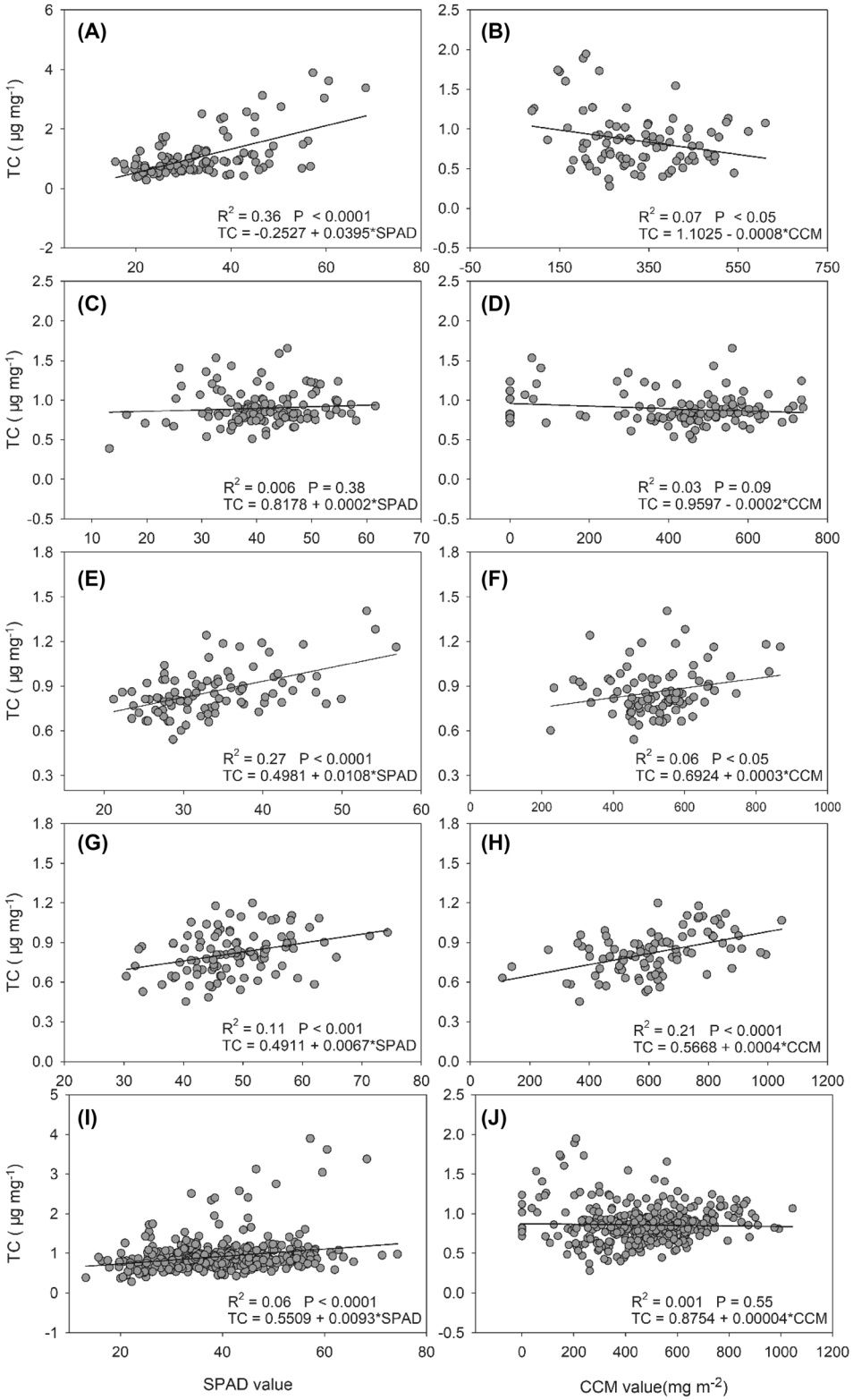


**Fig. 7** Correlation between total carotenoids extracted from cyanolichens and readings of SPAD-502 (left) and CCM-300 (right). **a, b** *Leptogium menziesii*; **c, d** *Lobaria retigera*; **e, f** *Sticta fuliginosa*; **g, h** *Sticta weigelii;* **i, j** all cyanolichens

largely because pigmented photosynthetic algae are unevenly distributed in lichen thalli, as shown in some lichen species using chlorophyll fluorescence imaging (Barták et al. 2000). Alternatively, the uneven distribution of algae could be attributed to the different growth patterns, such as apical/marginal, regular, and patchy intercalary growth (Honegger 1993; Barták et al. 2000), which result in irregular intrathalline gradients of physiological activity

at different points within a lichen thallus (Honegger 1993; Barták et al. 2000). Second, the accuracy of instruments was possibly confounded by physical (e.g., surface roughness, color, and water content) and chemical (e.g., secondary compounds) properties of the upper cortex of lichen thallus. For example, the degree of fitness of *S. nylanderiana* with smoother surface was 10–20% higher than that of other species. Color diversity of the upper cortex of lichen species, especially cyanolichens, may also reduce the degree of fit. Desiccated thallus reflects more irradiance than hydrated thallus (Gauslaa 1984; McEvoy et al. 2007; Solhaug et al. 2010), causing reduced transmittance through the desiccated upper cortices (Gauslaa and Solhaug 2001). Additionally, lichens also absorb and/or reflect specific wavelengths through secondary compounds (e.g., atranorin and parietin) deposited as crystals on fungal hyphae in the cortex (Solhaug et al. 2010); however, these may be washed off with acetone before pigment extraction (Barnes et al. 1992). Third, specific anatomical characteristics of certain cyanolichen species (e.g., the gel-like *L. menziesii*) may reduce the accuracy of instruments. Cyanobacteria often form thick extracelluar gelatinous sheaths outside the cells (Gauslaa and Coxson 2011), resulting in higher water-holding capacity (Honegger et al. 1996) and lower reflection of specific wavelengths (Solhaug et al. 2010). For example, the maximum water content of *L. menziesii* can reach 1104% of the dry mass in our studied area (Hu et al. 2016). Accordingly, *L. menziesii* showed a lower degree of fit and lowest measurable samples using CCM-300 (Table 1). Moreover, cyanobacteria lack chl *b*, which may also cause errors associated with the reflectance and/or absorbance of radiation by chlorophyll.

Furthermore, our results showed that CCM-300 is capable of measuring the pigment content of chlorolichens, which is consistent with the manufacturer's claims. To compare CCM-300 and SPAD-502 instruments, we analyzed only the foliose macrolichens. Considering the diversity in lichen morphology, such as crustose and fruticose types, CCM-300 may be more suitable for diverse lichen groups. However, our results also indicated that CCM-300 could not determine pigment content in samples with too low chlorophyll content because of extremely high light intensity or arid environments. Because both instruments showed a lower degree of fit for cyanolichens, we propose that both these instruments have limited application for measuring the chlorophyll content of cyanolichens, especially gel-like species. Although SPAD-502 showed a relatively low degree of fit with chlorolichens, it exhibited a certain advantage when measuring the total carotenoid content in cyanolichens (Fig. 6m, n). Moreover, SPAD-502 was able to analyze all foliose lichen samples and produced readable values. Overall, our data suggest that SPAD-502 may be applicable for a wider range of foliose species across various habitats than CCM-300.

In conclusion, this study is the first attempt to explore the relationship between the extracted pigment content and SPAD-502/CCM-300 chlorophyll meter readings in lichens. The degree of fit observed in this study suggests that these portable chlorophyll meters can be used for the measurement of pigment content of lichens, especially chlorolichens, in field experiments. Equations generated in this study will play an important role in predicting the absolute pigment content of the same lichen species or the same symbiotic algae lichen species, and facilitate further research in the field of lichen ecology.

**Acknowledgements** This study was supported by the National Natural Science Foundation of China (Nos. 31770494, 31400384 and 31600390), Yunnan Natural Science Foundation (2016FB056), Youth Innovation Promotion Association of the Chinese Academy of Sciences (CAS) (2017441), CAS 'Light of West China' Program and CAS 135 program (2017XTBG-T01 and 2017XTBG-F01). We thank Messrs. Fu-Ming Ke and Shi-Shun Li for their assistance with the field experiment. We thank the Biogeochemistry Laboratory of the Xishuangbanna Tropical Botanical Garden, Ailao Mountains National Nature Reserve and Ailaoshan Station for Subtropical Forest Ecosystem Studies for granting us permissions and facilitating this research work.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

Azia F, Stewart K (2001) Relationships between extractable chlorophyll and SPAD values in muskmelon leaves. J Plant Nutr 24:961–966

Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW (1992) A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a, and b, in lichens and higher plants. Environ Exp Bot 32:85–100

Barták M, Hájek J, Gloser J (2000) Heterogeneity of chlorophyll fluorescence over thalli of several foliose macrolichens exposed to adverse environmental factors: interspecific differences as related to thallus hydration and high irradiance. Photosynthetica 38:531–537

Beltman IH, Kok LJD, Kuiper PJC, Hasselt PRV (1980) Fatty acid composition and chlorophyll content of epiphytic lichens and a possible relation to their sensitivity to air pollution. Oikos 35:321–326

Berg AKVD, Perkins TD (2004) Evaluation of a portable chlorophyll meter to estimate chlorophyll and nitrogen contents in sugar maple (*Acer saccharum* Marsh.) leaves. For Ecol Manag 200:113–117

Bruinsma J (1961) A comment on the spectrophotometric determination of chlorophyll. Biochim Biophys Acta 52:576–578

Brunialti G, Frati L (2007) Biomonitoring of nine elements by the lichen *Xanthoria parietina* in Adriatic Italy: a retrospective study over a 7-year time span. Sci Total Environ 387:289–300

Bullock DG, Anderson DS (1998) Evaluation of the Minolta SPAD-502 chlorophyll meter for nitrogen management in corn. J Plant Nutr 21:741–755

Campbell RJ, Mobley KN, Marini RP, Pfeiffer DG (1990) Growing conditions alter the relationship between SPAD-501 values and apple leaf chlorophyll. HortScience 63:330–331

Cartelat A, Cerovic ZG, Goulas Y, Meyer S, Lelarge C, Prioul JL, Barbottin A, Jeuffroy MH, Gate P, Agati G, Moya I (2005) Optically assessed contents of leaf polyphenolics and chlorophyll as indicators of nitrogen deficiency in wheat (*Triticum aestivum* L.). Field Crop Res 91:35–49

Cerovic ZG, Masdoumier G, Ghozlen NB, Latouche G (2012) A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. Physiol Plant 146:251–260

Chapman SC, Barreto HJ (1997) Using a chlorophyll meter to estimate specific leaf nitrogen of tropical maize during vegetative growth. Agron J 89:557–562

Chettri MK, Cook CM, Vardaka E, Sawidis T, Lanaras T (1998) The effect of Cu, Zn and Pb on the chlorophyll content of the lichens *Cladonia convoluta* and *Cladonia rangiformis*. Environ Exp Bot 39:1–10

Coste S, Baraloto C, Leroy C, Marcon É, Renaud A, Richardson AD, Roggy JC, Schimann H, Uddling J, Hérault B (2010) Assessing foliar chlorophyll contents with the SPAD-502 chlorophyll meter: a calibration test with thirteen tree species of tropical rainforest in French Guiana. Ann For Sci 67:607

Dwyer LM, Tollenaar M, Houwing L (1991) A nondestructive method to monitor leaf greenness in corn. Can J Plant Sci 71:505–509

Dwyer LM, Anderson AM, Ma BL, Stewart DW, Tollenaar M, Gregorich E (1995) Quantifying the nonlinearity in chlorophyll meter response to corn leaf nitrogen concentration. Can J Plant Sci 75:179–182

Eisenberg D, Crothers D (1979) Physical chemistry with applications to the life sciences. Benjamin/Cummings, Menlo Park

Esposti MDD, de Sequeira DL, Pereira PRG, Venegas VHA, Salomao LCC, Filho JAM (2003) Assessment of nitrogenized nutrition of *Citrus* rootstocks using chlorophyll concentrations in the leaf. J Plant Nutr 26:1287–1299

Gauslaa Y (1984) Heat resistance and energy budget in different Scandinavian plants. Holarct Ecol 7:1–78

Gauslaa Y, Coxson DS (2011) Interspecific and intraspecific variations in water storage in epiphytic old forest foliose lichens. Botany 89:787–798

Gauslaa Y, Solhaug KA (2000) High-light-intensity damage to the foliose lichen *Lobaria pulmonaria* within a natural forest: the applicability of chlorophyll fluorescence methods. Lichenologist 32:271–289

Gauslaa Y, Solhaug KA (2001) Fungal melanins as a sun screen for symbiotic green algae in the lichen *Lobaria pulmonaria*. Oecologia 126:462–471

Gitelson AA, Gritz Y, Merzlyak MN (2003) Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. J Plant Physiol 160:271–282

Gonçalves JFDC, Silva EAD (2008) Evaluation of a portable chlorophyll meter to estimate chlorophyll concentrations in leaves of tropical wood species from Amazonian forest. Hoehnea 35:185–188

Gonzalez CM, Pignata ML (1994) The influence of air pollution on soluble proteins, chlorophyll degradation, MDA, sulphur and heavy metals in a transplanted lichen. Chem Ecol 9:105–113

Gratani L (1992) A non-destructive method to determine chlorophyll content of leaves. Photosynthetica 26:469–473

Hauck M (2010) Ammonium and nitrate tolerance in lichens. Environ Pollut 158:1127–1133

Hawkins TS, Gardiner ES, Comer GS (2009) Modeling the relationship between extractable chlorophyll and SPAD-502 readings for endangered plant species research. J Nat Conserv 17:72–86

Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334

Honegger R (1993) Tansley Review No. 60. Developmental biology of lichens. New Phytol 125:659–677

Honegger R, Peter M, Scherrer S (1996) Drought induced structural alterations at the mycobiont-photobiont interface in a range of foliose macrolichens. Protoplasma 190:221–232

Hu T, Li S, Liu S, Liu WY, Chen X, Song L, Chen Q (2016) Water relations and photosynthetic characteristics in different functional groups of epiphytic lichens in montane forest of Ailaoshan. Chin J Plan Ecolo 40:810–826

Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and b in N, N-dimethylformamide and 80% acetone. Plant Physiol 77:483–485

Jifon JL, Syvertsen JP, Whaley E (2005) Growth environment and leaf anatomy affect nondestructive estimates of chlorophyll and nitrogen in *Citrus* sp. leaves. J Amer Soc Hort Sci 130:152–158

Lang SI, Cornelissen JHC, Hölzer A, Braak CJFT, Ahrens M, Callaghan TV, Aerts R (2009) Determinants of cryptogam composition and diversity in *Sphagnum*-dominated peatlands: the importance of temporal, spatial and functional scales. J Ecol 97:299–310

Legaz ME, Vicente C, Ascaso C, Pereira EC, Filho LX (1986) Pigment analysis of sun and shade populations of *Cladonia verticillaris*. Biochem Syst Ecol 14:575–582

Li S, Liu WY, Li DW (2013) Epiphytic lichens in subtropical forest ecosystems in southwest China: species diversity and implications for conservation. Biol Conserv 159:88–95

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Ling Q, Huang W, Jarvis P (2011) Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. Photosynth Res 107:209–214

Marenco RA, Antezana-Vera SA, Nascimento HCS (2009) Relationship between specific leaf area, leaf thickness, leaf water content and SPAD-502 readings in six Amazonian tree species. Photosynthetica 47:184–190

Markwell J, Osterman JC, Mitchell JL (1995) Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynth Res 67:647–655

Marquard RD, Tipton JL (1987) Relationship between extractable chlorophyll and an in situ method to estimate leaf greenness. HortScience 22:1327

McEvoy M, Solhaug KA, Gauslaa Y (2007) Solar radiation screening in usnic acid-containing cortices of the lichen *Nephroma arcticum*. Symbiosis 43:143–150

Moran R, Porath D (1980) Chlorophyll determination in intact tissues using N, N-dimethylformamide. Plant Physiol 65:478–479

Nadkarni NM, Solano R (2002) Potential effects of climate change on canopy communities in a tropical cloud forest: an experimental approach. Oecologia 13:580–586

Netto AT, Campostrini E, Oliveira JGD, Bressan-Smith RE (2005) Photosynthetic pigments, nitrogen, chlorophyll a, fluorescence and SPAD-502 readings in coffee leaves. Sci Hortic 104:199–209

Palmqvist K (2000) Carbon economy in lichens. New Phytol 148:11–36

Parry C, Blonquist JM, Bugbee B (2014) In situ measurement of leaf chlorophyll concentration: analysis of the optical/absolute relationship. Plant Cell Environ 37:2508–2520

Peng S, García FV, Laza RC, Cassman KG (1993) Adjustment for specific leaf weight improves chlorophyll meter's estimate of rice leaf nitrogen concentration. Agron J 85:987–990

Phillips HC (1969) Annual growth rates of three species of foliose lichens determined photographically. Bull Torrey Bot Club 96:202–206

Purvis OW, Dubbin W, Chimonides PD, Jones GC, Read H (2008) The multi-element content of the lichen *Parmelia sulcata*, soil, and oak bark in relation to acidification and climate. Sci Total Environ 390:558–568

Qiu XZ, Xie SC (1998) Studies on the forest ecosystem in the Ailao Mountains, Yunnan. Yunnan Sciences and Technology Press, Kunming (in Chinese with English abstract)

Renhorn KE, Esseen PA, Palmqvist K, Sundberg B (1997) Growth and vitality of epiphytic lichens I. Responses to microclimate along a forest edge-interior gradient. Oecologia 109:1–9

Richardson AD, Duigan SP, Berlyn GP (2002) An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol 153:185–194

Schaper H, Chacko EK (1991) Relation between extractable chlorophyll and portable chlorophyll meter readings in leaves of eight tropical and subtropical fruit-tree species. J Plant Physiol 138:674–677

Shoaf WT, Lium BW (1976) Improved extraction of chlorophyll a and b from algae using dimethylsulfoxide. Limnol Oceanogr 21:926–928

Showman RE (1975) Lichens as indicators of air quality around a coal-fired power generating plant. Bryologist 78:1–6

Sibley JL, Eakes DJ, Gilliam CH, Keever GJ, Dozier WAJ, Himelrick DG (1996) Foliar SPAD-502 meter values, nitrogen levels, and extractable chlorophyll for red maple selections. HortScience 31:468–470

Simorte V, Bertoni G, Dupraz C, Masson P (2001) Assessment of nitrogen nutrition of walnut trees using foliar analysis and chlorophyll measurements. J Plant Nutr 24:1645–1660

Solhaug KA, Larsson P, Gauslaa Y (2010) Light screening in lichen cortices can be quantified by chlorophyll fluorescence techniques for both reflecting and absorbing pigments. Planta 231:1003–1011

Tretiach M, Brown DH (1995) Morphological and physiological differences between epilithic and epiphytic populations of the lichen *Parmelia pastillifera*. Ann Bot 75:627–632

Uddling J, Gelang-Alfredsson J, Piikki K, Pleijel H (2007) Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. Photosynth Res 91:37–46

Wang QB, Chen MJ, Li YC (2004) Nondestructive and rapid estimation of leaf chlorophyll and nitrogen status of peace lily using a chlorophyll meter. J Plant Nutr 27:557–569

Wellburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144:307–313

Xu W, Rosenow DT, Nguyen HT (2000) Stay green trait in grain sorghum: relationship between visual rating and leaf chlorophyll concentration. Plant Breed 119:365–367