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Anatomical constraints to nonstomatal diffusion conductance and photosynthesis in lycophytes and bryophytes

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Summary

- Photosynthesis in bryophytes and lycophytes has received less attention than terrestrial plant groups. In particular, few studies have addressed the nonstomatal diffusion conductance to CO_2 g_{nsd} of these plant groups.
- Their lower photosynthetic rate per leaf mass area at any given nitrogen concentration compared with vascular plants suggested a stronger limitation by CO_2 diffusion. We hypothesized that bryophyte and lycophyte photosynthesis is largely limited by low g_{nsd}. Here, we studied CO_2 diffusion inside the photosynthetic tissues and its relationships with photosynthesis and anatomical parameters in bryophyte and lycophyte species in Antarctica, Australia, Estonia, Hawaii and Spain.
- On average, lycophytes and, specially, bryophytes had the lowest photosynthetic rates and nonstomatal diffusion conductance reported for terrestrial plants. These low values are related to their very thick cell walls and their low exposure of chloroplasts to cell perimeter.
- We conclude that the reason why bryophytes lie at the lower end of the leaf economics spectrum is their strong nonstomatal diffusion conductance limitation to photosynthesis, which is driven by their specific anatomical characteristics.

Introduction

Bryophytes (the broad plant group that integrates Anthocerotophyta, Marchantiophyta and Bryophyta *sensu stricto*, i.e. hornworts, liverworts and mosses, respectively) and lycophytes (Lycopodiopsida) are land plants with simple organization that evolved in the Late Silurian (Edwards *et al.*, 1998; Graham & Gray, 2001). Bryophytes are represented by 13 000 extant species, making them the second most diverse group of land plants, surpassed only by angiosperms (Hedderson *et al.*, 1996; Goffinet *et al.*, 2001; Shaw & Renzaglia, 2004). Despite the modern prevalence of spermatophytes, the nonvascular community contributes substantially to primary productivity in high-latitude and high-altitude ecosystems, where vascular plants are largely constrained (Sjögersten *et al.*, 2006; Arndal *et al.*, 2009; Turetsky *et al.*, 2010, 2012; Porada *et al.*, 2013). Moreover, bryophytes coexist in spermatophyte-dominated habitats, but thanks to their poikilohydry they also co-dominate with lichens in niches where vascular plants are excluded by a lack of soil and/or nutrients, very low air temperatures and irregular or unpredictable water availability (Proctor *et al.*, 2007). Despite their importance, the number of studies centered on their comparative physiological performance is much smaller than those on tracheophytes.

Bryophytes are dominated by the gametophyte stage, which often lacks a differentiated vascular system, and never possess stomata. In addition, bryophytes lack a significant degree of foliage cuticularization (Edwards *et al.*, 1998; Renzaglia *et al.*, 2004, 2007; Shaw & Renzaglia, 2004), thus preventing control of leaf surface evaporation rates (Glime, 2007). Their poikilohydry imposes a high dependence on their photosynthetic rates on water availability, with limitations caused under conditions of both water deficit and excess (Proctor, 2001). The presence of surface water filling the pore space among neighboring foliage elements is also an important limitation, since the diffusion coefficient for CO_2 in water is 10^4 times lower than in air (Rice &

Giles, 1996; Proctor, 2001; Green *et al.*, 2011). Consequently, bryophytes can only attain maximum photosynthesis capacity at intermediate ranges of water content that might be species specific (Silvola & Aaltonen, 1984; Titus & Wagner, 1984; Maseyk *et al.*, 1999; Wagner *et al.*, 2013; Wang & Bader, 2018). However, even within this range of optimal hydration, the photosynthetic capacity per unit dry mass is much lower in bryophytes than in tracheophytes (Proctor, 2001; Brodribb *et al.*, 2007; Meyer *et al.*, 2008; Waite & Sack, 2010; Wang *et al.*, 2016). The mechanistic reasons for this have not been determined. In contrast to bryophytes, lycophytes are vascular plants that possess stomata on their microphylls, but like mosses they also show low photosynthetic capacity (Ruszala *et al.*, 2011; Brodribb *et al.*, 2017), which could be due to a poorly developed hydraulic system (Boyce, 2010) and diffusive limitations in the mesophyll (Tosens *et al.*, 2016; Veromann-Jürgenson *et al.*, 2017).

The leaf economics spectrum (LES) is a general set of interspecific trait relationships that reflects the cost and benefits of leaf investment, such that species with high leaf mass per area (LMA) tend to have longer lived photosynthetic organs with lower nitrogen (N) per dry mass and lower light-saturated photosynthetic rates. These trends have been shown for spermatophytes (Reich *et al.*, 1997; Wright *et al.*, 2004; Zhang *et al.*, 2015) and ferns (Tosens *et al.*, 2016). In bryophytes, LES relationships have been recently tested in moss species from tropical (Waite & Sack, 2010) and temperate climates (Wang *et al.*, 2017), and these relationships have been supported when considering projected canopy mass per area (CMA) instead of LMA, with CMA being considered as analogous to tracheophyte LMA (Proctor, 2000; Waite & Sack, 2010). Both studies observed that mosses followed the same relationships as tracheophytes, but with very different slopes and/or intercepts. Indeed, mosses had a much lower mass-based photosynthetic rate than tracheophytes for a given mass-based N concentration (Waite & Sack, 2010; Wang *et al.*, 2017), suggesting a diffusional limitation of their photosynthetic capacity or much greater investment in nonphotosynthetic biomass within foliage, including investment in cell walls (Onoda *et al.*, 2017). Furthermore, thick cell walls are directly associated with low mesophyll conductance due to the slow diffusion in the liquid phase (Niinemets *et al.*, 2009; Tosens *et al.*, 2012a,b; Tomás *et al.*, 2013; Onoda *et al.*, 2017; Veromann-Jürgenson *et al.*, 2017).

Photosynthetic limitations in tracheophytes can be divided among limitations imposed by stomatal conductance g_s, mesophyll conductance g_m (which we rename nonstomatal diffusion conductance g_{nsd} to account for plants lacking a true mesophyll, like those studied here), and leaf biochemistry/photochemistry. The relative importance of each limitation in bryophytes remained unexamined to our knowledge, and it has only been reported in three species of lycophytes (two Lycopodiales and one Selaginalles; Tosens *et al.*, 2016; Veromann-Jürgenson *et al.*, 2017). The existence of a phylogenetic trend towards increasing photosynthesis has been already suggested by Brodribb *et al.* (2007), and Flexas *et al.* (2012) noticed that this was linked to a trend towards increasing both g_s and g_m (or g_{nsd}) based on data for spermatophytes and a few hornwort and liverwort species. This hypothesis was further supported with recent data for ferns and fern allies (Gago *et al.*, 2013; Carriquí *et al.*, 2015; Tosens *et al.*, 2016). Whereas g_{nsd} data are available for a few hornwort and liverwort species (Meyer *et al.*, 2008), for mosses a value is available for only a single species (Hanson *et al.*, 2014), representing a significant gap of knowledge.

We hypothesized that, under optimum hydration, bryophytes would be mainly limited by diffusive resistance – that is, by the CO_2 diffusion through the tissues (hereinafter termed thallus for all nonleafy gametophytes and phyllidium for leaves of mosses and leafy liverworts) – rather than by biochemistry. Bryophytes are thought to have evolved mainly under selection pressure to support biomechanical stress associated with desiccation (Hanson *et al.*, 2014). These pressures have led, among other traits, to thick cell walls (Waite & Sack, 2010), which we hypothesize could result in a constitutively low g_{nsd}. Mesophyll conductance in a typical angiosperm leaf is the sum of gas- and liquid-phase pathways that CO_2 molecules have to pass in their pathway from the substomatal cavities to the carboxylation site in the chloroplast stroma. This gas-phase pathway is absent for unistratose mosses (i.e. with one cell layer thick phyllidium), theoretically enhancing CO_2 diffusion from the atmosphere to the cell surface, but present in Polytrichaceae species, which possess an air-filled 'pseudomesophyll' formed by lamellae columns (Clayton-Greene *et al.*, 1985). However, the main limitations to CO_2 diffusion are in the liquid phase, determined by the length and chemical characteristics of cell wall, plasmamembrane, cytoplasm and chloroplast envelope stroma. Also important are the mesophyll (or photosynthetic tissue in plants lacking mesophyll) and chloroplast surface areas exposed to cell perimeter – either intercellular (in multistratose photosynthetic organs) or ambient (in unistratose photosynthetic organs), S_m/S and S_c/S, respectively (Evans *et al.*, 2009; Terashima *et al.*, 2011). Our hypothesis of a low g_{nsd} in bryophytes rests on the following evidence: (1) mosses have the thickest cell walls reported for photosynthetic cells of land plants (Waite & Sack, 2010), a trait that negatively correlates with g_m in tracheophytes (Terashima *et al.*, 2011; Tosens *et al.*, 2016; Veromann-Jürgenson *et al.*, 2017); (2) their simple body structure could result in a lower S_m/S and S_c/S, negatively affecting g_m.

To test our hypothesis that the combination of thick cell walls and low S_c/S in bryophytes and lycophytes result in low g_{nsd}, we conducted a detailed analysis of gas exchange, anatomy and N content in a diversity of species. These measurements were designed, first, to assess the physiological and anatomical limitations on photosynthesis, second, to link morphoanatomical traits with variations in g_{nsd} and, third, to assess differences in the LES relationships for bryophytes compared with tracheophytes.

Materials and Methods

Experimental sites and growth conditions

Three lycophytes, 26 mosses and seven liverwort species (Table 1), adapted to different climates and microhabitats (Supporting

Information Table S1) from Antarctica, Australia, Estonia, Hawaii and Spain, were studied (Fig. 1; Methods S1). Moreover, the moss *Polytrichum juniperinum* and the liverwort *Marchantia polymorpha* were measured at two different sites, in Spain and Tasmania and in Estonia and Tasmania, respectively. Plants of all 36 species were measured at the time of the year when their growth conditions were most favorable (i.e. with least environmental stress). All plants (except for *M. polymorpha*, *Selaginella martensii* and *Selaginella uncinate*, which were grown in pots in a glasshouse) were collected in the field with the underlying substrate (i.e. soil or bark) and placed in plastic bags. Plants were watered as frequently as necessary to maintain hydration from collection to measurement (i.e. days to weeks depending on the species). Environmental conditions – including watering, fertilization and substrate – where plants were collected or kept until measurements were performed are specified in Table S2. Owing to logistic difficulties, all the traits could only be measured in a subset of species. Thus, species were divided into four categories depending on the completeness of the measurements (Table S3). Data for the Hawaiian species were already partially published (Waite & Sack, 2010), and new measurements were made from existing micrographs.

Photosynthesis measurements and estimation of nonstomatal diffusion conductance

For Hawaiian species, photosynthesis measurements were performed using an acrylic custom cuvette attached to an LI-6400 gas-exchange system (Li-Cor Inc., Lincoln, NE, USA), as detailed in Waite & Sack (2010). For all other species, simultaneous gas-exchange and fluorescence measurements were performed in young fully expanded microphylls (in lycophytes) and in healthy shoot or thalli (in mosses and liverworts) using either the gas-exchange system LI-6400XT coupled with a 2 cm^2 fluorimeter chamber (Li-6400-40) for Antarctic and five out of 14 Spanish species (changing the 2 cm^2 chamber to a 6 cm^2 chamber for respiration measurements) or the GFS-3000 gas-exchange system (Walz, Effeltrich, Germany) equipped with a leaf chamber fluorimeter with an 8 cm^2 cuvette for Australian,

Table 1 Phylogenetic classification following Kenrick & Crane (1996) for the 36 liverwort, moss and lycophyte species included in this study.

Phylum	Class	Order	Family	Species
Marchantiophyta (liverworts)	Jungermanniospida	Fossombroniales	Pelliaceae	*Pellia endiviifolia*
		Jungermanniales	Saccogynaceae	*Saccogyna viticulosa*
		Porellales	Porellaceae	*Porella canariensis*
	Marchantiopsida	Lunulariales	Lunuriaceae	*Lunularia cruciata*
		Marchantiales	Conocephalaceae	*Conocephalum conicum*
			Dumortieraceae	*Dumortiera hirsuta*
			Marchantiaceae	*Marchantia polymorpha*
Bryophyta (mosses)	Bryopsida	Polytrichales	Polytrichaceae	*Polytrichum formosum*
				Polytrichum juniperinum
		Fissidentales	Fissidentaceae	*Fissidens pacificus*
				Fissidens serrulatus
		Dicranales	Dicranaceae	*Dicranum polysetum*
				Dicranum scoparium
			Ditrichaceae	*Ceratodon purpureus*
				Campylopus hawaiicus
				Holomitrium seticalycinum
				Leucobryum seemannii
		Orthotrichales	Orthotrichaceae	*Macromitrium microstomum*
				Macromitrium piliferum
		Bryales	Mniaceae	*Plagiomnium elatum*
				Plagiomnium undulatum
			Briaceae	*Bryum pseudotriquetrum*
			Rhizogoniaceae	*Pyrrhobryum pungens*
		Hookeriales	Hookeriaceae	*Distichophyllum freycinetii*
				Hookeria acutifolia
		Hypnales	Thuidiaceae	*Thuidium tamariscinum*
			Entodontaceae	*Pseudoscleropodium purum*
			Hylocomiaceae	*Hylocomium splendens*
				Pleurozium schreberi
			Hypnaceae	*Ctenidium molluscum*
				Hypnum cupressiforme
			Amblystegiaceae	*Sanionia uncinata*
			Sematophyllaceae	*Acroporium fuscoflavum*
Tracheophyta	Lycopodiopsida (lycophytes)	Selaginalles	Selaginaceae	*Selaginella denticulata*
				Selaginella martensii
				Selaginella uncinata

Common names used throughout the paper for plant groups are described in parentheses after the scientific name.

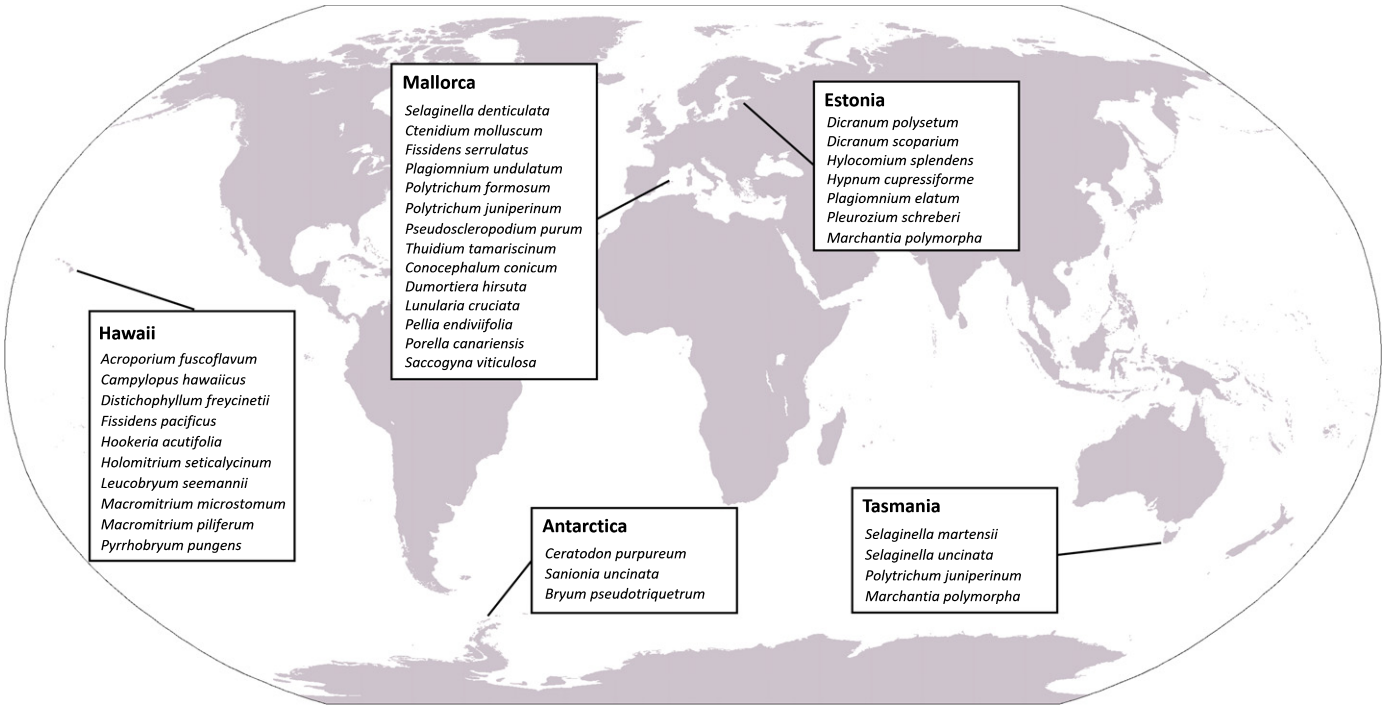

Fig. 1 Study sites of the species included in this study.

Estonian and nine out of 14 Spanish species. Details on how the measurements were performed, and the precautions taken, are given in Methods S1. Methods S1 also explains how the different photosynthetic parameters were estimated. Here, we just want to make explicit that the parameter defined as nonstomatal diffusion conductance g_{nsd} is analogous to the so-called mesophyll conductance g_m in higher plants, except that, first, most bryophytes do not show a true mesophyll, many of them consisting of a single layer of photosynthetic cells with no intercellular air spaces; and second, because of the approach used for its estimation, unavoidably g_{nsd} includes any potential residual component of diffusion through water film external to the photosynthetic tissue and/or cuticle, in addition to diffusion through the photosynthetic tissue itself.

LMA and CMA and N concentration

Shoots and thalli were dissected and an orthogonal photograph was taken, and dry mass was determined after oven drying at 70°C for 48 h. Total area was determined from photographs with ImageJ software (Schneider *et al.*, 2012) and LMA was calculated by dividing by dry mass. In mosses, CMA (see Notes S1 for a full list of trait abbreviations) was calculated by dividing the gametophyte dry mass by projected area. When testing the LES in mosses, CMA was used for mosses instead of LMA, except for *Polytrichum* species, as they have relatively large phillidia with an air-filled 'pseudomesophyll' (Clayton-Greene *et al.*, 1985) that resemble true leaves.

Total N concentration of oven-dried samples from Antarctica, Australia, Estonia and 9 out of 14 Spanish species was quantified with an elemental analyser (Truspec CN628; Leco Corp., St Joseph, MI, USA), and whole-sample average N concentration was estimated as the mass-weighted average. Total N concentration of 5 out of 14 Spanish species was quantified with an elemental analyzer (Thermo Flash EA 1112 Series; ThermoFisher Scientific, Bremen, Germany) at Universitat de les Illes Balears. Total N concentration of samples from Hawaii was determined by Kjeldahl digestion (Hue *et al.*, 2000; University Hawaii at Manoa Agricultural Diagnostic Center).

Anatomical measurements and modeling of nonstomatal diffusion conductance

Small pieces of microphylls, phyllidia or thalli from three to six individuals per species were taken after the gas exchange measurements. Samples were quickly fixed and prepared for light and transmission electron microscopy observation as detailed in Methods S1. All samples were photographed at ×100–×1000 magnification with a digital camera (U-TVO.5XC; Olympus, Tokyo, Japan; or DS-Fi1; Nikon Corp., Kyoto, Japan). Ultrathin sections for transmission electron microscopy (TEM H600; Hitachi, Tokyo, Japan) were contrasted with uranyl acetate and lead citrate. The electron micrographs were taken between ×1500 and ×30 000 magnification (see later Fig. 4). All images were analysed using ImageJ (Schneider *et al.*, 2012). Anatomical traits were estimated as described in detail in Tosens *et al.* (2016), considering the anatomical particularities of these phylogenetic groups, as detailed in Video S1. The curvature cell correction factor was measured and calculated for each species according to Thain (1983), using the average length/width ratio of three to five cells in two to four different fields of view for each tissue fraction. All traits were analysed in four to six fields of view in three

to six different sections. The micrographs were randomly selected in each section.

The fraction of photosynthetic tissue occupied by intercellular air space f_{ias} and photosynthetic tissue and chloroplast surface area exposed to air, either intercellular (in multistratose photosynthetic organs) or ambient (in unistratose photosynthetic organs) per unit of leaf area (S_m/S and S_c/S) were measured and calculated from light and transmission electron microscopy micrographs following Syvertsen *et al.* (1995) and Tosens *et al.* (2012a,b, 2016). Cell wall thickness T_{cw}, cytoplasm thickness T_{cyt} and chloroplast length L_{chl} and thickness T_{chl} were measured either from light or transmission electron microscopy micrographs, as deemed necessary for each species. These characteristics were measured for at least 10 cells per sampled individual per species.

The one-dimensional gas diffusion model of Niinemets & Reichstein (2003) was applied as modified by Tomás *et al.* (2013), with some particularities depending on the different foliar anatomies, some of which are very different compared with a typical angiosperm leaf (see later Fig. 4). The model allows estimation of the theoretical nonstomatal diffusion conductance supported by the given set of foliage anatomical characteristics (g_{nsd_ANAT}; see details in Methods S1).

Quantitative limitation analysis of A_N and partial limitations of g_{nsd}

Relative limitations on net assimilation per unit area A_{area} for lycophytes were calculated following Grassi & Magnani (2005) as described in Tomás *et al.* (2013). This analysis quantifies the relative importance of stomatal, nonstomatal diffusion conductance and biochemical limitations – the latter integrating light and carbon reactions, which co-limit photosynthesis; see Gallé *et al.* (2009) and Varone *et al.* (2012) for further explanation. In the bryophytes, the limitation was assumed to be only due to nonstomatal diffusion conductance and biochemistry (see Methods S1). Moreover, the partial limitation of the different anatomical traits to nonstomatal diffusion CO_2 conductance were calculated following the quantitative analysis described in Tomás *et al.* (2013). See Methods S1 for further details.

Statistical analyses

At least three individual plants were measured for each species for all measurements. Relationships among physiological and structural traits were explored by SPSS (SPSS Inc., Chicago, IL, USA) and all statistical tests were considered significant at $P < 0.05$. We used standardized major axis estimation and tested for a common slope among mosses and other plant groups using the likelihood ratio and then compared with a χ^2 distribution; if the mosses and other plant groups shared a common slope, we tested for a difference in intercepts by calculating the Wald statistic and comparing the χ^2 distribution between plant groups. These analyses were performed by means of the R library SMATR (Warton *et al.*, 2006, 2012).

Results

Photosynthesis, nonstomatal diffusion conductance and photosynthetic limitations

Net assimilation per unit area A_{area}, considering total area for lycophytes, *Polytrichum* and liverwort species and shoot projected area for all other moss species, varied 17.5-fold across species, from 0.43 ± 0.09 μmol CO_2 m^{-2} s^{-1} in *Fissidens serrulatus* to 7.55 ± 0.90 μmol CO_2 m^{-2} s^{-1} in *P. juniperinum*. If A_{area} based on total area is considered for mosses without intercellular airspace (i.e. except *Polytrichum*), A_{area} ranged between 0.044 ± 0.006 μmol CO_2 m^{-2} s^{-1} in *Leucobryum seemannii* to 0.774 ± 0.028 μmol CO_2 m^{-2} s^{-1} in *Plagiomnium undulatum* (Table S4). Nonstomatal diffusion conductance to CO_2 estimated from Chl fluorescence (g_{nsd_FLU}) varied 12-fold, from 1.86 ± 0.261 mmol CO_2 m^{-2} s^{-1} in *Pleurozium schreberi* to 23.8 ± 8.074 mmol CO_2 m^{-2} s^{-1} in *Selaginella denticulata* (Table S4). Maximum velocity of carboxylation $V_{c,max}$, chloroplastic CO_2 concentration C_c and electron transport rate ETR also varied strongly across species, but non-photorespiratory day respiration (R_d) varied only narrowly (Table S4). Despite potential uncertainties in the calculation of $V_{c,max}$, a strong correlation was found between ETR measured at 400 μmol CO_2 mol^{-1} air and $V_{c,max}$ (Fig. S1; $r^2 = 0.95$, $P < 0.0001$). Moreover, $V_{c,max}$ values remained within the same range when they were obtained with fast A_{area}–C_a (ambient CO_2 concentration) curves (e.g. without rehydrating the moss during the 10–15 min of the curve) or slow A–C_a curves (e.g. removing the moss from the cuvette and rehydrating it between each C_a point; Table S5). In mosses, A_{area} was strongly correlated with g_{nsd_FLU} (Fig. 2a; $r^2 = 0.93$, $P < 0.0001$) and with ETR (Fig. 2b; $r^2 = 0.83$, $P = 0.0006$), and not with $V_{c,max}$ (Fig. 2c). To discern the relative impact of stomatal limitation l_s (only in lycophytes), nonstomatal diffusion conductance limitation l_{nsd} and biochemical limitation l_b on A_{area} for each species, a quantitative limitation analysis was performed. For all bryophytes in which limitation analysis could be performed (one liverwort and nine mosses), l_m was the most important limitation of A_{area}. In lycophytes, the major limitations of photosynthesis were shared between l_{nsd} and l_b, and stomata had only a minor role in constraining A_{area} in the conditions in which they were measured (Fig. 3).

Leaf anatomy

Foliage of bryophytes presents structural features that make it vastly different from typical angiosperm leaves (Fig. 4), exhibiting a lack of stomata, presence of pores and/or a uni- or reduced multilayered cell structure. To resolve how these distinctive features influence nonstomatal diffusional limitations to photosynthesis, a structural and ultrastructural analysis of the photosynthetic organs was performed for each species. Groups and species varied strongly in their macroscopic and structural and ultrastructural characteristics (Video S1; Tables S5, S6). Dry LMA and CMA varied 12.4-fold and 6.5-fold, respectively, among species: from 6.4 ± 0.4 g m^{-2} in *Distichophyllum freycinetti* to 79.3 ± 4.7 g m^{-2} in *Polytrichum*

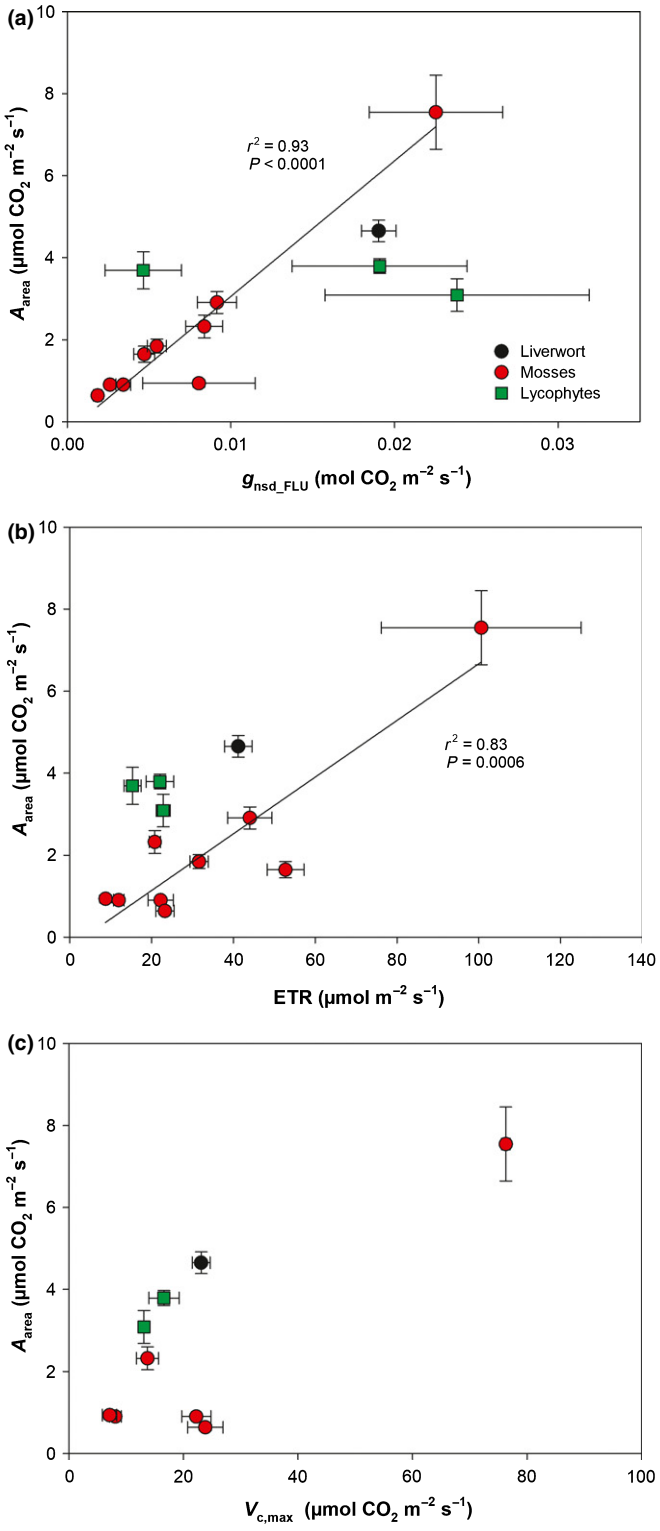

Fig. 2 Area-based net assimilation rate A_{area} in relation to (a) area-based nonstomatal diffusion conductance g_{nsd_FLU}, (b) electron transport rate ETR and (c) maximum velocity of carboxylation $V_{c,max}$. Values are means ± SE of 4–12 replicates per species. Data for moss species were fitted by linear regressions.

formosum for LMA, and from 48.1 ± 3.0 g m^{-2} in *P. schreberi* to 317 ± 49.3 g m^{-2} in *Hylocomium splendens*. Leaf density D_{leaf} and leaf thickness T_{leaf} varied 53.4-fold and 61.2-fold, respectively. f_{ias} varied from 51 ± 4% in *Selaginella uncinata* to zero in mosses with a single layer of cells (Table S6). Photosynthetic tissue and chloroplast surface area exposed to air, either intercellular (in multistratose photosynthetic organs) or ambient (in unistratose photosynthetic organs) – S_m/S and S_c/S, respectively – varied 5.4-fold and 8.6-fold, respectively: from 2.34 ± 0.02 m^2 m^{-2} in *Plagiomnium elatum* to 13.04 ± 0.58 m^2 m^{-2} in *P. juniperinum* for S_m/S, and from 0.59 ± 0.01 m^2 m^{-2} in *Pseudoscleropodium purum* to 5.18 ± 0.18 m^2 m^{-2} in *P. juniperinum*. T_{cw} varied 12.6-fold: from 0.270 ± 0.031 µm in *S. denticulata* to 3.40 ± 0.74 µm in *Distichophyllum freycinetii*. T_{chl}, L_{chl} and T_{cyt} varied 6.2-fold, 4.9-fold and 36.2-fold, respectively (Table S7). As a result of the extensive variation in anatomical characteristics, g_{nsd_ANAT} varied 45-fold: from 1.36 ± 0.26 mmol CO_2 m^{-2} s^{-1} in the moss *L. seemannii* to 45.09 ± 6.10 mmol CO_2 m^{-2} s^{-1} in the lycophyte *S. denticulata* when a cell wall porosity p_{cw} of 0.028 was considered (Table S8; see Methods S1). When scenarios were tested with a range of p_{cw} values, g_{nsd_ANAT} remained very low relative to the published values for vascular plants (Fig. S1; Table S8).

When pooling all species together, LMA (for lycophytes, *Polytrichum* and liverwort species) CMA (for the rest of moss species) did not correlate with either D_{leaf} or T_{leaf} (Fig. 5a,b). For the same LMA or CMA, bryophytes and lycophytes invested more dry mass to produce thicker cell walls compared with the spermatophyte dataset compiled by Onoda *et al.* (2017) (Fig. 5c), which comprises from herbs to evergreen angiosperms and a few gymnosperm species. Conversely, bryophytes and lycophytes had significantly lower S_c/S values for the same LMA or CMA range compared with the rest of embryophytes (Fig. 5d).

Anatomical determinants of nonstomatal diffusion conductance

A_{mass} was not correlated with T_{leaf} and D_{leaf} (Fig. 6a,b), but both A_{area} and A_{mass} were negatively correlated with T_{cw} (Fig. 6c,d), and positively with S_c/S in mosses (Fig. 6e,f). Comparing the estimates of g_{nsd_FLU} and g_{nsd_ANAT}, a highly significant positive correlation close to the 1 : 1 line was found when p_{cw} was considered as 0.028 ($r^2 = 0.73$, $P < 0.001$). When p_{cw} was considered as 0.1 or variable assuming a negative linear relationship (Fig. S2), positive relationships were still found, but the data shifted from the 1 : 1 line (Fig. S3).

To quantify the influence of subcellular liquid–phase limitations (gas-phase limitations in most mosses were zero due to the lack of intercellular airspace) in determining g_{nsd_ANAT}, a quantitative limitation analysis of liquid-phase limitations was also performed. Cell walls constituted the major component limiting CO_2 transfer into the chloroplast in the liverwort, mosses and lycophytes (Fig. 7). Inside each group, the different species presented similar values for each liquid-phase limitation (Fig. S4).

LES

The N content per area and mass (N_{mass} and N_{area}, respectively) varied 13.4-fold and 16.3-fold, respectively: from 0.24 ± 0.05%

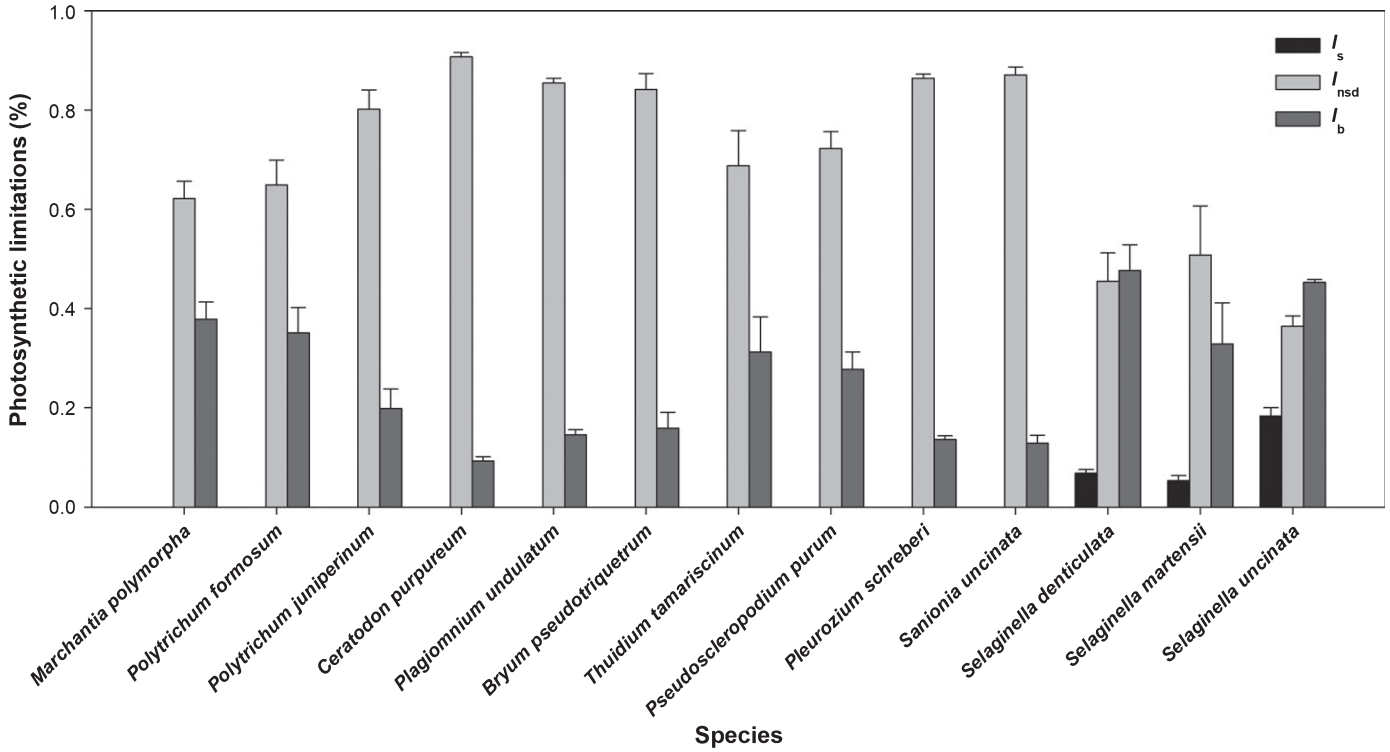

Fig. 3 The percentage of net assimilation limited by stomatal conductance l_s, nonstomatal diffusion conductance l_{nsd} and biochemistry l_b in the liverwort, mosses and lycophytes studied. Values are means ± SE of 3–10 replicates per species.

in *L. seemannii* to 3.22 ± 0.06% in *Ctenidium molluscum* for N_{mass}, and from 0.25 ± 0.01 g m^{-2} in *Pellia endiviifolia* to 4.09 ± 0.39 g m^{-2} in *Dicranum scoparium* for N_{area} (Table S9). The N_{mass} of the bryophytes and lycophytes studied was located at the low end of the spermatophyte range reported by Wright *et al.* (2004), ranging from 0.25% to 6.36%. To investigate potential differences in the LES relationships for bryophytes and lycophytes relative to angiosperms, gymnosperms and ferns, we compared trends with those described by Wright *et al.* (2004) for angiosperms, by Zhang *et al.* (2015) and Veromann-Jürgenson *et al.* (2017) for gymnosperms and by Tosens *et al.* (2016) for ferns. The low values of moss and liverwort species (with the exception of *Polytrichum* species) for A_{mass} for a given value of CMA (Fig. 8a), LMA (Fig. 8b) or – only in the case of mosses – N_{mass} (Fig. 8c) separated them from the rest of land plant groups. Although no significant differences were found between plant groups' slopes using a likelihood ratio test ($P = 0.19$ in Fig. 6a; $P = 0.51$ in Fig. 8c), intercepts between mosses and the other plant groups were different with a significance of $P > 0.0001$ in Fig. 8(a,c). Instead, lycophytes, a group in which no trendline was drawn due to the limited data available, were located in between the other groups of vascular plants.

Discussion

This study shows, from a survey of species from contrasting climates, that bryophytes constitute a unique trait space in the LES. Moreover, this study is the first to provide a comprehensive analysis of the photosynthesis-related and anatomical traits in bryophytes. They are revealed as the land plant group with the lowest nonstomatal diffusion conductance g_{nsd} values (confirmed by two independent techniques: the Chl fluorescence and the anatomical models), followed by lycophytes, thus confirming the hypothesis of a phylogenetic trend towards progressively increasing g_{nsd} from bryophytes to angiosperms. Whereas photosynthesis in mosses was mostly limited by g_{nsd} and in lycophytes co-limited by both g_{nsd} and biochemistry, g_{nsd} and photosynthesis in both primitive land plant groups were strongly determined by large cell wall thickness T_{cw} and low chloroplast surface area exposed to air per leaf area (S_c/S).

Low photosynthetic rates in bryophytes and lycophytes

Low values for maximum A_{area} (i.e. under optimum water content with no external water restricting CO_2 diffusion) were recorded for liverwort, mosses and lycophytes, and these were linked to low nonstomatal diffusion conductance estimated from Chl fluorescence (g_{nsd_FLU}). In fact, average g_{nsd_FLU} values for the mosses and the liverworts were the lowest within the land plant groups (Flexas *et al.*, 2012; Tosens *et al.*, 2016), followed by lycophytes. These data – within the rage of values previously reported for a few species of bryophytes (Meyer *et al.*, 2008; Hanson *et al.*, 2014) and lycophytes (Veromann-Jürgenson *et al.*, 2017) – support the suggested phylogenetic trend of g_{nsd}: bryophytes < lycophytes < pteridophytes < spermatophytes (Flexas *et al.*, 2012; Carriquí *et al.*, 2015).

The absolute values for both A_{area} and g_{nsd} must be taken with some caution. Even after taking all the precautions during

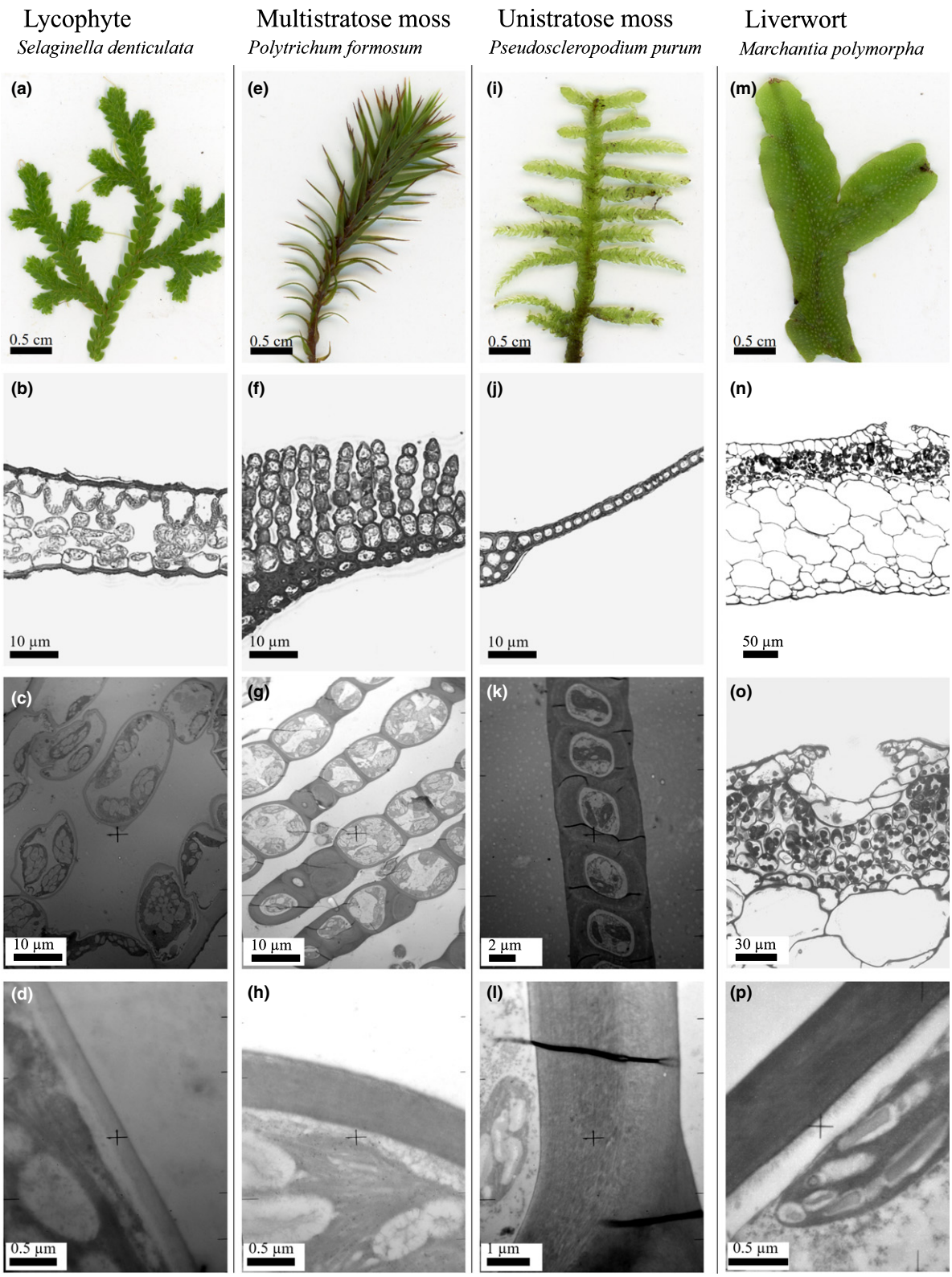

Fig. 4 Photographs and images of semithin and ultrathin microscopy cross-sections showing four representative morphologies of microphylls, phillidia and thalli of the species included in this study: (a–d) a lycophyte microphyll of *Selaginella denticulata*, (e–h) a phyllidium with multiple cell layers of the moss *Polytrichum formosum*, (i–l) a phyllidium with one layer of cells of the moss *Pseudoscleropodium purum* and (m–p) thallus of the liverwort *Marchantia polymorpha*.

measurements described in the Materials and Methods section, several issues can lead to misinterpretation of photosynthesis rates. For instance, we assume that the resistance to diffusion between CO_2 in the bulk air C_a and the leaf surface C_s is zero, which implies that there is no still air at the surface of the photosynthetic cells, which is unclear given the canopy and shoot

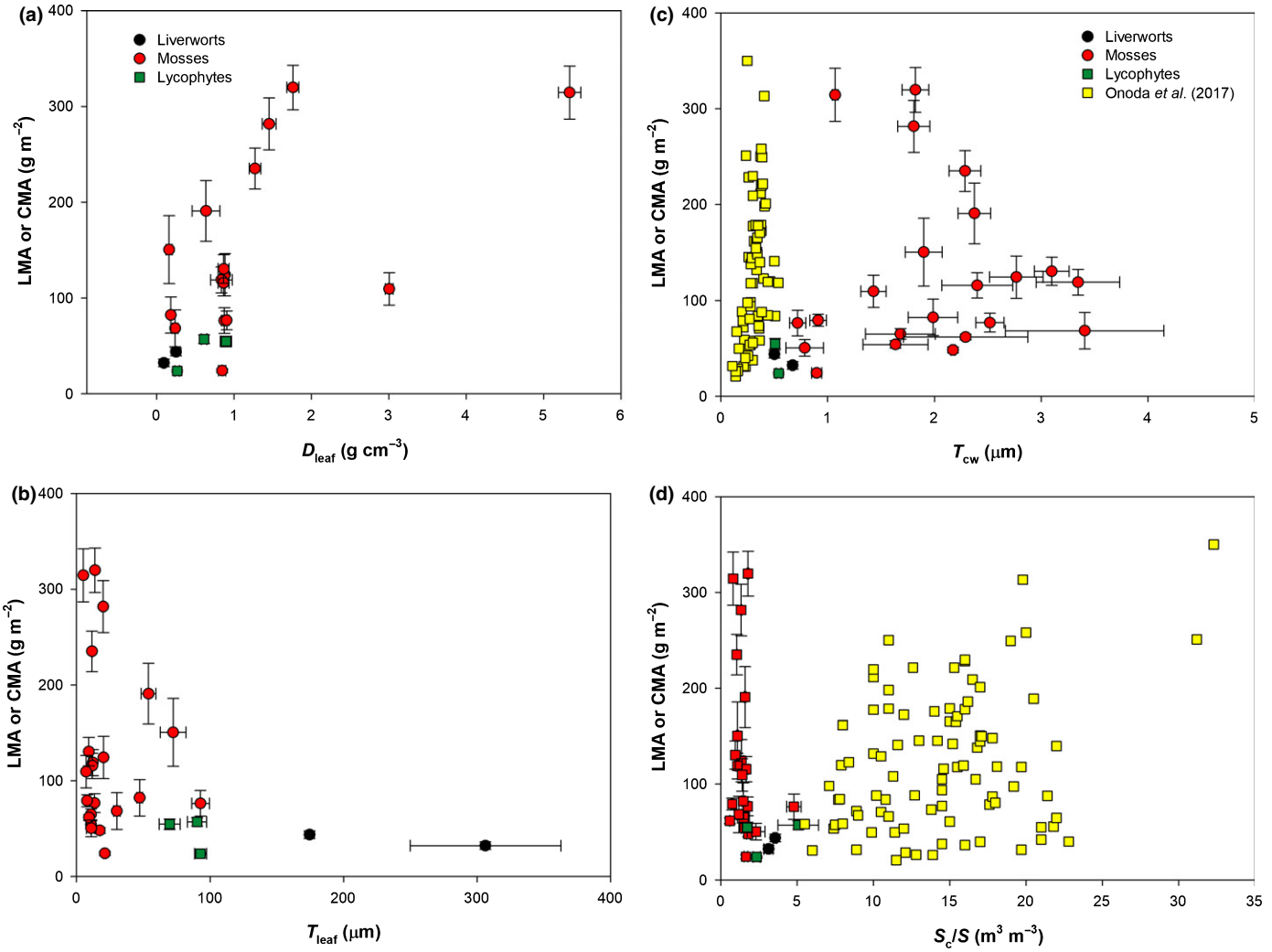

Fig. 5 Leaf or canopy dry mass per unit area (LMA or CMA, in lycophytes and bryophytes, respectively) in relation to (a) leaf density D_{leaf}, (b) leaf thickness T_{leaf}, (c) cell wall thickness T_{cw}, and (d) chloroplast surface area exposed to air S_c/S. Values are means ± SE of two to six replicates per species. Data from Onoda *et al.* (2017) for angiosperms and gymnosperms are presented for the available traits to allow a better comparison between groups.

complexity of mosses (Rice *et al.*, 2005; Rice & Cornelissen, 2014). Moreover, although we loosened the canopies and tried to place shoots next one another to avoid overlapping tissue (i.e. simulating a 'normal leaf'), some overlapping could have unavoidably occurred, thus leading to some underestimation of the actual photosynthetic area in the gas exchange cuvette, which would also be affected if there was some contribution of stem fractions (not considered to estimate the area) to total photosynthesis inside the cuvette. And, finally, the need for keeping humidity in the high range when measuring mosses creates a constant and large difference in water content between reference and sample air, and the equations to correct for direct water interference with the CO_2 signal are sensitive to errors in these conditions, which could result in biased estimates of A_{area}. There are even more concerns when estimating g_{nsd}. Besides the general limitations of the method for any species (Gu *et al.*, 2010; Gu & Sun, 2014), the potential uncoupling between gas exchange and Chl measurements due to the arrangements inside the cuvette, the use of hypothetical CO_2 compensation point without R_d (Γ*) values from angiosperms (due to the absence of published Γ* and Rubisco kinetics values for bryophytes; Griffiths *et al.*, 2004) and some potential influence of microbiome CO_2 methanogenic synthesis (Kostka *et al.*, 2016) add more uncertainties to g_{nsd} values. Despite all of these concerns, and recognizing that the absolute values presented should be taken with precaution, several lines of evidence suggest that the real values for A_{area} and g_{nsd} in bryophytes are at least in the range of the estimated ones. These include: (1) two parameters reflecting photosynthetic activity, acquired using two completely independent instruments and principles (ETR with a fluorometer and A_{area} with the infrared gas analyzer), showed a ratio ETR/$A_{area} \geq 7$ in all species but one (*S. uncinata*, 5.8); that is, the values expected for C_3 species (Flexas *et al.*, 2002); (2) a sensitivity analysis reveals that even assuming large errors in, for example, ETR, Γ*, or R_d, the estimated g_{nsd} values remain in the low range (Table S10); (3) despite all of the uncertainties related to the use of Chl fluorescence, the values obtained for bryophytes are well in the same range of those previously obtained using the isotopic method

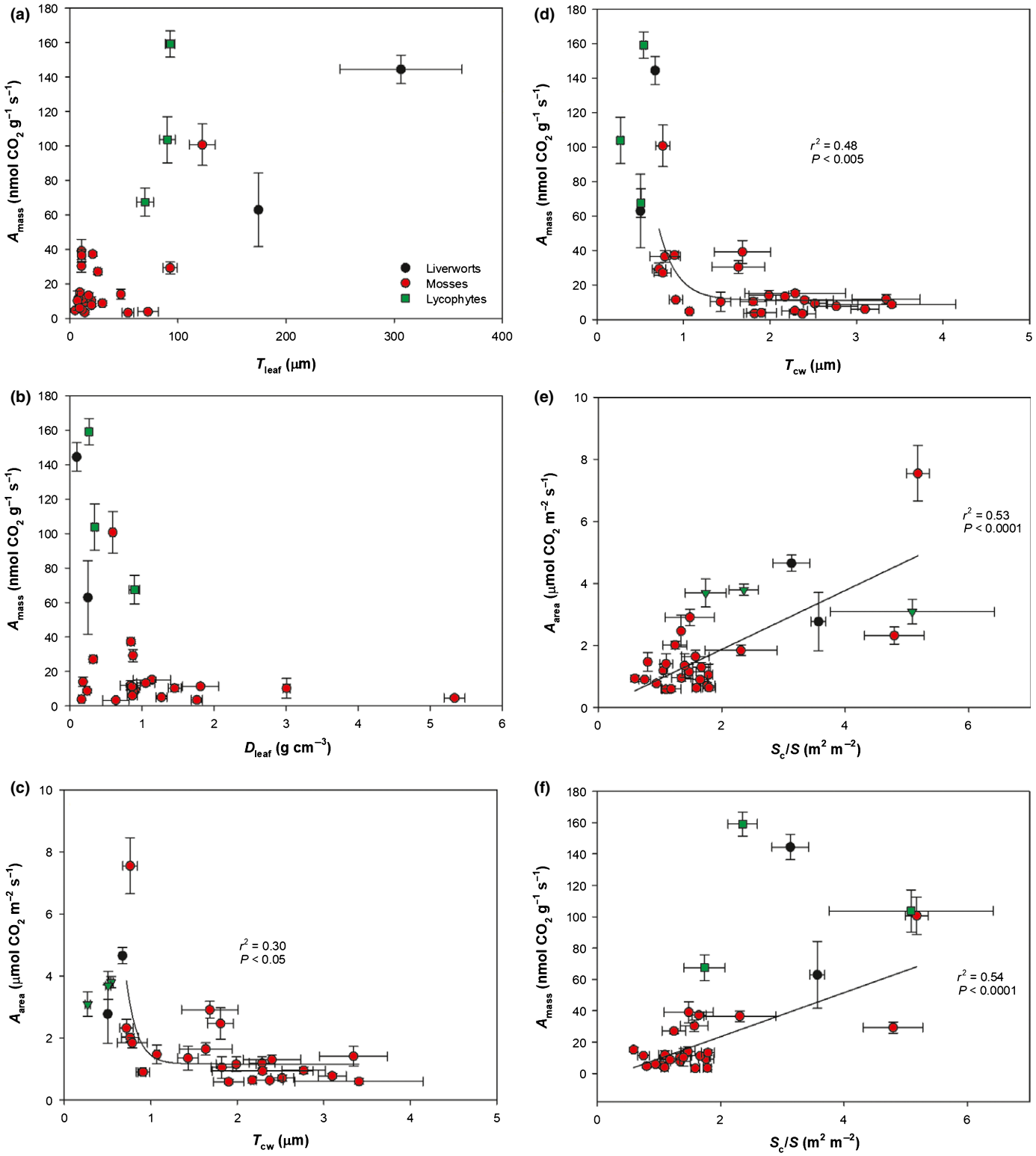

Fig. 6 Mass-based net assimilation rate A_{mass} in relation to (a) leaf thickness T_{leaf} and (b) leaf density D_{leaf}. (c) Area-based net assimilation rate A_{area} and (d) A_{mass} in relation to cell wall thickness T_{cw}. (e) A_{area} and (f) A_{mass} in relation to chloroplast surface area exposed to air S_c/S. Values are means ± SE of 3–12 replicates per species.

(Meyer *et al.*, 2008; Hanson *et al.*, 2014); and (4) using an absolutely independent method (i.e. anatomical modelling) similar values of g_{nsd} are obtained (see next section). In view of all this evidence, we consider that A_{area} and g_{nsd} in bryophytes (and lycophytes) are indeed as low as estimated, even if the precise absolute value for each species is subject to some limitations.

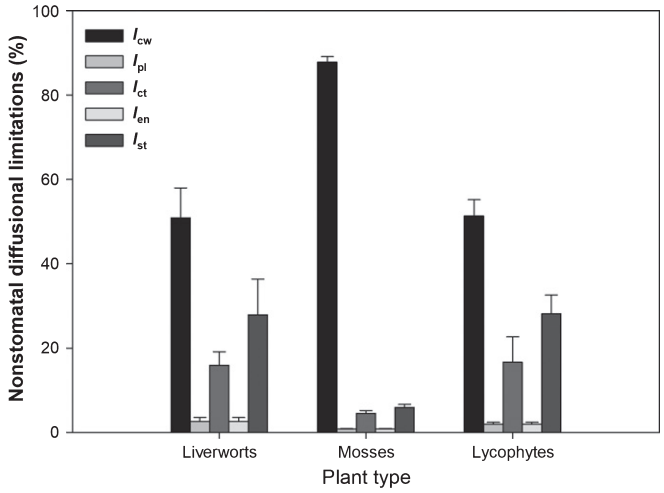

Fig. 7 The percentage of nonstomatal diffusion conductance per area limited by liquid-phase components in mosses, lycophytes and liverwort *Marchantia polymorpha*: cell wall l_{cw}, plasma membrane l_{pl}, cytoplasm l_{ct}, chloroplast envelope l_{en} and stroma l_{st}. Values are means ± SE per plant group.

The quantitative limitation analysis (Grassi & Magnani, 2005) revealed that bryophytes were mostly limited by g_{nsd} – from *c.* 60% to up to 90%, depending on the species – whereas biochemical limitations were of minor importance (Fig. 3). This confirms our hypothesis, based on the particularities of bryophytes' LES, of strong g_{nsd} limitation to photosynthesis. The phylogenetic explanation for this high g_{nsd} limitation to photosynthesis could be associated with different factors: first, that CO_2 transfer was relatively nonlimiting for photosynthesis under the elevated atmospheric CO_2 concentrations prevailing when bryophytes emerged on land (Graham & Gray, 2001; Berner, 2006); second, their adaptation to grow close to the soil, where CO_2 concentration can be high due to soil respiration (Hanson *et al.*, 2014); and/or third, their cell wall mechanical selection pressure to support desiccation rather than to maximize CO_2 diffusion (Hanson *et al.*, 2014). Conversely, lycophytes were mainly photosynthetically co-limited by both g_{nsd} and biochemistry (Fig. 3), in coincidence with what Veromann-Jürgenson *et al.* (2017) found in *S. uncinata*.

Anatomical determinants of CO_2 diffusion in bryophytes and lycophytes

With g_{nsd} being the most limiting factor for A_{area} in bryophytes, we aimed to determine whether this was related to anatomical traits being widely reported to constrain CO_2 diffusion in tracheophytes (Evans *et al.*, 2009; Terashima *et al.*, 2011). We found that cell wall thickness T_{cw} and chloroplast surface area exposed to the air, either intercellular (in multistratose photosynthetic organs) or ambient (in unistratose photosynthetic organs) per unit of leaf area (S_c/S), had extreme values in mosses. Although T_{cw} in lycophytes was similar to that of ferns (Tosens *et al.*, 2016), mosses had the largest T_{cw} reported for land plants. Hence, T_{cw} ranges from 0.170 to 0.81 µm in ferns (Tosens *et al.*, 2016), from 0.236 µm (Peguero-Pina *et al.*, 2016) to 1.22 µm in

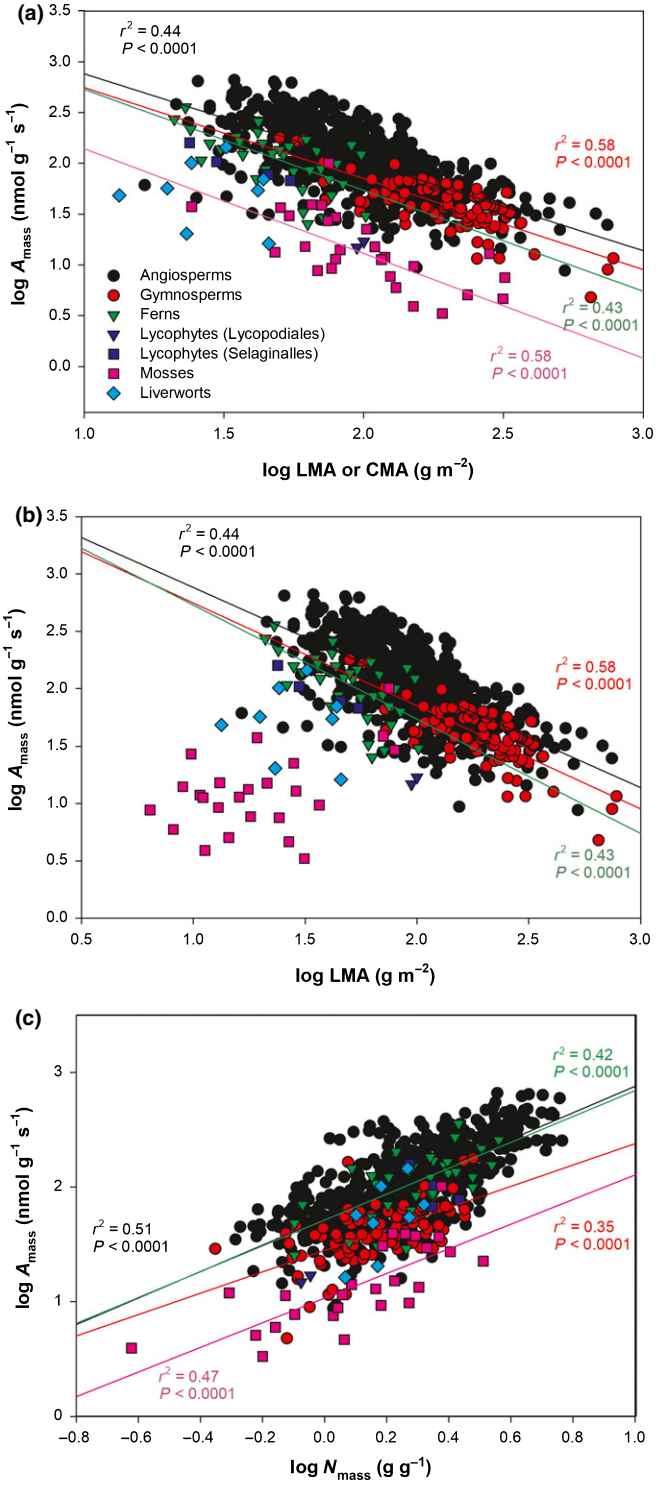

Fig. 8 Mass-based net photosynthesis as a function of (a) leaf dry mass per unit area (LMA), (b) canopy dry mass per unit area (CMA) for mosses and LMA for all other groups and (c), nitrogen content per mass N_{mass}. The axes are $\log_{10}$ scaled, and the data for each plant type were separately fitted by standardized major axis estimation. Common slopes across groups were tested using a likelihood ratio test ((a) $P = 0.19$; (c) $P = 0.51$); intercepts were compared between mosses and other plant groups by calculating the Wald statistic for (a) and (c); see the Materials and Methods section). The intercepts were different with a significance of $P < 0.0001$. Data for plant groups other than lycophytes, mosses and liverwort come from Wright *et al.* (2004), Zhang *et al.* (2015), Tosens *et al.* (2016) and Veromann-Jürgenson *et al.* (2017).

gymnosperms (Veromann-Jürgenson *et al.*, 2017), and from 0.096 μm (Han *et al.*, 2016) to 0.540 μm in angiosperms (Tomás *et al.*, 2013), whereas most mosses had a $T_{cw} > 1$ μm, the highest value being 3.4 μm in *D. freycinetti* (Table S6). Moreover, the values of S_c/S in mosses were among the lowest reported for ferns and spermatophytes (Onoda *et al.*, 2017). These two anatomical traits were strongly correlated with both area- and mass-based net photosynthesis (Fig. 6a,b and c,d, respectively), suggesting that these two traits explain a large part of variability of observed g_{nsd}. In addition, the other anatomical traits of these plant groups with particularly diverse photosynthetic organs (Fig. 4) also pointed to a high CO_2 diffusive resistance. Photosynthetic organs were especially thin in unistratose species, but within the pool of leaf thicknesses reported for tracheophytes in the species with multiple cell layers (Tosens *et al.*, 2012a,b, 2016; Tomás *et al.*, 2013; Xiong *et al.*, 2017; Veromann-Jürgenson *et al.*, 2017; Carriquí *et al.*, 2018; Table S6). The fraction of photosynthetic tissue occupied by intercellular air space f_{ias}, existing only in *M. polymorpha*, lycophytes and in the 'pseudomesophyll' of *Polytrichum* species, also ranged within the values reported for tracheophytes (Tosens *et al.*, 2012a,b, 2016; Tomás *et al.*, 2013). Moreover, all the species studied had a low S_c/S_m ratio (< 0.7), which suggests that many CO_2 molecules do not cross the cell wall specifically where there is a close chloroplast, implying that their CO_2 pathway (and resistance) would be larger. Neither leaf density D_{leaf}, T_{leaf}, T_{cw} nor S_c/S correlated with LMA (for lycophytes, liverworts and *Polytrichum* species) and CMA (for other moss species) (Fig. 5a–d).

Nonstomatal diffusion conductance modelled from anatomical traits g_{nsd_ANAT} was also very low in bryophytes and lycophytes, confirming with a fully independent method the estimations made from Chl fluorescence, g_{nsd_FLU} (Table S8; Fig. S4). The g_{nsd_ANAT} in these plants was low no matter which value was assumed for the cell wall porosity p_{cw}; see Methods S1.

Finally, a g_{nsd_ANAT} limitation analysis was performed to test the relative importance of each liquid-phase limitation. Whereas both cell wall and chloroplast thickness were the major components limiting g_{nsd_ANAT} in the single liverwort species, cell walls imposed on average more than the 65% of g_{nsd_ANAT} limitations in mosses, being three times greater than the limitation imposed by the chloroplast (Fig. 7). In the case of lycophytes, despite their gigantic chloroplasts (Table S7), g_{nsd_ANAT} was also limited mainly by cell walls (Fig. 7). Thus, bryophytes and lycophytes are revealed as the groups presenting the highest CO_2 diffusive resistance, which is mainly driven by extremely high T_{cw} and low S_c/S.

Impacts of anatomically reduced CO_2 diffusion on the LES in bryophytes and lycophytes

The LES is a set of relationships that reflect the physical influence of leaf structure and N content on net photosynthesis (Wright *et al.*, 2004). By characterizing climate-diverse species from around the world and with a wide range of morphologies, we demonstrate that mosses' LES diverges from all major vascular plant groups (Fig. 8). Mosses, as already reported for tropical (Waite & Sack, 2010) and temperate species (Wang *et al.*, 2014, 2017), possess a lower mass-based net assimilation rate A_{mass} for a given structural complexity, either considering the CMA or dry LMA than ferns (Karst & Lechowicz, 2007; Tosens *et al.*, 2016), gymnosperms (Wright *et al.*, 2004; Zhang *et al.*, 2015; Veromann-Jürgenson *et al.*, 2017) and angiosperms (Wright *et al.*, 2004; Fig. 8a, b). Despite sharing slopes ($P = 0.19$), differences within intercepts were highly significant ($P < 0.0001$), supporting the hypothesis for a strong anatomically determined g_{nsd} limitation to photosynthesis in mosses. Moreover, the stronger agreement of moss CMA, instead of LMA, with the other plant groups reinforces the analogous function of the moss canopy with the spermatophyte's leaf first suggested by Proctor (2000) and reported by Waite & Sack (2010). Liverworts had a behavior similar to mosses in the A_{mass}–LMA or –CMA relationship (Fig. 8a) but were aligned with tracheophytes (in the same way as lycophytes) in its A_{area} for a given N concentration (Fig. 8c). Even so, more data from these last two groups are needed to verify this. Similar to Wang *et al.* (2017), for the relationship between A_{mass} and the N concentration per mass N_{mass}, mosses had a lower photosynthesis than the rest of plant groups for the same content of N_{mass}, presenting the same slope ($P = 0.45$) but different intercept ($P < 0.0001$; Fig. 8c). This strongly suggests that biochemical limitation is not the main constraint to A_{mass} and/or that bryophytes allocate comparatively more N than tracheophytes to compounds other than Rubisco, but this is yet to be tested empirically.

Concluding remarks

In summary, this work confirms, using a large number of species, previous evidence that the net CO_2 assimilation capacity of bryophytes and lycophytes is low compared with that of ferns, gymnosperms and angiosperms. Although the absolute values might be viewed with caution due to limitations in the precision of measurements and estimations at these very low rates, several independent lines of evidence suggest that the low photosynthetic capacity exhibited by these species is largely due to low CO_2 diffusion conductance of their tissues, which in turn is largely explained by their anatomy, especially the very thick cell walls and low chloroplast exposure to intercellular air spaces. These characteristics have an effect in the trait relationships of bryophytes (i.e. the LES), so that bryophytes present a lower photosynthetic use efficiency of their leaf and canopy mass areas and N content.

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Author contribution

MC, MR-O, MR-C and JF designed the study; MC, MR-O, TJB, WG, RC, KM, AVP-C, LS, MW and JF conducted the experiments; MC, ÜN, TT and JF performed the analysis and MC and JF wrote the first version of manuscript; all authors contributed to the following versions of the manuscript to produce the final version.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 Maximum velocity of carboxylation ($V_{c,max}$) in relation to electron transport rate (ETR).

Fig. S2 Hypothetical relationships between cell wall porosity and thickness.

Fig. S3 Comparison of g_{nsd_FLU} estimated with the variable J method and modelled from anatomy.

Fig. S4 Limitation analysis on g_{nsd_ANAT}.

Methods S1 Detailed materials and methods.

Notes S1 List of abbreviations for traits.

Table S1 Climate, range of dispersal and microhabitat.

Table S2 Environmental conditions.

Table S3 Measured data available.

Table S4 Average values for diffusional and biochemical photosynthetic characteristics.

Table S5 Agreement between maximum velocity of carboxylation ($V_{c,max}$) obtained from fast and slow A_{area}–C_a curves.

Table S6 Average values for morphological and structural traits.

Table S7 Average values for ultrastructural characteristics.

Table S8 Average values for non-stomatal diffusion conductance modelled from anatomy.

Table S9 Average values for nitrogen content.

Table S10 Sensitivity analysis of the effects of biases in Γ*, α, and R_d on the estimation of g_{nsd_FLU}.

Video S1 Measurement of anatomical foliage traits in two species.

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