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Leaf thermal regulation strategies of canopy species across four vegetation types along a temperature and precipitation gradient

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Abstract

The ecophysiological processes of leaves are more related to leaf temperature (Tl) than air temperature (Ta). Transpiration and leaf physical traits enable plants to maintain Tl within a thermal range. However, it is challenging to quantitatively study leaf thermal regulation strategies, due to the complex interaction between thermal effects of transpiration and leaf physical traits. We utilized a 3-T method that compares Tl, Ta, and Tn (the temperature of non-transpiring leaves) investigate thermal regulation strategies of dominant canopy species in four vegetation types, including a savanna woodland, a tropical rain forest, a subtropical evergreen broad-leaved forest, and a temperate mixed forest. Our results indicate that the difference between Tl and Ta decreased as the site mean temperature increased. Transpirational cooling was strongest in savanna woodland, and decreased from the hottest site to the coldest site. Without transpiration, sun-exposed leaves were consistently hotter under sunshine than air. This physical warming effect increased from the hottest site to the coldest site. We observed leaf area, water content and leaf angle played a significant role in physical thermal regulation. The present research quantitatively measured leaf thermal regulation strategies across a temperature and precipitation gradient, which advances our understanding of how plants adapt to their thermal environments.

KEYWORDS: leaf temperature, leaf traits, physical thermal effect, transpirational cooling, thermal regulation, thermal response

INTRODUCTION

Leaf temperature (Tl) is the direct micro-environment governing plant ecophysiological processes (Gates, 1968; Slot and Winter, 2017), and further determines ecosystem energy, water and carbon budgets (Rey-Sánchez et al., 2017; Sánchez et al., 2009). However, leaf temperatures often deviate from air temperature (Ta). Previous investigation across 62 species have revealed that in a 5℃ environment, leaf temperatures can be elevated by up to 10℃ compared to the surrounding air temperature. Conversely, in a 55℃ environment, leaf temperatures can be approximately 7℃ lower than the ambient air temperature (Michaletz et al., 2015). The temperature difference between leaf and air has also been observed to reached 18.3 ℃ in the Atlantic forest, Brazil (Fauset et al., 2018). Similar temperature deviations from air temperature are also common in inanimate materials such as water or metal due to their distinct physical properties, for example heat capacity, reflectivity, and size. However, these properties of inanimate materials remain constant regardless of the environment. In contrast, plant traits can adapt or acclimate to various environments, enable them to maintain their leaf temperatures within a specific range. Although the time required for traits to change may vary widely, ranging from a few seconds (e.g. stomatal conductance and transpiration) to hundreds of years, plant traits are flexible. The combination of all the physical traits (e.g., morphological traits, optical traits, material properties) and physiological leaf traits (e.g., transpiration) that contribute to maintaining leaf temperatures within the optimal temperature range for photosynthesis is referred as "thermal regulation" (Jones and

Rotenberg, 2011; Monteiro et al., 2016). It includes cooling effects in hot habitats and warming effects in cool habitats. Thermal regulation, thermal tolerance, and thermal avoidance together constitute thermal adaptation strategies of plants.

Thermal regulation capacities of leaves differ among species, and vary with the environment (Fauset *et al*., 2018). According to the regression slope of Tl vs. Ta (β), three types of leaf thermal response have been identified: limited homeothermy ($\beta < 1$), poikilothermy ($\beta = 1$), and megathermy ($\beta > 1$) (Blonder and Michaletz, 2018). Homeothermic leaves maintain Tl below Ta when Ta exceeds a certain threshold, while poikilothermic leaves closely track Ta (Tl = Ta), and megathermic leaves exhibit a faster increase in temperature compared to Ta. Generally, stronger thermal regulation, including both warming and cooling is found under extreme thermal environments (John-Bejai et al., 2013; Körner, 2016; Smith, 1978; Vogel, 2005), while weaker thermal regulation is found in more optimal thermal environments (Drake et al., 2020). However, the mechanisms underlying leaf thermal regulation strategies across different environmental gradients have not been fully explored.

Leaf temperature is determined by a combination of leaf physical and physiological traits and environmental conditions (Campbell and Norman, 1998; Monteith and Unsworth, 2013; Nobel, 2005). Leaf traits related to radiation loading and heat exchange impact leaf temperature. For example, optical traits, leaf size and orientation determine radiation loading (Jones and Rotenberg, 2011; Lambers et al., 1998), while material properties such as water content and density affect heat capacity (Jones, 2014; Lambers et al., 1998); leaf shape and area are related to heat

conductance (g_{Ha}) (Leigh et al., 2012; Muller et al., 2021);Stomatal conductance (g_s) and water vapor transport conductance (g_{va}) influence transpirational cooling (Gates, 2003; Jones and Rotenberg, 2011; Monteith, 1973; Muir, 2019). However, it is challenging to study thermal effects of leaf traits in the field due to the high variability of leaf traits and their interactions (Blonder et al., 2020; Kitudom et al., 2022). For example, small leaves with dark color also have thin boundary layers, which facilitates heat exchange, meanwhile their dark colors also enables them to absorb more radiation. In reality, it is the coordination of multiple leaf traits that improves plant adaptation to the primary stress under its specific environment. Not all leaf traits contribute directly to thermal regulation. Therefore, some previous studies used artificial leaves to quantitatively evaluate thermal effects of leaf traits under controlled environments (Daudet et al., 1998; Fetcher, 1981; Vogel, 2009). Lin et al. (2017) employed a method to quantitatively distinguish thermal effects of transpiration and leaf physical traits *in situ*. This technique, called "3-T method" requires three temperatures: the temperature of a control leaf (Tl), the temperature of a non-transpiring leaf (Tn), and air temperature (Ta). Although the combination of Tl, Tn and Ta has often been used to evaluate water stress, transpiration and stomatal conductance (Jones, 1999; Jones et al., 2018; Jones and Rotenberg, 2011; Qiu et al., 2002), Lin et al. (2017) firstly used it to quantify thermal regulation in the field. By employing this method, researchers can effectively monitor the thermal effects of transpiration and the physical traits of leaves separately, thereby revealing leaf thermal regulation strategies and their response to the natural environment *in situ*.

Upper-canopy species are fully exposed to air and solar radiation. Compared with shaded plants, they are more influenced by radiative heating, turbulent exchange, and longwave radiation loss at night (Miller et al., 2021). Transient “lulls” in wind can cause leaf temperature to rise by > 5 °C in just a few seconds (Vogel, 2005). The highly exposed and fluctuating environment makes canopy leaves more susceptible to temperature extremes. Maximum temperatures of upper-canopy leaves have been shown to exceed photosynthetic thermal optima in several tropical forests (Doughty and Goulden, 2008; Mau et al., 2018; Miller et al., 2021; Pau et al., 2018). In such a fluctuating environment, traits associated with temperature regulation should incur greater selective advantage than in understory conditions. In addition, understory species are distributed in buffered micro-environments due to shading which might be very different from the canopy environment (Vinod et al., 2023). Therefore, canopy species more strongly reflect the interaction between plants and the local environment (Still et al., 2021).

In the present research, we used the 3-T method to study thermal regulation strategies of upper canopy species in a savanna woodland, a tropical rain forest, a subtropical evergreen broad-leaved forest and a temperate mixed forest. Thermal regulation strategies depend on the temperature and water status of the habitat (Fauset et al., 2018; Gates, 2003; Jones and Rotenberg, 2011). We hypothesize that the savanna species mainly depend on leaf physical traits to cool leaves due to limited transpirational cooling under dry conditions; tropical rain forest species can utilize both transpirational cooling and leaf physical traits to avoid high leaf temperatures;

while subtropical forest species exhibit weaker thermal regulation due to limited thermal stress; the species in the temperate forest primarily rely on physical warming to cope with cold stress.

MATERIALS AND METHODS

Study sites and plant species

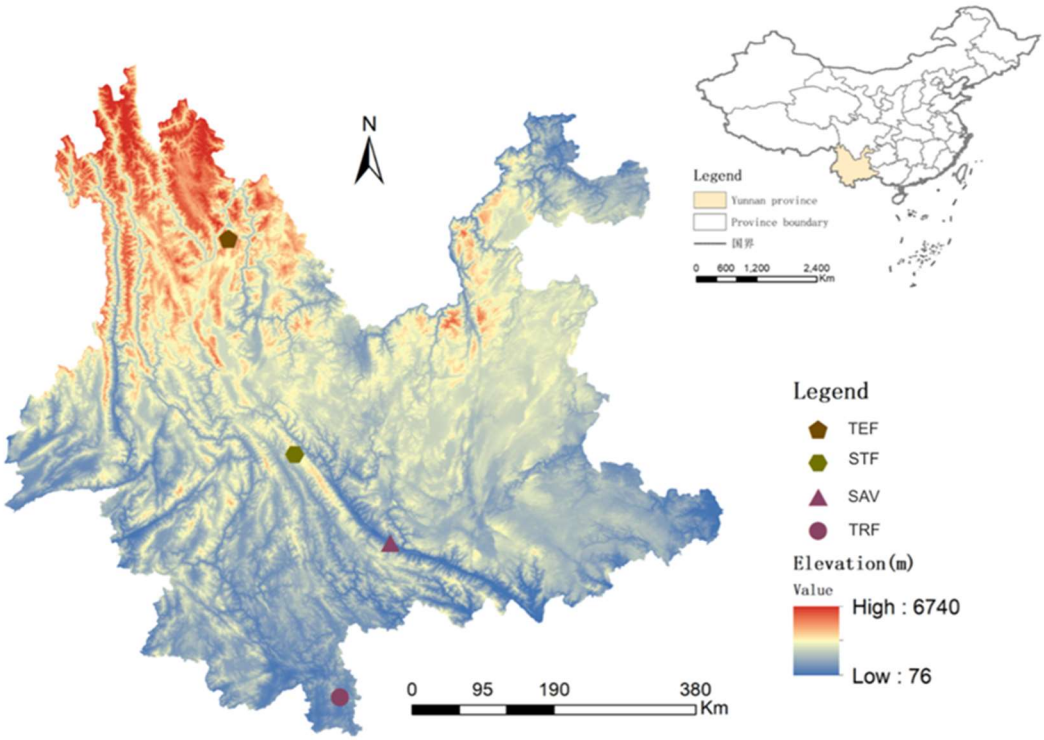

Figure 1 Site distribution.

We selected four vegetation types from the south to the north of Yunnan province, China, including a savanna woodland (SAV), a tropical rain forest (TRF), a subtropical broad-leaved forest (STF), and a temperate mixed forest (TEF) (Table 1 and Fig. 1). Four dominant upper canopy species were chosen in each site. These species covered all the emergent species in TRF, all the canopy species in TEF, the most abundant three species and the sixth most abundant canopy species in STF, and the most abundant three species and the seventh most abundant species that grow

closely and under similar micro-habitat in SAV. Considering data balance across sites, the replicate individuals were determined by the species with the smallest number of individuals, thus three individuals were selected for each species. Detailed information of the species can be found in Table S1.

Measurement of thermal regulation strategies

The 3-T method was used to measure thermal regulation strategies. This method needs three temperatures: leaf temperature (Tl), leaf temperature of non-transpiring leaf (Tn), and air temperature (Ta). The thermal effect of transpiration was calculated by Tl – Tn (Gates, 1968), and thermal effect of leaf physical traits compared with air was calculated by Tn – Ta. Note that transpirational cooling refers to Tn – Tl, which is therefore positive when there is a cooling effect. All the temperatures were measured by T-type thermal couples (TT-T-30-SLE-1000, OMEGA, USA; diameter = 0.25mm). To guarantee the accuracy of temperature measurements, we compared leaf temperature difference (dT) between thermal couple and PT-100 (YAGEO Nexensos GmbH, Germany) in the field. PT-100 is a platinum resistance temperature detector (RTD) that utilizes the electrical resistance of platinum to measure temperature. The accuracy of PT-100 is reported drift of 0 ℃ is 0.04% (0.16 ℃) after 1000 hours at 400 ℃ in the field. The results showed thermocouple temperature measurements are slightly higher, with an average dT of 0.6%. The maximum dT reached 2.7% which happened after noon (Notes S3). Temperatures were recorded by data logger (UX-120-04, HOBO, USA) every one minute from May 13 to May 16 at TRF, May 19 to May 23 at STF, May 25 to May 28 at SAV, June 4 to June 7 at TEF

in 2019. This period was the most severe heatwave in Yunnan province from 1961 to 2019 (Kitudom *et al*., 2022). At TRF and STF, crowns were accessed using canopy crane infrastructure, whereas at the lower canopy sites SAV and TEF, they were accessed from the ground or using a ladder. We used heat-conducting glue to fix the thermal couple head on the adaxial side of the leaves. With this method, thermal couples can be tightly fixed on leaves without impacting on stomata conductance and avoid irradiation effects on the sensor head (Kitudom *et al*., 2022). We put Vaseline on the abaxial side of leaves to get non-transpiring leaves (all the leaves are hypostomatous) (Gates, 1968; Jones, 1999). A thin layer of Vaseline on the abaxial side of the leaf had negligible impacts on leaf physical thermal effects ((Thorpe and Butler, 1977); Notes S1 and S2 for experimental tests and sensitivity analysis of the impact of the Vaseline application). Temperatures of four mature, sun-exposed and healthy control leaves, and two non-transpiring leaves of similar traits to the control leaves were measured. Air temperatures beside these leaves were measured simultaneously with thermal couples on the abaxial sides of leaves to avoid direct solar radiation. Ten-minute average temperatures were used for analysis. Tn might be lower than Tl due to water adhering to the Vaseline surface. This situation often happened at night and in the early morning. However, Tn < Tl also occasionally happened during daytime in TEF, which might be because of weak transpirational cooling. Therefore, a small change of leaf angle, wind and radiation loading of the non-transpiring and the control leaf could induce negative Tn - Tl. We assumed transpirational cooling was zero (Tn = Tl) when Tn < Tl.

198 **Leaf traits measurement**

199 We selected leaf traits that might be related to leaf temperature including
200 morphological, optical, anatomical, physiological traits and material properties (Table
201 2). Leaves for morphological traits measurement were collected adjacent to the leaves
202 for temperature measurements for 8–10 leaves per individual and 3 individuals per
203 species. All the leaves were scanned on a flatbed-scan scanner. Leaf area (Area), leaf
204 perimeter (P), perimeter/area ratio (P/A), leaf length (Length), and leaf width (Width)
205 were analyzed by ImageJ 1.52q based on the scanned image. Leaf angle was
206 measured using the "Measure" app on Apple's iPhone (Apple Inc.). The horizontal
207 position is set at 0 degrees, with the leaf facing downwards, the angle is negative, and
208 the angle is positive when the leaf is facing upwards. Ten leaves of each individual
209 were used to measure reflectivity (R), transmissivity (T), and absorptivity (A) at
210 wavelength between 400nm and 700nm with an integrated sphere connected to a
211 spectrometer (USB2000, Ocean Optics, USA), and greenness with a chlorophyll
212 meter (SPAD-502, Minolta, Japan). Leaf water content (WC) and leaf density was
213 measured by weighing 3–8 leaves (more leaves for low-weight leaves) for each
214 individual. WC was calculated by the ratio of weight difference between fresh and dry
215 leaves to the dry mass (Pérez-Harguindeguy et al., 2013). Leaf fresh mass density
216 (Density.f) and leaf dry mass density (Density.d) were calculated as the ratio of leaf
217 fresh and dry mass to leaf volume respectively (Pérez-Harguindeguy et al., 2013).
218 Leaf volumes were determined by the water displacement method. Four leaves of
219 each individual were used to measure anatomical traits. Leaf thickness (Thickness),

220 the thickness of upper and lower epidermis (Epidermis_up, Epidermis_low), palisade
221 mesophyll (Thickness_palisade) and spongy mesophyll (Thickness_spongy) were
222 measured using paraffin cross section. Paradermal sections were cut from the middle
223 part of the leaf avoiding major veins. Paradermal sections for stomata measurement
224 were boiled in water for 10–15 min, then immersed in a 1:1 mixture of 30% H_2O_2 and
225 glacial acetic acid aqueous solution until being soft and disintegrated, after which we
226 carefully separated the epidermis. Paradermal sections for vein analysis were
227 bleached with 5% NaOH until they became transparent. Paradermal sections for
228 stomata and vein analysis were stained in 1% safranin diluted with ethanol for 15 min
229 before taking photos under a light microscope. Stomatal density was calculated by the
230 number of stomata divided by the area of view. Vein density was calculated by the
231 total length of veins per area. Diurnal patterns in transpiration rate, photosynthesis
232 rate, and stomatal conductance were measured with a Portable Photosynthesis system
233 (LI-6400, LICOR, USA) using a transparent leaf chamber. Temperature, light,
234 relative humidity and CO_2 concentration during the measurements were maintained at
235 ambient conditions (Fig. S1) and not controlled. The flow rate was 500 $\mu mol \cdot s^{-1}$. For
236 each individual, three leaves next to the leaves for temperature measurements, were
237 measured repeatedly from morning to afternoon depending on solar radiation and the
238 availability of the canopy crane at each site (SAV: 8:00–17:00; TRF: 9:20–14:40;
239 STF: 9:30–16:30; TEF: 8:30–17:40). Gas exchange measurements were conducted for
240 two days in SAV, STF and TEF, and one day in TRF due to the unavailability of the
241 canopy crane. The maximum transpiration rate (Tr_{max}), photosynthesis rate (A_{max}) and

242 stomatal conductance (g_{max}) were extracted from the diurnal measurements of leaf gas
243 exchange. These physiological traits might change with environment of measurement,
244 while the trend among species should be stable. Photosynthetic thermal tolerance was
245 measured with a PlanTherm PT100 (PSI, Czech Republic) based on the response of
246 basal chlorophyll *a* fluorescence to temperature (F_o-T curve) with three sun leaves for
247 each individual (details can be found in Kitudom et al., 2022). Leaf segments (2cm×
248 1cm) were heated by water bath from 25 ℃ to 70 ℃. Heating rate was 2 ℃ min^{-1}.
249 T_{crit} was calculated as the intersection of lines extrapolated from the slow and fast
250 rising portions of F_o-T curve (Knight and Ackerly, 2002).

251 **Meteorological measurements**

252 Meteorological data were obtained from the measurements on towers above the
253 canopy in SAV, TRF, and STF. Meteorological data of TEF were obtained from a
254 weather station installed in the open land at a distance of 10 meters from the forest.
255 Air temperature, relative humidity, wind speed, and downward solar radiation (DR)
256 were sampled at 0.5 Hz using CR1000 dataloggers (Campbell Scientific, Inc, USA) at
257 each site. Ten-minute averages were used in the present study. The details of
258 mounting heights and instruments are shown in table S2.

259 **Data analysis**

260 Individual tree averages of leaf temperature, leaf thermal effects and leaf traits were
261 used for analysis. Considering that transpirational cooling only happened during
262 daytime and the variance of nighttime physical thermal effects were small among
263 species in each site and nighttime cooling is more effected by environmental factors

264 and canopy characteristics rather than physiological processes of plants, the following
265 analysis only used daytime values (DR > 100 $w \cdot m^{-2}$).

266 • Patterns of parameters across biomes

267 Differences in leaf temperature, thermal effects of leaf physical traits and
268 physiological traits, and leaf traits among sites were analyzed by multiple
269 comparisons of least significant difference (LSD) (Steel et al., 1997).

270 • Leaf thermal response type

271 We calculated β as the slope of the regression line between Ta and Tl for each species.
272 We then used the "slope. Test" function in R package "smart" to test the difference
273 between β and 1. A β value is significantly smaller than 1 ($P < 0.05$) indicates
274 limited homeothermy; β that is not significantly different from 1 ($P > 0.05$) indicates
275 poikilothermy; and if β is significantly larger than 1 ($P < 0.05$), this species exhibits
276 megathermy.

277 • The relationship between thermal adaptation strategies

278 We used Pearson correlation to analyze the relationships between leaf temperature
279 regulation strategies (transpirational cooling and physical warming during daytime)
280 and photosynthetic thermal tolerance.

281 • The impact of microclimate and leaf traits on thermal regulation strategies

282 To analyze the different impacts of climate on leaf temperature regulation, the
283 correlations between leaf temperature metrics (Tl, dT, physical warming effect, and
284 transpirational cooling) and climate parameters (Ta and DR) were calculated using
285 Spearman's rank correlation. Here, dT is the temperature difference between leaf and

air. Correlation coefficients were expressed as r.Tl_Ta, r.Tl_DR, r.dT_Ta, r.dT_DR, r.physic_Ta, r.physic_DR, r.trans_Ta and r.trans_DR, which were put into a PCA. This separated species out according to their relationships between leaf temperature metrics with Ta and DR, and allowed us to analyze which of these relationships are most important for this separation. This was performed using the "prcomp" function in base R. We further assessed how the position of the species is related to the species' traits. This was performed using "env_fit" in the "vegan" package.

To identify key traits related to transpirational cooling and physical warming during daytime (DR > 100 $w \cdot m^{-2}$), Bayesian mixed effects models were used. For the model examining the relationship between the maximum transpiration rate and transpirational cooling (trans), the fixed effect was the maximum transpiration rate (Tr_{max}) and the random effects were site and species (Eq. 1).

$$\text{trans} = Tr_{max} + 1|\text{Site} + 1|\text{Species} \quad (1)$$

In the model exploring the relationship between the physical traits and physical warming (physic), many physical leaf traits could impact physical warming. To avoid high correlated traits in the regression model, these traits were categorized into four groups: morphological traits, optical traits, material properties, and anatomical traits. Pearson correlation was used to identify which traits that had significant correlations with leaf physical warming. The traits with the strongest or significant correlations with physical warming in each group were selected. We further checked collinearity among these traits using pairwise Pearson correlation. If the correlation coefficient was higher than 0.7, one of the two traits was removed. The retained leaf traits (Angle,

P, Greenness, WC, Density.d, Vein density and Palisade) were set as fixed effects for the full model (Eq. 2), with site and species serving as random effects. The fixed effects were center scaled to a mean of 0 with standard deviation of 1. According to the correlation between leaf angle and physical warming, the absolute values of leaf angle had a higher correlation with physical warming, therefore we used the absolute values of leaf angle.

physic = |Angle| + P + Greenness + WC + Density.d + Vein density + Palisade + 1|Site + 1|Species (2)

We constructed linear mixed regression models in a Bayesian framework using R package "brms" (Burkner, 2017). We fit models with student_t priors for all the coefficients, because the sample size was small and the population variance was unknown. Four Markov Chain Monte Carlo (MCMC) chains were used to sample from the posterior distribution of the regression parameters for each model, with 3000 iterations per chain. Half of the iterations were used for warming up. Chains convergence was diagnosed by Rhat values equal to 1. For the full model of the association of leaf physical traits to physical warming, no coefficient was significant. To identify the best model, we dropped traits from the full model one by one, and used WAIC values for model selection. Conditional R^2 and Marginal R^2 were calculated using the r^2 function in the "performance" R package (Lüdecke et al., 2021). Effect size was calculated by the following equation (Le Provost et al., 2020):

$$\text{Effect size} = \frac{\alpha_i}{\sum_1^i \alpha} mR^2 \quad (3)$$

Where α_i is the coefficient of the fixed effect i, mR^2 is the marginal R^2 of the model. If the model had no random effects, mR^2 equals the R^2 of the model.

We found a non-linear relationship between leaf area and leaf physical warming. To investigate this relationship, we separately analyzed two ranges of leaf area (< 50 cm^2 and ⩾ 50 cm^2) using Pearson correlation. In addition, we employed Pearson correlation was used to examine the relationship between leaf traits and microclimate parameters with leaf physical warming at each site. Correlations were considered significant at $P < 0.05$.

RESULTS

Leaf temperature patterns across and within sites

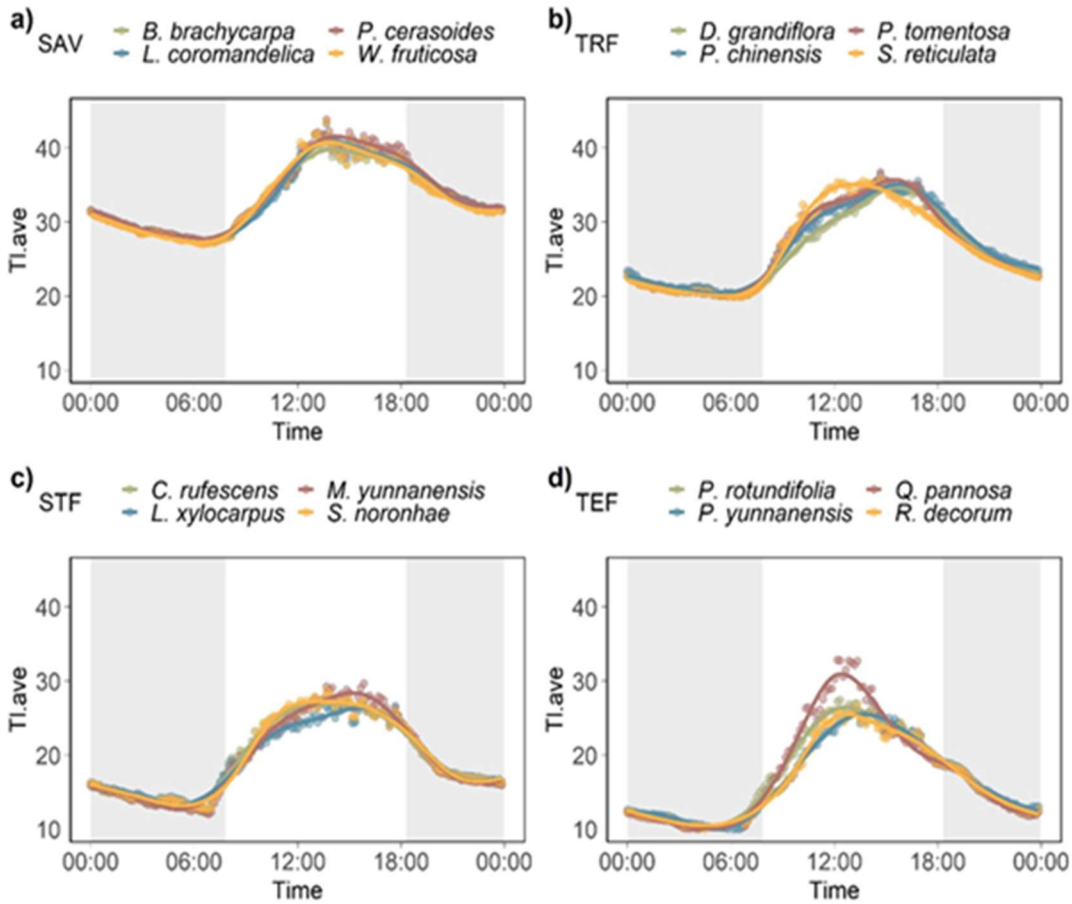

Figure 2 Diurnal leaf temperatures (10 min average). Shading areas indicate nighttime. SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temp mixed forest.

Leaf temperatures increased from TEF to SAV. The minimum leaf temperatures ranged from 8.4 ± 0.11 °C in TEF to 26.2 ± 0.11 °C in SAV. The maximum leaf temperatures ranged from 33.3 ± 2.07 °C in TEF to 46.0 ± 0.51 °C in SAV (Table 3). Daily leaf temperature ranges of TRF and TEF species were higher than STF and SAV species (P = 0.003). Of all the species, *Q. pannosa* in TEF had the highest daily leaf temperature range (25.9 ± 1.85 °C), and *B. brachycarpa* in SAV had the lowest daily leaf temperature range (16.4 ± 0.52 °C) (Fig. 2). The maximum leaf

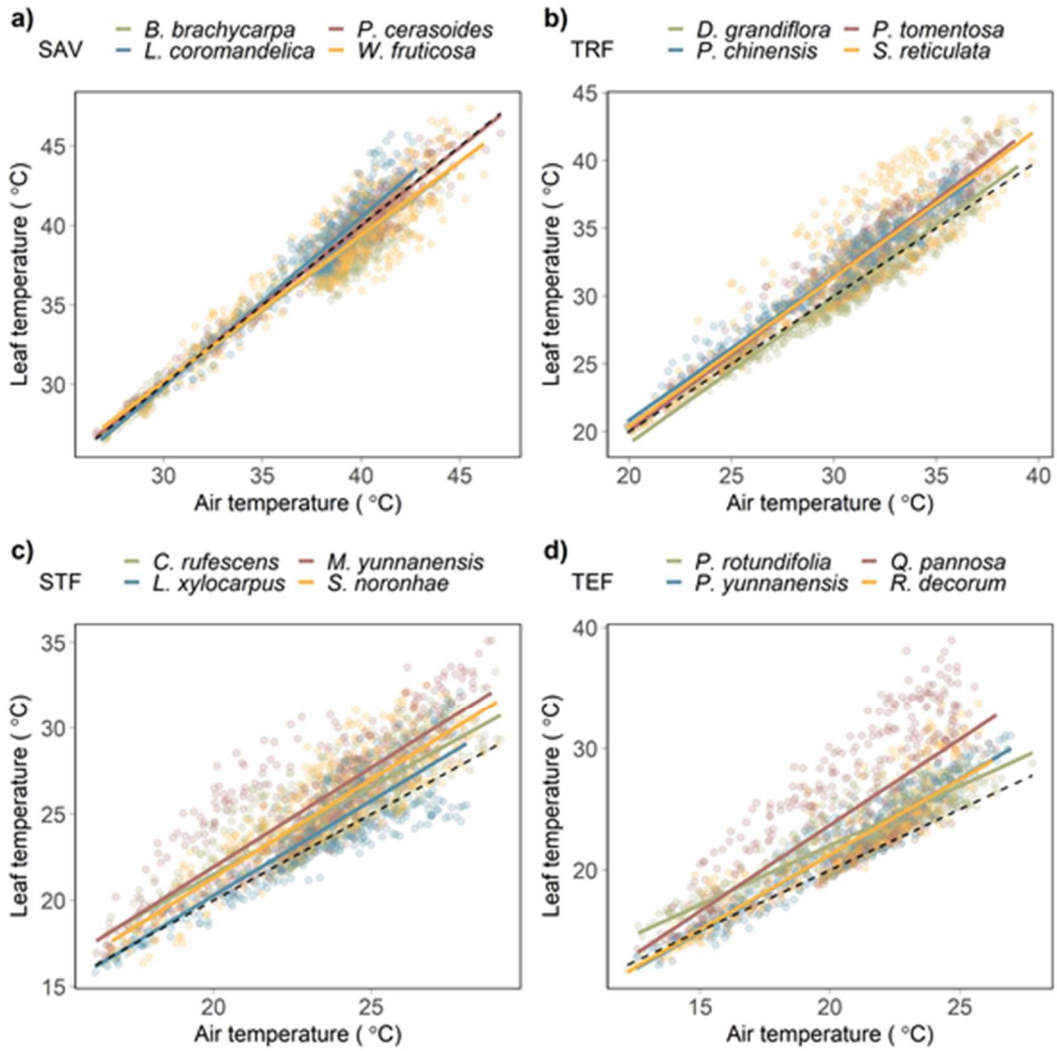

Figure 3 Linear regression between air temperature and leaf temperature. The slope of the dashed line is 1. SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest.

348 temperatures also varied among species within sites. Within each site, the maximum
349 leaf temperature variances among species were 1.2 °C, 1.8 °C, 2.9 °C and 13.6 °C in
350 SAV, TRF, STF, and TEF respectively.

351 Leaf temperatures of SAV species were closest to air temperature. Site mean
352 temperature difference between leaf and air (dT) decreased with site mean
353 temperature ($P < 0.001$) (Table 3). SAV species *P. cerasoides* and *W. fruticosa*
354 exhibited poikilothermic characteristics ($\beta = 1$); SAV species *B. brachycarpa* showed
355 limited homeothermy ($\beta < 1$); and all the other species displayed megathermy ($\beta > 1$)
356 (Fig. 3). Although β of TEF species *P. rotundifolia* was below 1, its leaf temperatures
357 were consistently higher than air temperature.

358 **Thermal regulation strategies across and within sites**

359 Compared with air temperature, physical traits had warming effects on leaves during
360 daytime and cooling effects during nighttime (Fig. S2). All the species showed the
361 strongest transpirational cooling before or around the time of peak air temperature,
362 except for *L. coromandelica* in SAV (Fig. S3). The physical daytime warming and
363 nighttime cooling effects were positively correlated across sites (Pearson correlation =
364 0.74, $P = 0.001$), however nighttime cooling was very weak and differences among
365 species were small. Thus, the following analysis only includes physical warming and
366 transpirational cooling during daytime (DR > 100 $w \cdot m^{-2}$). Generally, the plants in the
367 hotter sites exhibited stronger transpirational cooling and less physical warming. For
368 three of the SAV species (*B. brachycarpa*, *W. fruitcosa*, and *P. cerasoides*) and one
369 TRF species (*D. grandiflora*), the main thermal regulations were transpirational

370 cooling. Among them, *B. brachycarpa* and *W. fruitcosa* had the strongest
371 transpirational cooling of all the species (Fig. 4a). In contrast, the species in the cold
372 sites tended to have limited transpirational cooling, with *P. rotundifolia* and *P.*
373 *yunnanensis* in TEF forest showing the weakest transpirational cooling. Physical
374 warming dominated thermal regulation strategies for the species in TRF, STF and
375 TEF, except for *D. grandiflora* in TRF (Fig. 4a). The contribution of physical
376 warming to thermal regulation increased from the hot sites to the cold sites. TEF
377 species *Q. pannosa* had the strongest physical warming (Fig. 4b).

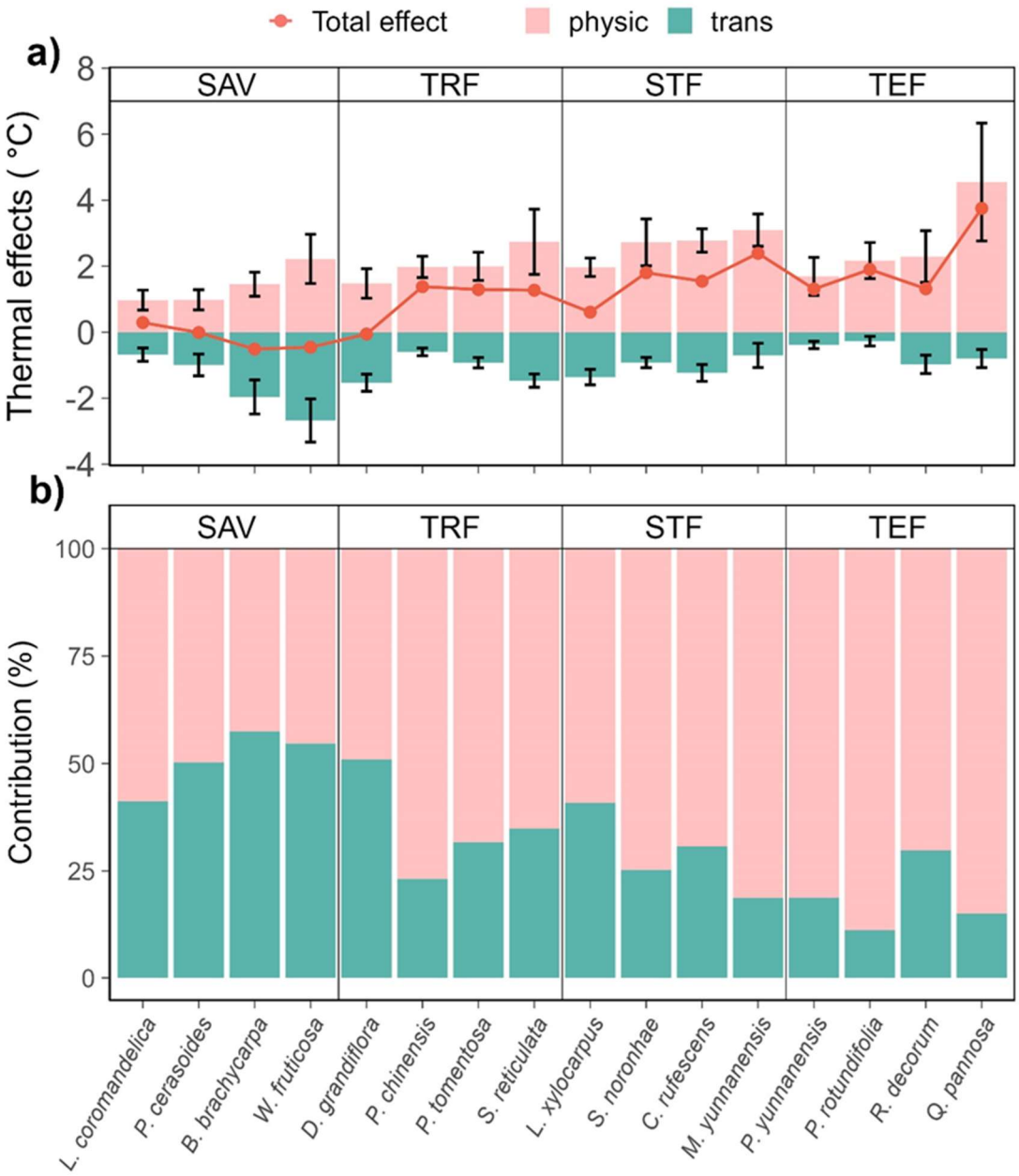

Figure 4 Leaf temperature regulation strategies. a). Thermal effects of transpiration and leaf physical traits during daytime; b). The contribution of transpirational cooling and leaf physical warming to the temperature difference between leaf and air. SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest.

The impact of microclimate and leaf traits on thermal regulation strategies

The correlation of leaf temperature metrics (Tl, dT, transpirational cooling, and physical warming) with Ta and DR separated species out. PC1 explained 39% and

382 PC2 explained 29% of the variance. PC1 was dominated by the positive relationship
383 between Tl and DR, and the negative relationships between transpirational cooling
384 and both Ta and DR. Species with high scores on this axis (*P. rotundifolia*, and *M.*
385 *yunnanensis*) had stronger positive relationships of Tl with DR, dT with Ta and DR,
386 and negative relationship of transpirational cooling with Ta and DR, hence displayed
387 less transpirational cooling and high leaf temperature under hot and bright conditions.
388 Species with low scores on this axis (*P. cerasoides*, *W. fruticosa*, and *B. brachycarpa*)
389 had stronger positive relationships of transpirational cooling with Ta and DR, and
390 therefore displayed stronger transpirational cooling under hot and bright conditions,
391 and accordingly, the leaf temperature did not increase strongly under increasing light
392 intensity. PC2 was dominated by the positive relationship between physical warming
393 and DR. The species with high scores on this axis (*P. tomentosa*, and *P. chinensis*) had
394 stronger positive relationships between physical warming and DR, therefore displayed
395 more physical warming under bright conditions. Species with low scores on this axis
396 (*P. rotundifolia*, and *L. xylocarpus*) showed weaker positive relationships between
397 physical warming and DR, therefore displayed less physical warming compared with
398 other co-existing species under bright conditions (Fig. 5).

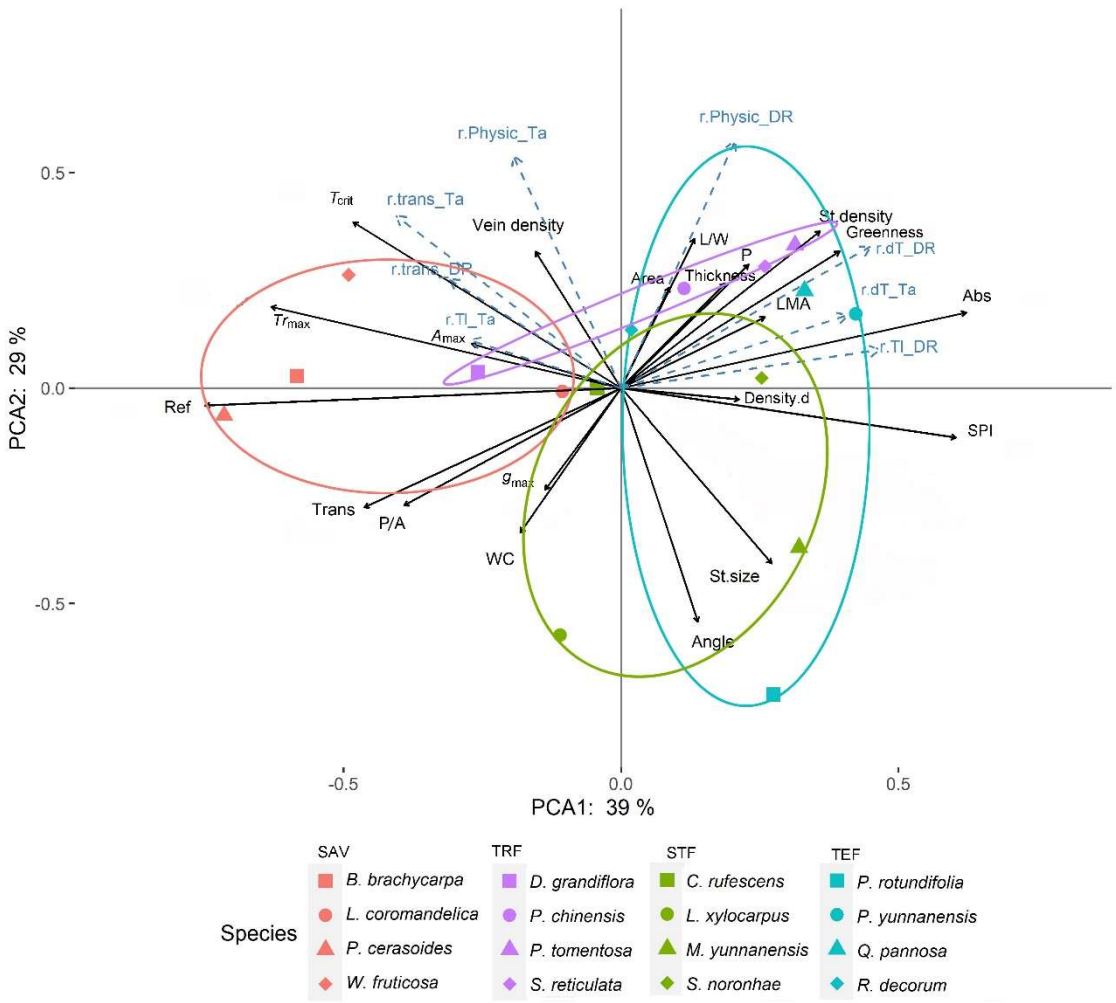

Figure 5 Principal component analysis of the relationship between thermal regulation strategies and climate factors and leaf traits. r.trans_DR, Pearson correlation coefficient between transpirational cooling and downward solar radiation (DR); r.trans_Ta, Pearson correlation coefficient between transpirational cooling and air temperature (Ta); r.physic_DR, Pearson correlation coefficient between physical warming and DR; r.physic_Ta, Pearson correlation coefficient between physical warming and Ta; r.dT_Ta, Pearson correlation coefficient between temperature difference between leaf and air (dT) and Ta; r.dT_Ta, Pearson correlation coefficient between dT and DR; r.Tl_DR, Pearson correlation coefficient between leaf temperature (Tl) and DR; r.Tl_Ta, Pearson correlation coefficient between Tl and Ta. SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest.

399

With reference to plant traits, PC1 was dominated by gas exchange. Species with low values canceled out their heating with transpirational cooling (Fig. 4b), potentially giving them a photosynthetic advantage. These species also had high reflectance. PC2 was positively related to leaf size (Area and L/W), greenness and stomatal density, while negatively related to WC and g_{max} (Fig.5). Therefore, large leaves had stronger physical warming under bright conditions, however, this warming effect can be balanced by WC. The maximum stomatal conductance was coupled with leaf shape and water content (Fig. 5).

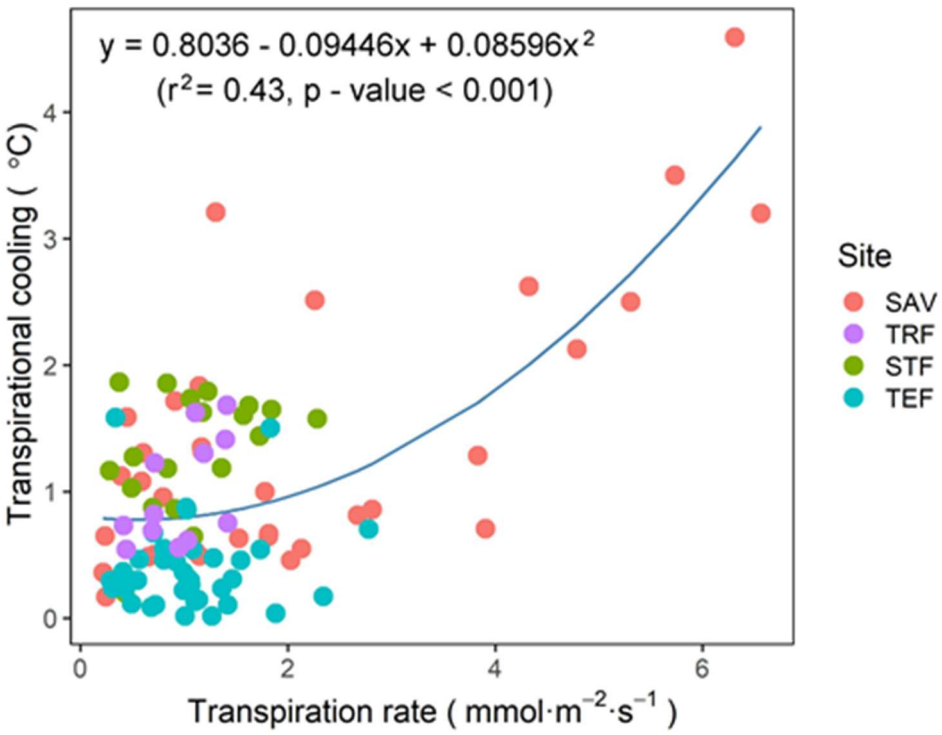

Figure 6 The relationship between transpirational cooling and transpiration rate. SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest.

Bayesian linear mixed regression showed that the marginal R^2 between the maximum transpiration rate and transpirational cooling was 0.461. Instantaneous

410 transpiration rates and transpirational cooling presented a quadratic relationship,
411 however, the relationship was weak when the transpiration rate was below 2.5
412 $mmol \cdot s^{-1} \cdot m^{-2}$ (Fig. 6). The best Bayesian mixed regression model showed that only
413 WC or Angle had significant negative relationships with physical warming effects,
414 with a marginal R^2 0.213 and R^2 0.114 respectively. There was a significant positive
415 correlation between leaf areas and physical warming effects for leaves smaller than 50
416 cm^2 (Pearson correlation = 0.52, P = 0.005), whereas the correlation turned negative
417 for leaves larger than 50 cm^2 (Pearson correlation = -0.74, P = 0.03).

418 The leaf traits and microclimate parameters that had high correlation with leaf
419 physical warming differed among sites. The significantly correlated leaf traits and
420 microclimate parameters were WC, optical traits (Trans, Ref, Abs and Chl),
421 physiological traits (Tr_{max}, A_{max}, and g_{max}), Vein density and Ta_{max} in SAV; WC, L/W,
422 leaf physiological traits (Tr_{max}, A_{max}, and g_{max}, T_{crit}), Epidermis_up,and Ta_{max} in TRF;
423 leaf material property (density.d and WC) and SPI in TEF. No significant correlations
424 between leaf traits and microclimate parameters and leaf physical warming were
425 found in STF (Fig. S4).

426 **The relationship between thermal adaptation strategies**

427 Transpirational cooling and physical warming effects showed positive correlation
428 across sites, but these correlations were significant only for SAV species within sites
429 (Pearson correlation = 0.96, P = 0.04) (Fig. 7a). Thermal tolerance was negatively
430 correlated with physical warming effects across sites (Pearson correlation = - 0.31, P
431 = 0.03), while a significant positive correlation was found for TRF species (Pearson

correlation = 0.67, P = 0.02) (Fig. 7b). Photosynthetic thermal tolerances increased with transpirational cooling, asymptoting when thermal tolerance reached 46 °C (Fig. 7c). All four SAV species were deciduous, and shed leaves during dry season, which enables them to avoid heat stress when water is limited. Therefore, thermal regulation, thermal tolerance and thermal avoidance support thermal adaptation of SAV species together. In the two hot forests, thermal tolerance was positively correlated to leaf temperature (Pearson correlation = 0.77, P = 0.003 in SAV, and Pearson correlation = 0.7, P = 0.02 in TRF) (Fig. 6d-e).

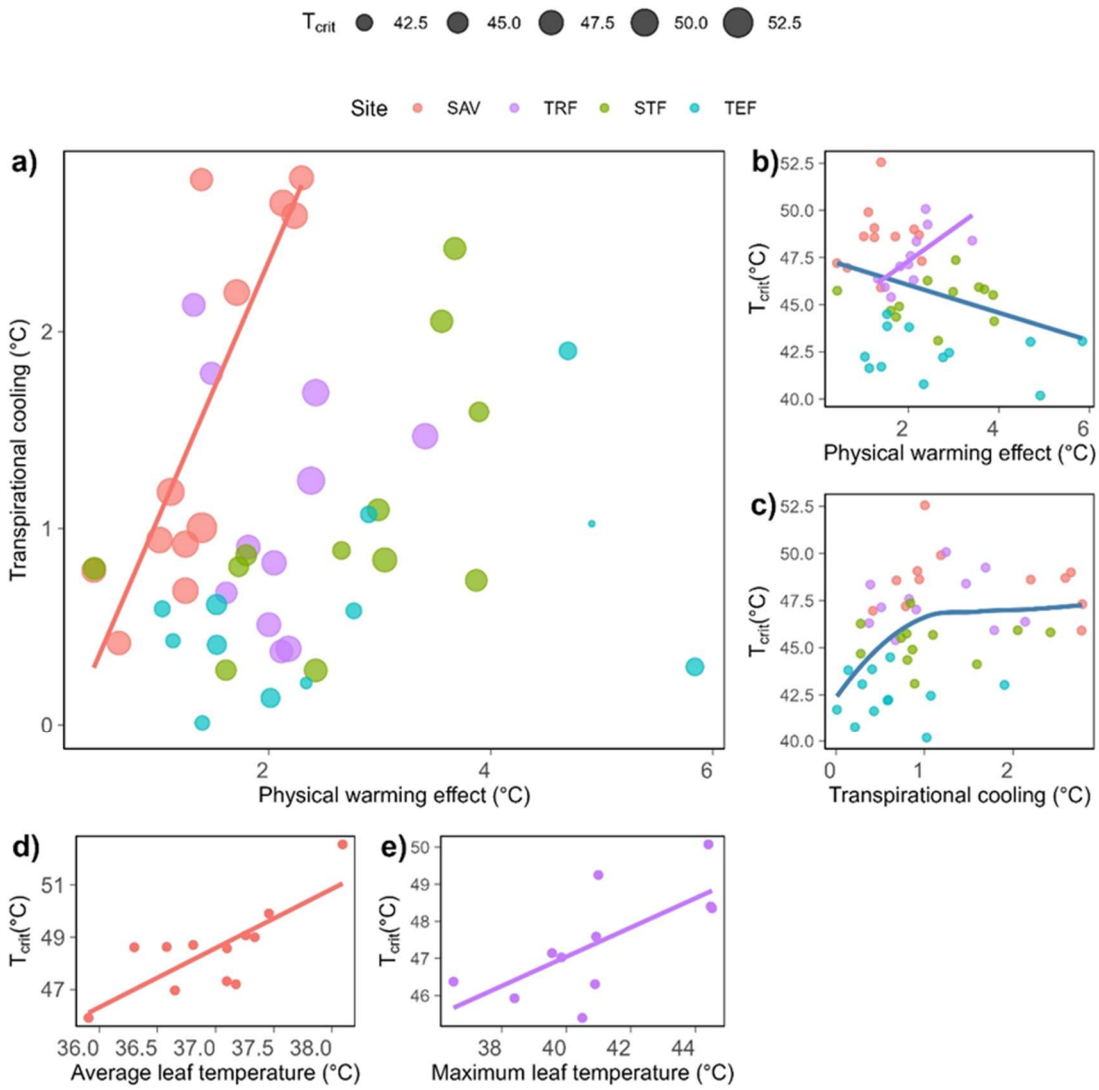

Figure 7 The relationship between thermal adaptation strategies. T_{crit}, photosynthetic thermal tolerance; SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest.

DISCUSSION

Thermal regulation strategies across a temperature and precipitation gradient

Transpirational cooling and physical warming effects of leaves varied with vegetation types along a temperature and precipitation gradient. The first two hypotheses that leaf physical cooling dominates leaf regulation strategies for the savanna species, and that both transpirational cooling and leaf physical traits are important for leaf cooling for the tropical rain forest species, were not fully supported by our results. Instead, transpirational cooling prevailed in thermal regulation of SAV species, in addition to leaf physical traits to reduce physical warming. TRF species presented moderate transpirational cooling and physical warming. The hypotheses regarding thermal regulation strategies of STF and TEF species were supported by our results.

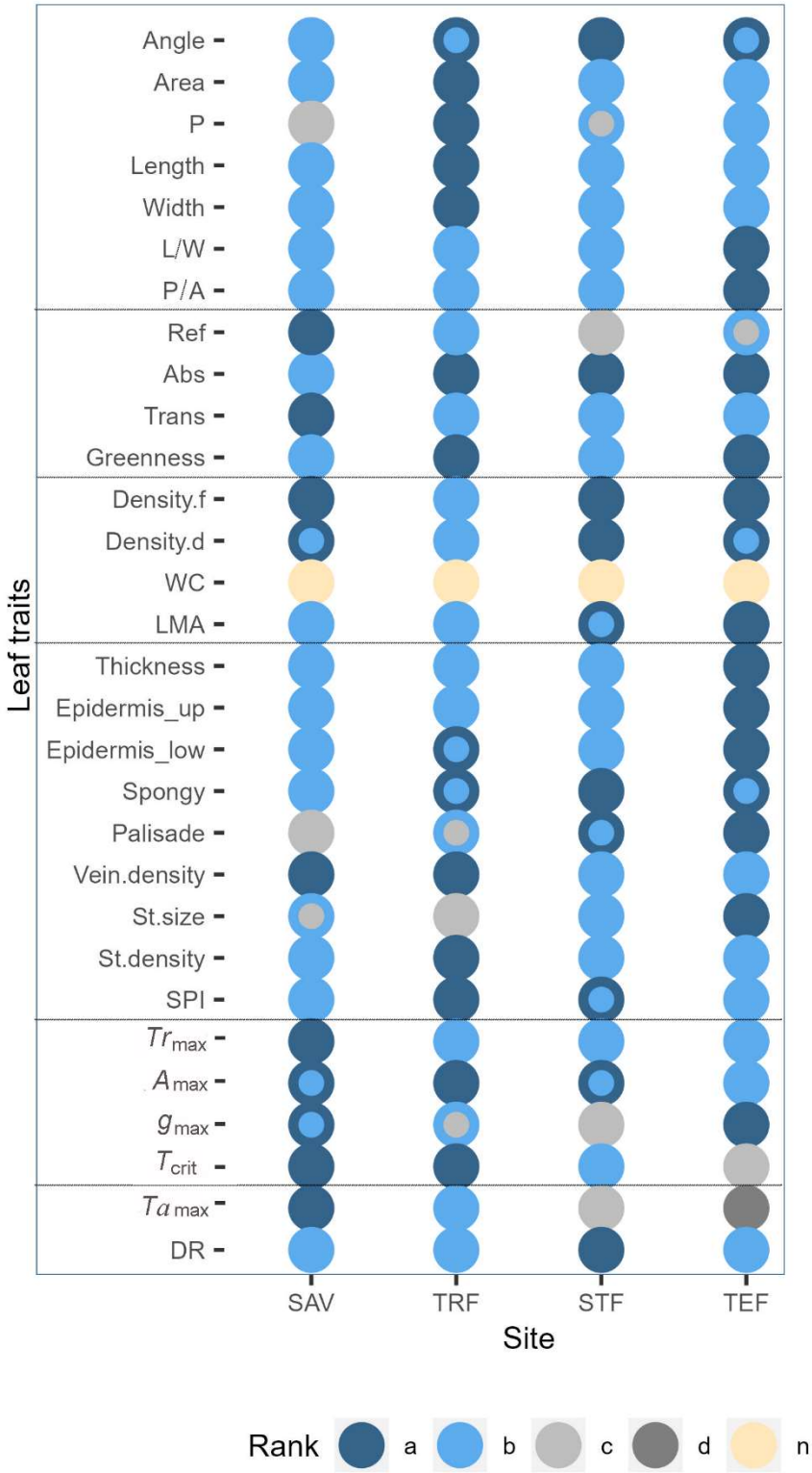

Figure 8 Multiple comparison of leaf traits and microclimate parameters among sites. SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest. Different color means significant difference. The same color means no significant difference. Values decreased from a to d.

454

455 The results showed that plants in the hot environment mainly relied on transpirational
456 cooling to avoid high leaf temperatures (Fig. 4). An increasing number of studies have
457 reported increased transpiration rates at high temperature (Crawford et al., 2012; Lin
458 et al., 2017; Sadok et al., 2021; Slot et al., 2016; Urban et al., 2017), even under
459 drought conditions (Aparecido et al., 2020; Smith, 1978; Urban et al., 2017).
460 Transpirational cooling may thus be even more important in arid and hot
461 environments due to its high cooling efficiency and its greater plasticity than leaf
462 physical traits. High VPD in a dry environment can facilitate transpirational cooling
463 as long as the stomata remain open. Although high thermal tolerance can partially
464 compensate for weak transpirational cooling, we did not find this pattern in our
465 research. On the contrary, both transpirational cooling and thermal tolerance increased
466 with environment temperature until thermal tolerance reached a saturation point (Fig.
467 7c). In addition, reducing physical warming is necessary for the plants in hot-dry
468 environment. SAV species had the lowest absorptivity and the highest reflectivity (Fig.
469 8), so that they can alleviate radiation loading, and hence showed low values of
470 physical warming (Fig. 4a). In addition to thermal regulation, species in the hot sites
471 (SAV and TRF) also had high photosynthetic thermal tolerance (Kitudom et al. 2022),
472 which would enable them to operate within their thermal safety margin. Most species
473 in SAV are deciduous species. On one hand, shedding leaves during the dry season
474 can further avoid heat damage (Zhang et al., 2012). On the other hand, deciduous
475 species usually had higher water and carbon exchange rate than evergreen species, so
476 that they can grow fast during the growing season and reduce leaf temperature with

477 high transpiration rates (Tomlinson et al., 2013). This demonstrates that plants can
478 utilize multiple methods to alleviate heat stress in extremely hot environments,
479 therefore there is no trade-off between thermal regulation, thermal tolerance and
480 thermal avoidance.

481 In cooler forests, the primary stress may shift from heat to other elements such
482 as coldness, light, or herbivory. Consequently, the strategies for adapting to heat
483 become weaker or shift towards cold adaptation. For instance, the offset of physical
484 warming through transpirational cooling diminishes (Fig 7a), and the relationships
485 between thermal tolerance and leaf temperatures vanish in cool forests (Fig 7d and e).
486 TEF species have developed mechanisms to increase leaf temperature to adapt to low
487 temperatures. Transpiration always cools leaves; generally, only leaf physical traits,
488 except for thermogenesis, can have warming effects. Accordingly, physical warming
489 dominated thermal regulation for the species in cold regions. Take *Q. pannosa* as an
490 example, it has the lowest water content and high absorptivity and density, as well as
491 being covered by dense brown trichome on the abaxial side of the leaf. As a result, *Q.*
492 *pannosa* showed the strongest physical warming among all the species. In addition to
493 leaf traits at the leaf level, some other traits at branch and canopy level also contribute
494 to leaf temperature regulation. For instance, the emergent trees in TRF promote
495 convection compared to a more even canopy; most of the TEF species have short
496 petioles and clustered leaves, which increases the thickness of the insulating boundary
497 layer (Michaletz and Johnson, 2006; Smith and Carter, 1988). For the species without
498 specific traits to resist the cold, shedding leaves during winter is a final solution. The

499 ambient temperature in STF is cool, with few extreme temperatures, therefore we did
500 not find any leaf physical trait significantly related to physical warming (Fig. S4).
501 Even so, the dense and even canopy at STF could provide a heat buffer to extreme
502 temperatures. In brief, plants under extreme thermal environment can utilize all means
503 to optimize performance and survive. Integrating studies at leaf, branch and canopy
504 levels can reveal the mechanisms for plant adaptations to the thermal environment.

505 **Leaf regulation strategies among species**

506 Even under the same environment, plants might adopt different leaf thermal
507 regulation strategies. Pioneer species typically show more active metabolism (Bazzaz,
508 1979), hence stronger transpirational cooling. In SAV, *W. fruticosa* and *B.*
509 *brachycarpa* are shrubs. They have much higher photosynthetic and transpiration
510 rates, shorter leaf life span (Zhang, 2007; Zhang et al., 2019) and high branch die
511 back ratio compared with the other two tree species (Chen et al., 2021). They showed
512 strong transpirational cooling. To balance transpirational cooling and water shortage,
513 *W. fruticosa* develops few small leaves; *B. brachycarpa* folds leaves under strong
514 solar radiation (Crawford et al., 2012; Lin et al., 2017). Blonder & Michaletz (2018)
515 and Blonder et al. (2023) proposed that stomatal optimization models should consider
516 additional optimization criteria related to avoiding thermal mortality under extreme
517 hot environment. Generally, photosynthesis and transpiration are coupled because
518 both CO_2 and water vapor enter and exit through the stomata. However, under
519 extreme high temperature, transpiration might increase regardless of photosythesis
520 (Drake et al., 2018; Feng et al., 2023; Urban et al., 2017). *W. fruticosa* and *B.*

521 *brachycarpa* represented low water use efficiency when exposed to high temperatures
522 (Fig. S5), indicating that they may adjust their stomata to prioritize leaf cooling over
523 carbon gain. Plants from various functional groups can employ a wide range of water
524 use strategies (Aparecido et al., 2020; Bueno et al., 2019; Gong et al., 2023). The
525 other two SAV species adopt more conservative water use strategies. The TRF
526 species *D. grandiflora* presented similar thermal regulation strategies to SAV species,
527 which involve high levels of transpirational cooling. However, *D. grandiflora* showed
528 higher water use efficiency during the daytime (Fig S5), suggesting that it may adjust
529 its stomata to maximize carbon gain instead. This species is characterized by
530 unusually large and evergreen leaves which had a disadvantage in heat dissipation.
531 Nonetheless, the high transpiration rate accompanied by high photosynthesis rate and
532 large leaves, benefit the maximization of carbon gain, enabling *D. grandiflora* to
533 quickly reach the canopy as a pioneer tree (Mo et al., 2013). In addition, heat stress in
534 TRF was not as strong as it is in SAV, allowing growth to be prioritized over avoiding
535 heat stress. Although *D. grandiflora* had the largest leaf size and absorptivity, its
536 physical warming was weakest among TRF species. Self-shading, more vertical leaf
537 angles, and high water content might play important roles in reducing and buffering
538 leaf temperature.

539 **The relationship between thermal response and thermal regulation**

540 Energy balance theory predicts that limited homeothermy ($\beta < 1$) occurs when
541 stomatal conductance is high and convective resistance is low; poikilothermy ($\beta = 1$)
542 occurs when convective resistance is low; megathermy ($\beta > 1$) occurs when

microclimate or trait parameters co-vary in certain ways with Ta, e.g. when incident radiation or relative humidity increase with Ta (Blonder and Michaletz, 2018). However, the relationships between β and the parameters of leaf traits and environment are too complex to be simulated by a simple model. We can evaluate β from the perspective of thermal regulation. When transpirational cooling is stronger than physical warming, plants present homeothermy; when transpiration cooling equals physical warming, plants present poikilothermy; when transpirational cooling is weaker than physical warming, plants present megathermy. In the present study, most species were megathermic; only two poikilothermic (*P. cerasoides* and *W. fruticosa*) and one limited homeothermic species (*B. brachycarpa*) were found in SAV. Our results suggest that plants present limited homeothermy at the biome scale, as cooling effects were stronger in hotter environments and warming effects were stronger in colder environments. However, at the species level, megathermy is more typical for sun leaves in field conditions, which is in accordance with the finding from Blonder & Michaeletz' leaf energy balance model (Blonder & Michaeletz, 2018). A growing number of studies reports megathermy of sun leaves under sunshine (Fauset et al., 2018; Still et al., 2022). Air is almost transparent to solar radiation, while leaves can absorb more radiation than air, therefore thermal effects of leaf physical traits always have a warming effect under solar radiation, if there is convective resistance. Only when leaves are small and under strong wind, convective resistance becomes insignificant (Muller et al., 2021). However, this situation was not common under field conditions in our study. Reducing solar radiation loading is indeed another

565 mechanism to alleviate physical warming. For example, the desert plant *Welwitschia*
566 *mirabilis* achieves relatively low leaf temperature by high reflectivity and casting
567 shadow above the ground (Schulze et al., 1980). Although high reflectivity and low
568 absorptivity can reduce radiation loading, these factors are unlikely to reduce β below
569 1 without transpiration.

570 The limited homeothermy of *B. brachycarpa* can be a result of its high stomatal
571 conductance and small leaves (low convective resistance), and its capacity to fold
572 leaves to avoid radiation loading. Although *W. fruticosa* also had high stomatal
573 conductance, its strong physical warming balanced the cooling effect of transpiration,
574 therefore it presented poikilothermy. *P. cerasoides* and *L. coromandelica* had low
575 absorptivity, relatively small leaves, and more vertical leaf angles which can reduce
576 physical warming. However, transpirational cooling of *P. cerasoides* was stronger
577 than *L. coromandelica*, thus *P. cerasoides* presented poikilothermy, while *L.*
578 *coromandelica* presented megathermy. In TEF, wind speed was the highest among the
579 four sites. *P. rotundifolia*, which had $\beta < 1$, has long petioles. They can swing with
580 wind and leaf angle becomes steeper under high temperature, thus convection and
581 reducing radiation loading might be the main causes of low β for *P. rotundifolia.*

582 **3-T method for studying thermal regulation**

583 The 3-T method provided an effective and convenient way to study thermal regulation
584 strategies. It can be used to continuously monitor transpirational cooling and physical
585 thermal effects in the field, and it enables us to disentangle the potentially interacting
586 effects of transpiration and leaf physical traits. This method is not restricted to

application at the leaf level; it can also be used at the stand or community level. The development of technology for achieving non-transpiring leaves is ongoing. Coating leaves with Vaseline is a traditional way (Lin et al., 2017; Thorpe and Butler, 1977; Wallace and Clum, 1938; Zhang et al., 2020). The main artifact of Vaseline coating is the changes of the boundary layer (see Notes S1), which means that the coating must be applied thinly and evenly ($\leqslant 3$ mg • cm^{-2}). Developing new materials and using high-precision modeling to calculate Tn can further improve the accuracy of the 3-T model.

There are some notes for the 3-T method. First, the leaf with Vaseline must have similar leaf physical traits to the control leaf to minimize the influence on leaf physical thermal effects; Second, if there was condensation or rain on leaves, water would be retained longer on the Vaseline surface than leaves. Tn might be lower than Tl when control leaves were dry while the Vaseline coated leaves were wet; Third, the high temperature of Tn might damage leaf, the damaged leaf should be replaced in time.

CONCLUSION

The present research used 3-T method to study thermal regulation strategies of leaves along a temperature and precipitation gradient. We found higher transpirational cooling in hotter sites and stronger physical warming in cooler sites. The results highlight the key role of transpirational cooling in hot sites, even in an arid region. Although leaf physical traits can relieve heat damage, no physical traits at the leaf level can reduce leaf temperature equal to or below air temperature under solar

radiation. Among leaf physical traits, water content, leaf area and leaf angle play significant role in regulating leaf physical thermal effects. The present research revealed a relatively comprehensive scenario of leaf regulation strategies under four distinct environments, thereby enhance our understanding of how plants adapt to thermal environments.

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Table 1 Site information (Kitudom et al., 2022)

Site	Abbreviation	Location	Elevation (m)	MAP (mm)	MAT (°C)	Ta_{max} (°C)	Ta_{min} (°C)	RH (%)	Canopy height (m)
Savanna woodland	SAV	23°28′N, 102°10′E	481	733	25.0	45.1	26.5	62 (53)	4-6
Tropical rain forest	TRF	21°22′N, 101°34′E	704	1415	22.7	38.6	18.6	80 (65)	>50
Subtropical broad-leaved forest	STF	24°32′N, 101°02′E	2501	1931	11.8	28.7	12.2	81 (60)	25-30
Temperate mixed forest	TEF	27°00′N, 100°13′E	3240	1300	8.7	26.8	9.1	65 (57)	25-30

Ta_{max} and Ta_{min}, the maximum and minimum air temperature above the canopy during measurement, averaged by all the measure points on the canopy; MAP, mean annual precipitation; MAT, mean annual temperature; RH, average relative humidity in 2019, the values in brackets are the average RH during measurement.

Table 2 The investigated leaf traits (Kitudom et al., 2022).

Class	Leaf traits	Abbreviation (unit)
Morphological trait	Leaf Area	Area (cm^2)
	Perimeter	P (cm)
	Leaf length	Length (cm)
	Leaf width	Width (cm)
	Length/Width	L/W
	The ratio of perimeter to area	P/A (cm^{-1})
	Angle	Angle (°)
Optical trait	Reflectivity	Ref (%)
	Absorptivity	Abs (%)
	Transmissivity	Trans (%)
	Greenness	Greenness
Material property	Leaf fresh mass density	Density.f (g cm^{-3})
	Leaf dry mass density	Density.d (g cm^{-3})
	Water content	WC (%)
	Leaf mass per area	LMA (g cm^{-2})
Anatomical trait	Leaf thickness	Thickness (μm)
	Thickness of upper epidermis	Epidermis_up (μm)
	Thickness of lower epidermis	Epidermis_low (μm)
	Thickness of spongy tissue	Spongy (μm)
	Thickness of palisade tissue	Palisade (μm)
	Leaf vein density	Vein density (mm^{-1})
	Stomata size	St.size (μm)
	Stomata density	St.density (No mm^{-2})
	Stomata $size^2$ × Stomata density	SPI (mm^{-1})
Physiological trait	Maximum photosynthesis rate	A_{max} (μmol m^{-2} s^{-1})
	Maximum transpiration rate	Tr_{max} (mmol m^{-2} s^{-1})
	Maximum stomatal conductance	g_{max} (mol m^{-2} s^{-1})
	Photosynthetic thermal tolerance	T_{crit} (°C)

Table 3 Range of leaf temperature (Tl) and temperature difference between leaf and air (dT).

Site	Tl range (°C)	dT range (°C)
	Mean ± SE	Mean ± SE
SAV	26.2 ± 0.11 ~ 46.0 ± 0.51	-1.3 ± 0.27 ~ 1.1 ± 0.43
TRF	18.2 ± 0.05 ~ 42.4 ± 0.91	-0.9 ± 0.11 ~ 2.5 ± 0.52
STF	11.1 ± 0.20 ~ 33.3 ± 0.66	-1.2 ± 0.11 ~ 2.9 ± 0.44
TEF	8.4 ± 0.11 ~ 33.3 ± 2.07	-0.8 ± 0.12 ~ 4.9 ± 1.44

SAV, savanna woodland; TRF, tropical rain forest; STF, subtropical evergreen broad-leaved forest; TEF, temperate mixed forest.