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1 Original article:

2 **Soil microorganisms behave like macroscopic organisms: patterns in the global distribution**
3 **of soil euglyphid testate amoeba**

4

5 Running header: Forest soil euglyphid diversity

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18

19 **ABSTRACT**

20 **Aim**

21 Patterns of alpha and beta diversity of soil protist communities and the factors that shape them
22 remain largely unknown. We undertook a worldwide survey of forest litter to investigate the patterns
23 of diversity in a group of testate amoebae. We aimed to assess: (1) whether there is a latitudinal
24 gradient in alpha diversity, and (2) whether beta diversity was correlated solely with environmental
25 factors commonly used in soil biology research or if it was also independently explained by
26 geographical barriers.

27 **Location**

28 Worldwide.

29 **Methods**

30 We studied the diversity of Euglyphida, a common group of testate amoebae, in 35 samples of forest
31 litter and moss samples from a global survey, using small subunit rRNA gene sequences. We assessed
32 the relationship between sample alpha diversity and latitude using generalized additive models
33 (GAM). Furthermore, we determined the relationships between community composition and
34 geographical models (distance-based Moran's Eigenvector Maps - db-MEM) using Generalized
35 UniFrac distances (GUniFrac). We also investigated the relationship between individual measured soil
36 parameters, WORLDCLIM data and diversity (alpha plus beta) using both raw data and synthetic
37 variables obtained through principal components analysis.

38 **Results**

39 We recorded 245 phylotypes belonging to 6 out of 7 known Euglyphida families, plus four novel deep
40 clades. Euglyphid alpha diversity was positively correlated with temperature and negatively with
41 latitude and litter C/N ratio. Euglyphida community structure was correlated with the spatial

42 eigenvector Db-MEM31, independently of all measured environmental variables. Db-MEM31
43 corresponds to a natural barrier constituted by the Northern hemisphere desert belt. Beta diversity
44 was correlated with other environmental variables, such as pH, isothermality and temperature in the
45 coldest month of the year.

46 **Main conclusions**

47 Soil euglyphid alpha diversity displays a latitudinal gradient, and beta diversity is not only correlated
48 with climatic and physicochemical parameters but also with geographic barriers. Such patterns of
49 diversity were until recently believed to be characteristic only for macroscopic organisms.

50 **Keywords:** alpha diversity, beta diversity, climatic gradient, cosmopolitanism, environmental filters,
51 geographical isolation, latitudinal gradient of diversity, protists, testate amoebae.

52 INTRODUCTION

53 Microbial biogeography has suffered from the difficulty of isolating and identifying species, and has
54 traditionally been considered separately from macroscopic organisms biogeography (Martiny *et al.*,
55 2006). However, recently, observational and experimental studies on microorganisms have become
56 commoner, and are increasingly using molecular approaches to attempt to overcome limitations
57 inherent to morphology-based taxonomy (Lara & Acosta-Mercado, 2012). The result is that some
58 paradigms are now being repeatedly questioned - especially the longstanding but provocative idea
59 that different rules govern the diversity and distribution of microbes and macroscopic organisms (e.g.
60 Finlay *et al.*, 2004). Indeed, recent studies have revealed the existence of a high diversity of
61 microorganisms, often with limited distributions and showing evidence for historical contingencies
62 and habitat preferences (Porazinska *et al.*, 2010; Heger *et al.*, 2011b; Naff *et al.*, 2013; Tedersoo &
63 Smith, 2013; Lueke *et al.*, 2014). Identification of factors that affect the composition and diversity of
64 soil microbial communities can improve our understanding of the impact of environmental changes
65 on soil ecosystems; these have started to be investigated in bacteria, where communities were
66 shown to be correlated with pH (Jackson & Fierer, 2006; Laubner *et al.* 2009). Protists, as major
67 consumers of decomposers such as many bacteria and fungi (Old & Oros, 1980; Ekelund & Rønn,
68 1994) play a pivotal role in soil food webs and are therefore an excellent group for assessing these
69 questions. Moisture has been suggested to influence global soil microeukaryotic diversity (Bates *et*
70 *al.*, 2013), but no study has yet focussed in detail on a single, functionally homogeneous, group of
71 protists at a global scale.

72 A well-documented pattern in many groups of macroscopic organisms is the positive
73 correlation of diversity with latitude. Clearly latitude is a human construct – part of a grid system
74 which can be used to define the position of a particular location and cannot in itself explain species
75 richness. However, latitude is correlated with many other potentially significant environmental
76 features, such as energy input into the system, which may potentially explain these patterns

77 (Sherratt and Wilkinson, 2009). This latitudinal pattern is often mirrored by a similar decrease in
78 diversity with elevation, as observed in many plant and animal taxa, although patterns showing a
79 peak in species richness at mid-elevations are also common (Adams & Woodward, 1989; Willig *et al.*,
80 2003; Hillebrand, 2004; Jablonski *et al.*, 2006; Cox & Moore, 2010). A range of factors can plausibly
81 affect these patterns in diversity as well as energy input; for example water availability and
82 evapotranspiration influence β -diversity in most animal and plant communities (Hawkins *et al.*, 2003)
83 and historical process may also be important (Sherratt & Wilkinson, 2009). Currently, data supporting
84 latitudinal diversity gradients are very limited for most groups of protists (Sherratt & Wilkinson,
85 2009), with the exception of large marine forms such as foraminiferans (Allen *et al.*, 2006; Yasuhara
86 *et al.*, 2012) and polycystine radiolarians (Boltovskoy *et al.*, 2010). Using the excellent fossil record
87 provided by benthic foraminiferans, Buzas *et al.* (2002) provided support for the idea that higher
88 temperatures favoured faster speciation events through geological time. However, given the
89 diversity of lifestyles encountered in protists, these conclusions cannot be reasonably extended to all
90 other taxa.

91 The influence of distance and geographical barriers on microbial diversity, or in other terms
92 the existence of geographically limited distributions, was hotly debated around the turn of the 21st
93 century. Baas Becking's tenet "everything is everywhere, but, the environment selects" (Baas
94 Becking, 1934), frequently referred to as "EiE" postulates that barriers to dispersal are not effective
95 in preventing organisms from dispersing. Therefore, they do not play any role in the distribution of
96 microorganisms, and only environmental filters operate on microbial communities. This viewpoint
97 has been developed further and applied specifically to protists (Fenchel *et al.*, 1997; Finlay, 2002;
98 Finlay *et al.*, 2004). However, the existence of "flagship" (i.e., morphologically conspicuous), species
99 of microorganisms that have geographically restricted distributions, contradicts this view (Foissner,
100 2006; Smith & Wilkinson, 2007; Vyverman *et al.*, 2007; Heger *et al.*, 2011a). In addition, increasing
101 evidence for the existence of substantial cryptic protist diversity suggests that even supposedly

102 cosmopolitan free-living taxa might in fact correspond to complexes of genetically distinct biological
103 species, each of which may potentially have a restricted distribution (Darling *et al.*, 2007; Aurahs *et*
104 *al.*, 2009; Casteleyn *et al.*, 2010; Watts *et al.*, 2011; Heger *et al.*, 2013). The idea that barriers to
105 dispersal do not have any effect on diversity as suggested by the EiE hypothesis is at one extreme of
106 a range of possible scenarios: in this case only ecological filtering would affect community
107 composition. The question remains open whether pure spatial contingencies also have an effect, and
108 if they do, to what extent.

109 To address this general question, we used as a model group the Euglyphida Cope, a
110 monophyletic clade of predominantly bacterivorous testate amoebae that build a self-secreted
111 siliceous test (shell) (Meisterfeld, 2002). Euglyphids are ubiquitous soil protists, found under a wide
112 range of environmental conditions, some species even being psychrophilic (Smith, 1992; Santibañez
113 *et al.*, 2011). We surveyed their molecular diversity in forest litter and mosses from 35 sites covering
114 a broad range of climates, from all continents except Antarctica, using a specific PCR protocol to
115 amplify selectively euglyphid SSUrRNA genes from environmental DNA extracts, an approach coined
116 metabarcoding (Pompanon *et al.*, 2011). These molecular methods provide an alternative approach
117 to the long-running debates on the validity of morphological criteria that have bedevilled the study of
118 protist diversity (Finlay *et al.*, 2004; Mitchell & Meisterfeld, 2005; Heger, 2009). We evaluated the
119 phylotype composition obtained at the different sites and determined to what extent it could be
120 predicted based on soil characteristics, macroclimatic variables and the geographical position of the
121 sampling sites. In addition, we determined which variables were most correlated with diversity and
122 community structure and compared these patterns to existing data and theory derived from the
123 study of macroscopic organisms.

124 **MATERIALS AND METHODS**

125 *Sampling and environmental data*

126 Samples of soil litter (upper 3 cm composed essentially of organic matter) and mosses (growing on
127 the soil surface) were collected from 35 sites in forest ecosystems covering most biogeographical and
128 bioclimatic regions of the world (Fig. 1; see also Appendix S1 in Supporting Information). The
129 coordinates of each sampling location were recorded using a field GPS. We used two key metrics to
130 characterize soil chemistry: pH as a key factor explaining soil testate amoeba biodiversity and
131 community structure (Bonnet, 1964), and the C/N ratio as a measure of organic matter
132 decomposition (Bardgett, 2005). Total C and N contents determined by CHN analyser (CHN EA1109-
133 Elemental analyser, Carlo Erba Instruments) were used to calculate the C/N ratio and pH was
134 measured in a 1:1 aqueous slurry.

135 We computed spatial variables to investigate the geographical patterns of euglyphid diversity
136 and their possible relationship with known biogeographical barriers. These variables represent
137 spatial structures, from local to global scales, and can be easily used as spatial explanatory variables
138 in models and regression analyses. We computed these variables, based on the geographical
139 coordinates of the sampling points, following the method of Borcard & Legendre (2002) and Borcard
140 *et al.* (2004). We decomposed a Euclidean distance matrix among sites into 41 distance-based
141 Moran's Eigenvector Maps (db-MEM). We then chose the two db-MEMs that were most strongly
142 correlated with community composition using forward selection. These two variables - db-MEM6 and
143 db-MEM31 - were kept for further analyses. We used db-MEM as spatial descriptors because this
144 approach uses a multi-scale decomposition of a distance matrix that is more likely to capture the
145 relevant spatial structures (e.g. biogeographical barriers) than methods using distances based on raw
146 coordinates alone (Dray *et al.*, 2006).

147 We used the plot coordinates to extract biologically relevant bioclimatic variables from the
148 30 arc-second resolution grids of the WorldClim project (Hijmans, 2005). These variables comprise
149 various metrics based on monthly temperature and precipitation data that are biologically relevant
150 for fauna and flora (Elith *et al.*, 2006), protosteloid amoebae (Aguilar *et al.*, 2011), and, as

151 hypothesized here, for Euglyphida. In order to reduce dimensionality, we then conducted a principal
152 components analysis (PCA) of these data and used the obtained principal components as climatic
153 variables in subsequent analyses (clim_PC1 and clim_PC2). Using PCA axes instead of environmental
154 variables enables the consideration of multiple climatic variables at the same time and summarizes
155 them into two synthetic variables that cover a greater part of the variance in climatic conditions than
156 temperature and precipitation alone. Overall, we had three groups of two environmental variables
157 representing spatial (db-MEM6 and db-MEM31), climatic (clim_PC1 and clim_PC2) and soils (pH and
158 C/N) aspects, respectively.

159 Here Figure 1

160 *DNA extraction, PCR, sequencing and phylogenetic analysis*

161 DNA was extracted using, in combination, a MoBio Power Soil™ DNA extraction kit (Carlsbad, CA,
162 USA) and a bead-beating apparatus (FP120 FastPrep™ cell disruptor, Savant Instruments, Inc.,
163 Hotbrook, NY). A total of 0.25g of sample and 60 µl of C1 solution were added to the Powerbead
164 tube. The tube was inverted several times and then shaken for 30s at $5.5\text{m}\cdot\text{s}^{-1}$ in the FastPrep™ cell
165 disruptor as an alternative to the vortexing step recommended by the manufacturer. The other steps
166 of the protocol followed the manufacturer's instructions. We chose to use a "classical" PCR, cloning
167 and sequencing approach rather than next generation sequencing methods because longer
168 sequences, and higher quality control, were deemed essential for building an accurate phylogenetic
169 tree and to discriminate closely related phylotypes. This approach is optimal for the study of a given
170 phylogenetic group such as the Euglyphida.

171 Amplification of the small subunit rRNA gene was performed in two steps; a first PCR was
172 achieved with the specific primers EuglySSUF (forward) (5' GCGTACAGCTCATTATATCAGCA 3') and
173 EuglyLSUR (reverse) (5' GTTGGCACCTTAACTCGCG 3'), the latter primer placed on the LSU rRNA
174 gene. The cycling profile was as follows: an initial denaturation at 94°C for 5 minutes, and then 40

175 cycles with 94°C for 15 seconds as the denaturation step, 62°C for 15 seconds as the primer
176 annealing step, with a touchdown of 1°C per cycle for the eight first cycles, and 72°C for 150 seconds
177 as an elongation step. The final elongation step was of 10 minutes at 72°C. A second, semi-nested
178 PCR was carried out, again using EuglySSUF in combination with EuglySSUR (reverse) (5'
179 GCACCACCACCCATAGAATCWAGAAAGATC 3'), with an initial denaturation at 94 °C for 3 minutes, and
180 then 30 cycles with a denaturation step of 94°C for 30 seconds, then an annealing step at 59°C for
181 30s and an elongation step at 72°C for 60 seconds, followed by a final elongation at 72°C for 10
182 minutes. PCR reactions were carried out in 50 µl of reaction buffer containing 1 µl DNA template
183 (around 1–5 ng), 1.5mM MgCl₂, dNTPs (10 nmol each), 20 pmol of each primer, and 1 U TaqDNA
184 polymerase (Promega). The resulting amplicon was 1100 bp long and comprised the variable region
185 v4; it spanned approximately the two first thirds of the entire SSU rRNA gene.

186 Amplicons were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into
187 *E. coli* TOP10' One Shot cells by heat shock (Invitrogen). Cells were spread onto LB agar medium
188 containing 50µg/ml ampicillin and X-gal and IPTG according to the manufacturer's instructions for
189 blue-white screening. Colonies were picked and the insert was amplified using PCR primers
190 EuglySSUF and EuglySSUR. The presence of the expected size insert was checked on the white
191 colonies by PCR amplification. Clone inserts were amplified with vector primers M13F and M13R and
192 inserts from the expected size were sequenced directly with the specific primers EuglySSUF and
193 EuglyLSUR. Between 18 and 73 clones per site were sequenced depending on the diversity
194 encountered.

195 The obtained sequences were trimmed for ambiguities and aligned manually using the
196 software BioEdit v. 7.0.9.0 (Hall, 1999). Chimerical sequences were eliminated by careful observation
197 of group-specific signature sequences, as suggested by Berney *et al.* (2004). Each difference in a
198 single nucleotide was considered as yielding a distinct phylotype in further analyses; community
199 composition was expressed as a percentage of the total sequences, which has been shown to be well

200 correlated with individual abundances in other protist groups, such as marine Stramenopiles MAST-4
201 (Rodriguez-Martinez *et al.*, 2009) and rotaliid foraminiferans (Pawlowski & Weber, 2013). Clones
202 were sequenced until saturation was reached: this was established using the software DOTUR
203 (Schloss & Handelsman, 2005). Phylotype sequences were aligned with sequences derived from
204 GenBank (for accession files please refer to Fig. 3). The phylogenetic tree was built using
205 RAxMLv7.2.8 (Stamatakis *et al.*, 2008), as proposed on the Black Box portal ([http://phylobench.vital-
206 it.ch/raxml-bb/](http://phylobench.vital-it.ch/raxml-bb/)) using the GTR+ Γ +I model and was performed on 994 characters. The tree was rooted
207 with non-euglyphid Cercozoa taken from a wide array of Monadofilosa. Clone sequences have been
208 deposited in GenBank under the names KT272446-KT272698, and KP892886-KP892888.

209 *Euglyphid α and β diversity*

210 We characterized euglyphid testate amoeba α -diversity using phylotype richness (total number of
211 phylotypes per site) and the Shannon (Shannon & Weaver, 1949) and Simpson (Simpson, 1949)
212 indices of diversity. We chose these measures because they are broadly used in ecological research
213 and describe complementary aspects of α -diversity.

214 To characterize β -diversity, we measured the pairwise phylogenetic distances among
215 euglyphid communities using a generalized version of UniFrac (Lozupone & Knight, 2005), called
216 GUniFrac (Chen *et al.*, 2012), which allows modulation of the relative weight given to both rare and
217 over- represented sequences. While UniFrac is now used routinely in the field of environmental
218 microbiology (Lozupone & Knight, 2007; Lauber *et al.*, 2009), GUniFrac is its latest development and
219 has been shown to have higher detection power than UniFrac or other measures of β -diversity (Chen
220 *et al.* 2012). We calculated GUniFrac for $\alpha = 0$, $\alpha = 0.5$, $\alpha = 1$ as well as the unweighted and variance
221 adjusted weighted version of UniFrac to detect any changes in euglyphid communities diversity.

222 *Numerical analyses*

223 To investigate the general biodiversity of euglyphid testate amoebae, we first computed rank
224 abundance and species accumulation curves. From the species accumulation curve, we estimated the
225 potential total size of the phylotype pool by comparing the obtained curve to simulated curves
226 computed using various models (i.e. Chao, first and second order jack-knife (Smith, 1984; Chao,
227 1987)). Moreover, we computed rank-abundance curves and fitted commonly used models of
228 abundance distribution (null or broken-stick, pre-emption, lognormal, and Mandelbrot; (Wilson,
229 1991)). The curves generated were compared visually.

230 We then investigated the correlation of euglyphid phylotype richness and Simpson and
231 Shannon diversity with each selected environmental variable using generalized additive models
232 (GAM). These models allow the discrimination of non-linear relationships and are commonly used in
233 ecological research (Guisan *et al.*, 2002). These analyses allowed us to determine which variables
234 were correlated with the α -diversity of euglyphids.

235 We finally used Permutational multivariate analysis of variance (PermanovaG), using the
236 multiple distance matrices produced by GUniFrac, to test whether the soil, climatic and spatial
237 variables influenced euglyphid β -diversity (Chen *et al.*, 2012). PermanovaG combines multiple
238 distance matrices in a single test and thereby does not require an *a priori* knowledge of the type of
239 changes of community composition.

240 All calculations were carried out within the R framework (R Development Core Team 2011)
241 using packages “GUniFrac” (<http://cran.r-project.org/web/packages/GUniFrac/index.html>), “PCNM”
242 (<http://R-Forge.R-project.org/projects/sedar/>) and “vegan” ([http://cran.r-](http://cran.r-project.org/package=vegan)
243 [project.org/package=vegan](http://cran.r-project.org/package=vegan)).

244 RESULTS

245 *Euglyphid diversity*

246 We obtained a total of 245 different euglyphid phylotypes. The number of phylotypes per sample
247 varied between 1 (DR1, Dominican Republic) and 31 for the most diverse location (I2, India). The
248 species accumulation curve and rarefaction analyses indicated that the global diversity of phylotypes
249 did not attain saturation (Fig. 2a and b), indicating that total euglyphid diversity in forest litters is
250 significantly higher than the total number of phylotypes recovered in this study. The flattening of the
251 rarefaction curves was reached at 266, 351, and 557 phylotypes, using the Bootstrap, first order
252 Jackknife, and Chao estimates, respectively. The rank abundance curve appeared different from the
253 predicted null model curve (i.e. that should have been observed if the community composition was
254 under neutral selection; see Fig. 2b), suggesting strong niche effects.

255 *-Here Figure 2a and b-*

256 The phylogenetic tree built on the clone sequences together with sequences retrieved from
257 GenBank showed that the 245 phylotypes included representatives of all known euglyphid families
258 except Cyphoderiidae (Lara *et al.*, 2007), a group that is associated with freshwater and marine
259 intertidal habitats (Meisterfeld, 2002). Three phylotypes (CH2_2_11, Ma_44 and CD3_3) clustered
260 together within the marine (and marginally freshwater) family Paulinellidae (Meisterfeld, 2002), a
261 clade that has been reported recently for the first time from soils (Tarnawski & Lara, 2015). In
262 addition, four well-supported clades (named here EEC1 to EEC4) appeared as new families
263 represented only by environmental clone sequences obtained from this and previous studies (Fig. 3).
264 Additional sequences retrieved from GenBank appear also on Fig. 3.

265 *-Here Figure 3-*

266 *Environmental variables*

267 For spatial aspects, db-MEM6 and 31 were retained by the forward selection procedure. For climatic
268 aspects, principal components clim_PC1 and clim_PC2 represented ~70% of the total variance of the
269 climatic data. Clim_PC1 (40.1% of variance) was explained mostly by variables associated with

270 temperature seasonality, including isothermality and coldest month temperature, whereas clim_PC2
271 (28.5%) was correlated with warmest month temperatures and mean yearly temperature (see
272 Appendix S2 in Supporting information). Moreover, pH and C/N were negatively correlated with each
273 other (see Appendix S3 in Supporting information).

274 *Biodiversity patterns (α -diversity)*

275 Richness, defined as the number of phylotypes, and diversity as expressed as either Shannon or
276 Simpson indices were strongly correlated ($r=0.92$, $P<0.001$, Pearson correlations), therefore only
277 phylotype richness will be discussed hereafter. Variations in phylotype richness were explained by
278 the eigenvector map db-MEM6, a pattern that follows closely a latitudinal division of the Earth ($P<$
279 0.01 , $r^2=54.2\%$), but also by the climatic principal component clim_PC 2 ($P=0.02$, $r^2=42.9\%$) and by
280 C/N ratio ($P=0.02$, $r^2=32.8\%$, Table 1, Fig. 4). Among the variables selected for clim_PC2, temperature
281 of the warmest months was highly correlated with α -diversity ($P>0.01$, $r^2=52.9\%$: Fig 5).

282 *-Here Figure 4-*

283 *Community patterns and environmental drivers (β -diversity)*

284 Euglyphid community composition was most strongly correlated with climatic principal component
285 clim_PC1, which was in turn explained mostly by isothermality, and temperatures in the coldest
286 month of the year (see Appendix S2 in Supporting information). The two distance-based Moran's
287 Eigenvector Maps (db-MEM) models showing the best fit in the RDA selection procedure were db-
288 MEM6 and db-MEM31.

289 The GUniFrac analysis showed that db-MEM6 and db-MEM31 (Table 1) were significantly
290 correlated with the communities. While db-MEM6 (i.e. equivalent to a latitudinal gradient; Fig. 5)
291 was obviously correlated with climate, db-MEM31 (i.e. showing a separation between North and

292 South of the Cancer tropic desert belt; Fig. 5) was not. Community composition was also significantly
293 correlated with climatic PC1 (i.e. precipitation seasonality; $P < 0.01$), as well as with pH ($P < 0.01$).

294 *-Here Figure 5- and -Table 1-*

295 **DISCUSSION**

296 *General considerations about euglyphid diversity in forest soils*

297 Our data support the view that protists are highly diverse. Eukaryotic diversity still contains major
298 clades only revealed by environmental DNA (eDNA) surveys (López-García *et al.*, 2001; Massana *et*
299 *al.*, 2004; Lara *et al.*, 2010), that have barely been investigated: most of these clades are protists.
300 Indeed in this study we discovered four new major (family level, *sensu* Lara *et al.*, 2007) clades of
301 euglyphids for which no data exist, either from cultured or from isolated taxa. In addition, as the
302 barcoding gene used here, SSU rRNA, is highly conserved, the real specific diversity is very likely
303 higher than the 245 phylotypes identified in our study. For instance, the mitochondrial cytochrome
304 oxidase gene (COI) commonly used in animal studies is 3–5 times more variable than SSU rRNA in
305 Euglyphida and has been shown to perform better in species level discrimination (Lara *et al.*, 2011;
306 Heger *et al.*, 2011). Although some of the new clades may include taxa previously described
307 morphologically, but lacking molecular data (e.g. family Psammonobiotidae), we consider it unlikely
308 that all four clades belong to such taxa. The discovery in terrestrial habitats (i.e. non-wetland forests)
309 of sequences related to the mostly marine or freshwater Paulinellidae (Nicholls, 2009) is also
310 noteworthy and further illustrates the usefulness of eDNA surveys. This is a surprising finding,
311 because the salinity barrier that separates freshwater and marine environments is difficult to cross
312 for protists (Logares *et al.*, 2009), and soil protist communities are very distinct from those of aquatic
313 environments (Foissner, 1987).

314 In this study, we chose to use a “conventional” molecular approach to screen richness, i.e.
315 cloning and (Sanger) sequencing, in order to retrieve more phylogenetic information that could then

316 be used to build trees. The use of newer, high throughput sequencing strategies would probably have
317 yielded higher richness. However, we believe that the observed patterns of diversity would have
318 been the same, as our approach allowed the retrieval of the most dominant taxa.

319 *Latitudinal gradient of diversity*

320 Our data clearly support the existence of a latitudinal gradient of diversity in soil euglyphid testate
321 amoebae. When richness per sample is plotted against latitude (and if sites above 500 m a.s.l. are
322 removed as having atypical climates for their latitude, c.f. Ju *et al.*, 2014), these data show a
323 unimodal distribution peaking at low latitudes (Fig. 4). In addition, the eigenvector map db-MEM6
324 (mostly a broad scale latitudinal pattern) was highly correlated with diversity and explained a large
325 proportion of the distribution of phylotypes. The BIOCLIM variable that was best correlated with
326 diversity was temperature of the warmest month (Fig. 4). This may seem counterintuitive, as soil
327 protists are expected to be stressed during the hottest part of the year. However, as our sampling
328 design included only forest soil sites that were shaded by trees, soil temperatures probably do not
329 reach values that can be harmful to euglyphids. Possibly more importantly, it is also likely that
330 humidity is generally sufficient in soil forest litter, thus promoting euglyphid activity even when
331 absolute temperatures are high. Indeed, as long as water availability is not a limiting factor, high
332 temperatures can increase enzymatic activity and, therefore, primary production. This is a likely
333 explanation why only temperature, and not precipitations amount and/or regularity were correlated
334 with species richness, as opposed to the predictions of the water-energy theory (O'Brien, 2006). The
335 species–energy theory (Hawkins *et al.*, 2003) postulates that warm climates support higher individual
336 numbers because of higher productivity, and therefore extinction rates are lower than in colder
337 climates, thus diversity accumulates.

338 In addition, richness appears to be inversely correlated with C/N values, which is a commonly
339 used measure of the speed of organic matter turnover, which is directly related to soil productivity

340 (Bardgett, 2005). Therefore, a low C/N value is associated with high biochemical energy, which
341 suggests that the species–energy model may apply to soil euglyphid diversity. It has previously been
342 argued that the species–energy paradigm convincingly explains latitudinal diversity gradient in
343 marine organisms (Tittensor *et al.*, 2010). A convincing latitudinal gradient has also been found in
344 marine bacteria (Fuhrman *et al.*, 2008) but not in a recent study of aquatic testate amoebae across
345 China (Ju *et al.*, 2014). In soil bacteria, however, a latitudinal effect was not found; instead higher
346 bacterial diversity was correlated with low C/N ratio (Fierer & Jackson, 2006). Among soil microbial
347 eukaryotes, a similar latitudinal gradient was observed for dictyostelid amoebae (Swanson *et al.*,
348 1999; Perrigo *et al.*, 2013; Stephenson & Feest, 2013) and fungi (Treseder *et al.*, 2014), and higher
349 yeast diversity was correlated with high temperatures (Vishniac, 2006). Protist-sized Metazoa
350 (rotifers) from genus *Keratella* also exhibit higher diversity in the tropics, together with a high degree
351 of local endemism (Seger & De Smet, 2008). Therefore a provisional conclusion would be that the
352 latitudinal gradient of diversity applies to some microbial-sized organisms, but not to others. Life
353 history traits probably explain these differences, but geographically uneven sampling efforts may also
354 be important (Fontaneto *et al.*, 2012).

355 *Non-cosmopolitanism of euglyphid phylotypes*

356 Geographical variables such as the Equator/Cancer tropic desert belt (db-MEM31; Fig. 5) and a
357 latitudinal gradient (db-MEM6; Fig. 5) significantly explained the geographical distribution of
358 phylotypes (see also Table 1). While db-MEM6 is strongly correlated with temperatures (Fig. 3) and is
359 therefore not independent of environmental parameters, db-MEM31 is not correlated with the other
360 measured variables (i.e. pH and C/N). As pH and the C/N ratio often explain best the distribution of
361 soil organisms (Ponge *et al.*, 1997; Ponge, 2003), db-MEM31 is therefore likely to be a primarily a
362 purely spatial variable; it corresponds to the barrier of deserts surrounding the tropic of Cancer,
363 which is also a major biogeographical limit for arcellinid testate amoeba genera such as *Apodera*,
364 *Alocodera* and *Certesella* (Smith & Wilkinson, 2007; Smith *et al.*, 2008). This barrier is arguably

365 caused by the main wind regimes, which permit passive dispersal only along similar latitudes but
366 prevent the easy crossing of the equator, even for small-sized organisms (Wilkinson *et al.*, 2012).
367 These different lines of evidence therefore suggest that environmental barriers prevent testate
368 amoeba phylotypes from spreading worldwide, thus allowing allopatric speciation to occur.

369 The distribution of euglyphid phylotypes was also shown to be correlated with other
370 variables. The correlation between β -diversity and climate evenness (PC1) probably indicates that
371 only certain phylotypes can tolerate extremes such as deep frost (and indeed minimum temperature
372 of the coldest month was also strongly correlated with PC1). In this case, the ability of fast
373 encystment (i.e. to enter a dormant stage) may play a crucial role in survival. Likewise, pH was
374 significantly correlated with community composition, indicating the existence of specialists in acidic
375 and/or alkaline substrates. Soil or water pH is known as one of the major factors explaining the
376 structure of testate amoeba communities, together with moisture (Lamentowicz & Mitchell, 2005;
377 Mitchell *et al.*, 2008). For example, in our survey, phylotype CH4_I120 (whose sequence is identical to
378 *Assulina muscorum* AJ418791) has been found only in sites with marked seasonality, where it
379 sometimes represents a large part of all phylotypes (in some sites more than 70% of the total
380 number of sequences). The existence of specialist phylotypes is further corroborated by the rank-
381 abundance curve of soil euglyphid data, which significantly diverged from the null model (Fig. 1). This
382 shows that deterministic forces influence community composition, in contrast to the pattern found in
383 some microbial Metazoa such as moss-dwelling rotifers (Fontaneto *et al.*, 2011). If niche effects
384 influence community composition, evolving towards specialisation could be a winning strategy for
385 euglyphids.

386 **CONCLUSION**

387 Our study revealed the existence of an unexpectedly high diversity of euglyphid testate amoebae,
388 both at deep phylogenetic levels (i.e. presence of undetected clades) and within individual clades.
389 Phylotype richness was significantly higher in low latitude, high-energy environments (i.e. with high

390 temperatures and fast nutrient cycling). In addition, soil euglyphid diversity and community structure
391 were explained by a combination of both geographic isolation and ecological specialization (i.e.
392 niche-driven community patterns). The geographical isolation observed in euglyphid testate
393 amoebae is in clear contradiction to the cosmopolitan distribution models, which were believed to
394 apply to free-living microorganisms. Our results from forest litter euglyphid testate amoebae thus
395 suggest that the patterns of diversity and community structure of certain protists are rather similar
396 to those observed for multicellular organisms. We predict that a more thorough investigation of the
397 ecology of different groups of protists, taking into account their true diversity (i.e. beyond
398 morphotypes) will reveal many similar cases. More generally, our study suggests that the rules that
399 govern soil protist diversity are similar to those for larger organisms. There may be a unity to ecology
400 that crosses the boundary created by the limitations of human vision that separates the macroscopic
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414

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643

644 **SUPPORTING INFORMATION**

645 Additional Supporting Information may be found in the online version of this article:

646 **Appendix S1:** List of all sampled sites with coordinates, country, climatic values and measured soil
647 variables (pH and C/N)

648 **Appendix S2:** PCA of BIOCLIM data extracted from the sampling sites.

649 **Appendix S3:** Correlations between spatial, climatic and soil variables.

650

651 **BIOSKETCH**

652 **Enrique Lara** is a researcher at the University of Neuchâtel, Switzerland. His research interests
653 encompass various aspects of microbial eukaryote evolution and ecology, with a special focus on
654 testate amoebae.

655 Author contributions:

656 E.L. designed the study, L. R.-D. and E.L. performed cloning and sequencing, B.F. and E.L. analysed the
657 data, and all authors discussed the results and wrote the manuscript.

658 Editor: Walter Jetz

659 **Figure legends**

660 Figure 1: Location of forest litter and mosses sampling sites for a worldwide study of euglyphid
661 environmental diversity, and codes used to identify them.

662 Figure 2a: Accumulation of soil euglyphid phylotypes based on 35 forest litter samples from a
663 worldwide sampling. The box and whiskers show the median, inter-quartile and 95%
664 confidence intervals of phylotype richness based on resampling of the data (100 iterations).

665 Figure 2b: Rank-abundance curves of soil euglyphid phylotype data compared to five models of
666 species rank-abundance, null or broken-stick, pre-emption, lognormal, and Mandelbrot. Note
667 the log scale for the abundance axis of the rank-abundance graph.

668 Figure 3: Maximum likelihood phylogenetic reconstruction of the euglyphid clades recovered from
669 clones obtained through metabarcoding from a worldwide study of forest moss and litter soil
670 samples. The tree was built using sequences of the SSUrRNA gene obtained in this study, plus
671 data from GenBank (both environmental clones and sequences derived from isolates or
672 cultures). A diverse panel of Monadofilosea were chosen to root the tree. A total of 994
673 characters were used in the analysis.

674 Figure 4: Euglyphid phylotype richness as determined by a worldwide survey of euglyphid genetic
675 diversity in forest litter and mosses plotted against (a) maximum temperature of the
676 warmest month of the sites, (b) latitude and (c) sample C/N ratio. Residuals of fitted General
677 Additive Models are plotted against the latter variables. Twice-standard-error curves are
678 shown using dashed lines.

679 Figure 5: Distance-based Moran's eigenvector maps (db-MEM) generated on a world map based on
680 euglyphid community composition obtained through metabarcoding from a worldwide
681 sampling of forest litter and moss. Variables db-MEM6 and 31 are correlated with phylotype
682 distribution. Variable db-MEM6 corresponds generally with a latitudinal gradient, in contrast
683 with db-MEM31. The pattern shown by db-MEM31 suggests strong dissimilarities in

684 communities North and South from the desert belt around the Cancer tropic and/or the
685 inter-tropical convergence.
686

687 **Table**

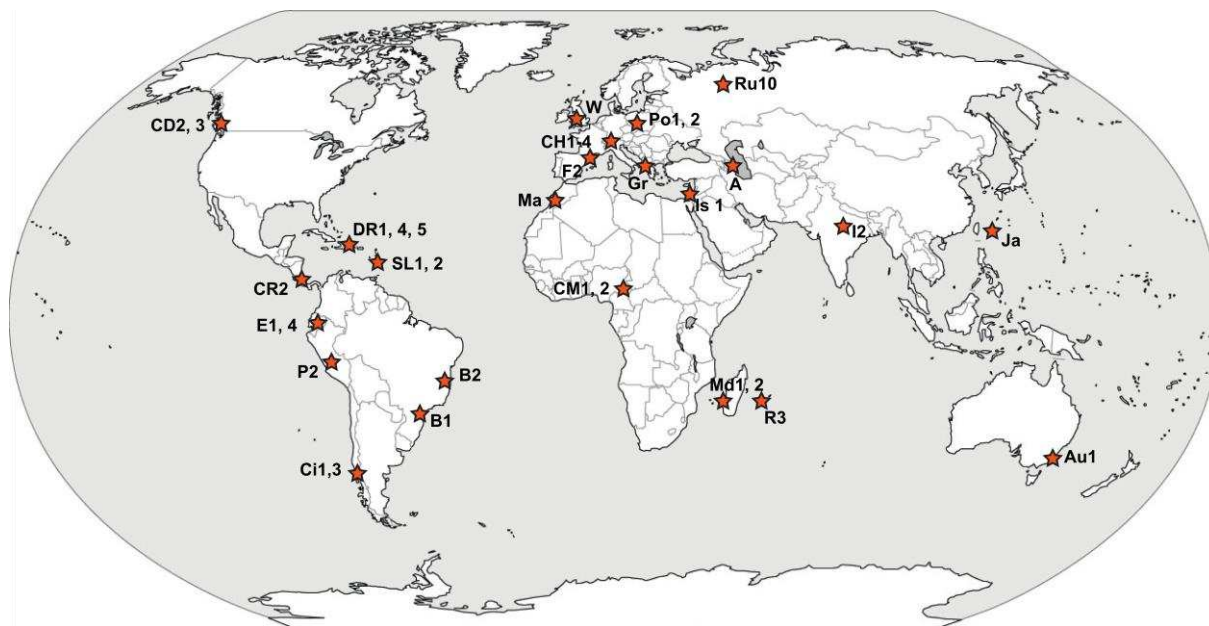
688 Table 1: Summary of statistics derived from GUniFrac and GAM analyses based on euglyphid
 689 community composition obtained through metabarcoding from a worldwide sampling of
 690 forest litter and moss. Significant values are indicated in bold. Spatial 1 and 2 are distance-
 691 based Moran's eigenvectors that have been retained in the analysis, Climate 1 and 2 are the
 692 first two principal components from the principal components analysis performed on data
 693 extracted from the BIOCLIM dataset (values are shown in Appendix S1 in Supporting
 694 Information).

		GAM - changes in species richness (alpha diversity)		GUniFrac - changes in community composition (beta diversity)
		r^2	<i>P</i> value (Wald test)	<i>P</i> value after 9999 permutations
Spatial 1	db-MEM31	0.01	0.95	0.01
Spatial 2	db-MEM6	54.2	< 0.01	0.04
Climate 1	clim_PC1 (PCA BIOCLIM)	16.5	0.08	0.02
Climate 2	clim_PC2 (PCA BIOCLIM)	42.9	0.02	0.15
Soil 1	pH	24.2	0.07	< 0.01
Soil 2	C/N	32.8	0.02	0.07

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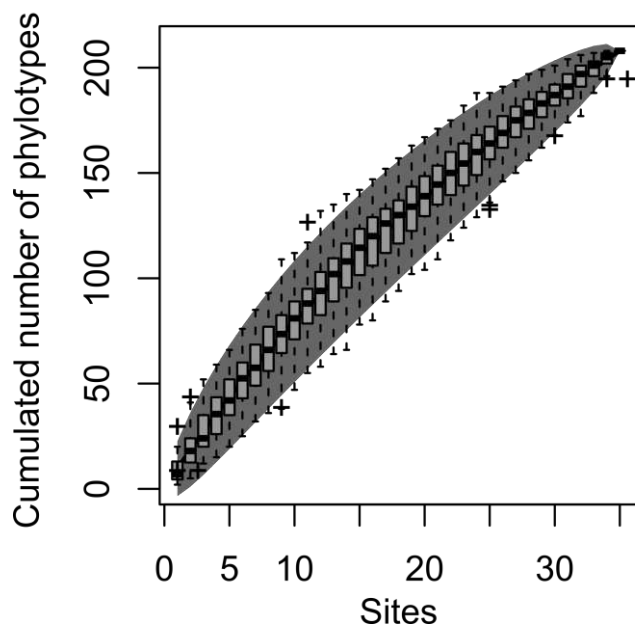


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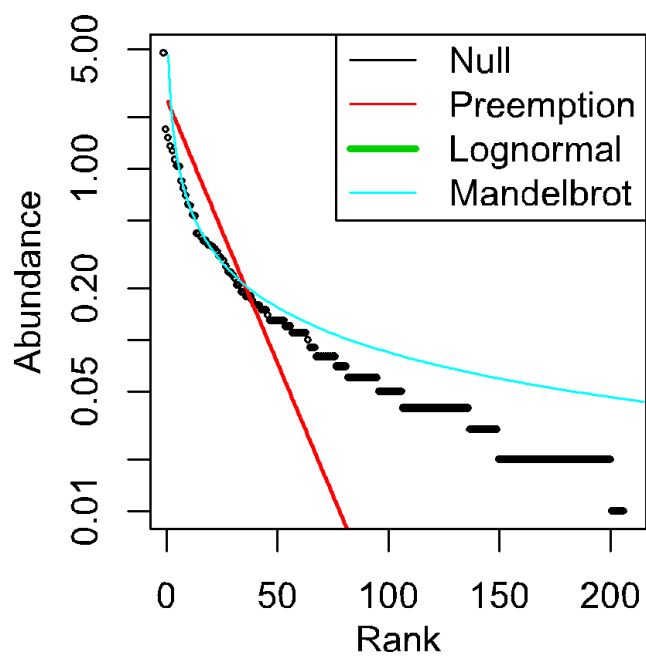
699 Figure 1 Grey scale in print, colour online.

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a Phylotype accumulation curve

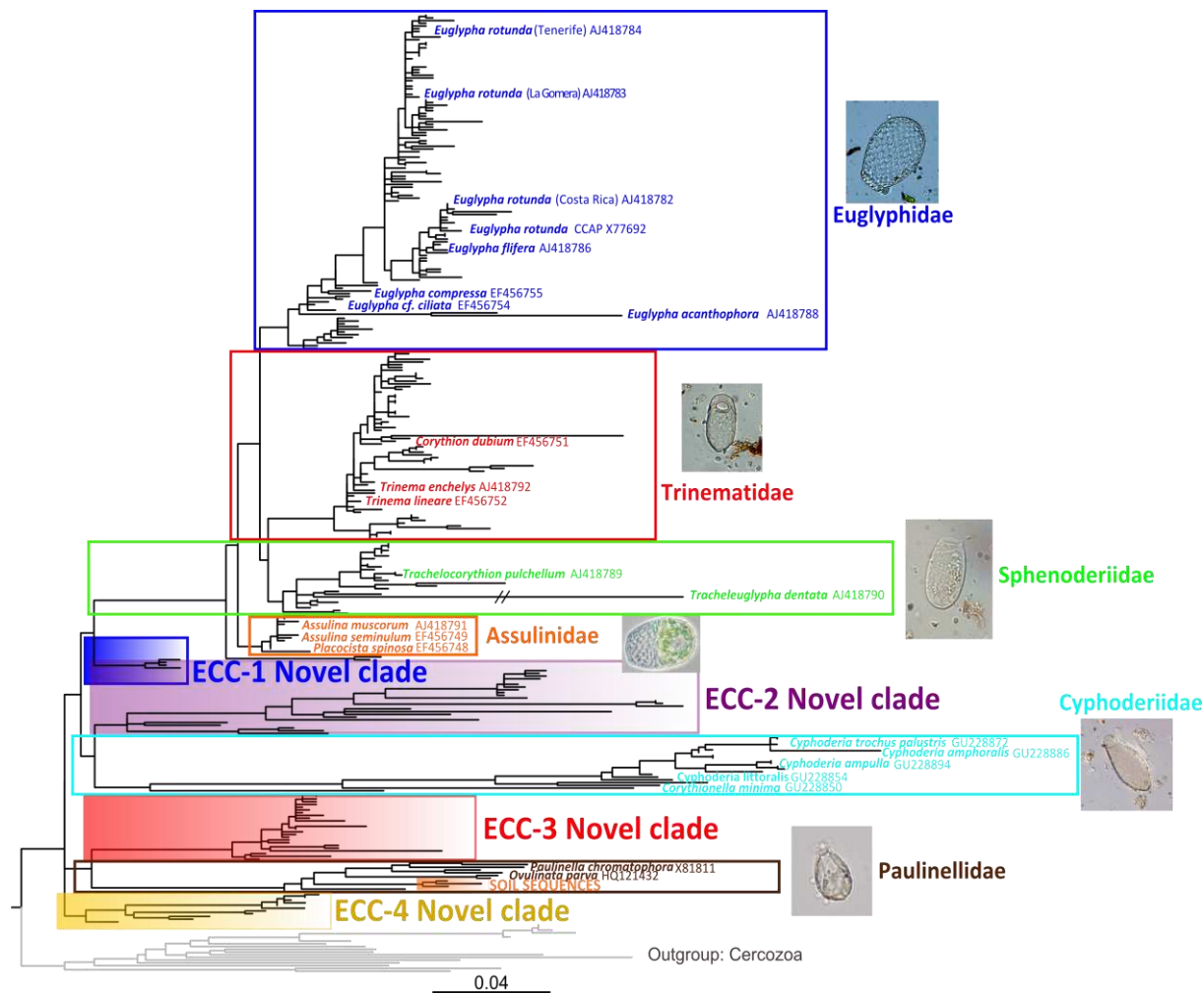
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b Rank-Abundance curve

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704 Figures 2 a and b Grey scale in print, colour online.

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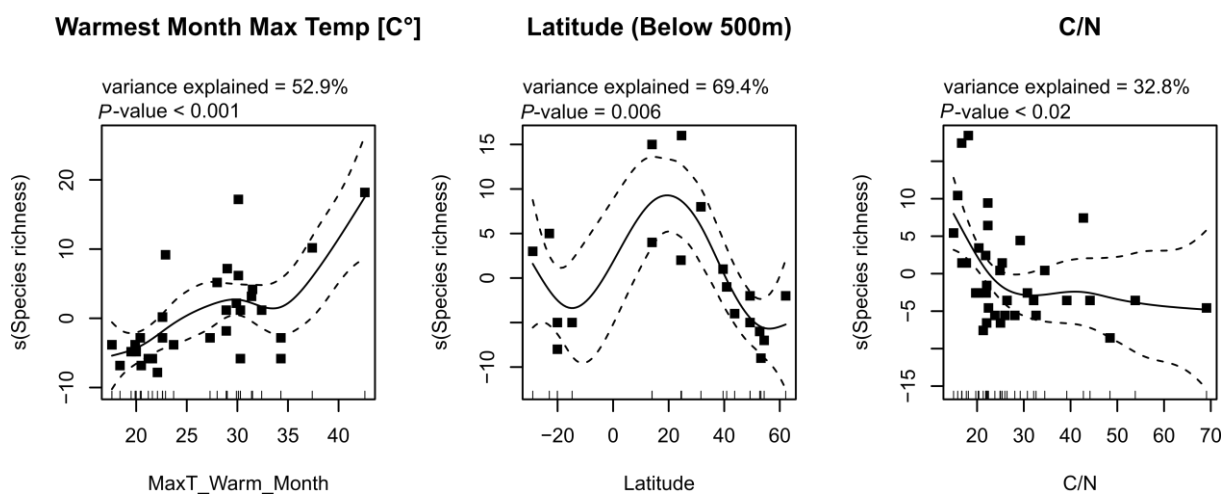


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707 Figure 3 Grey scale in print, colour online.

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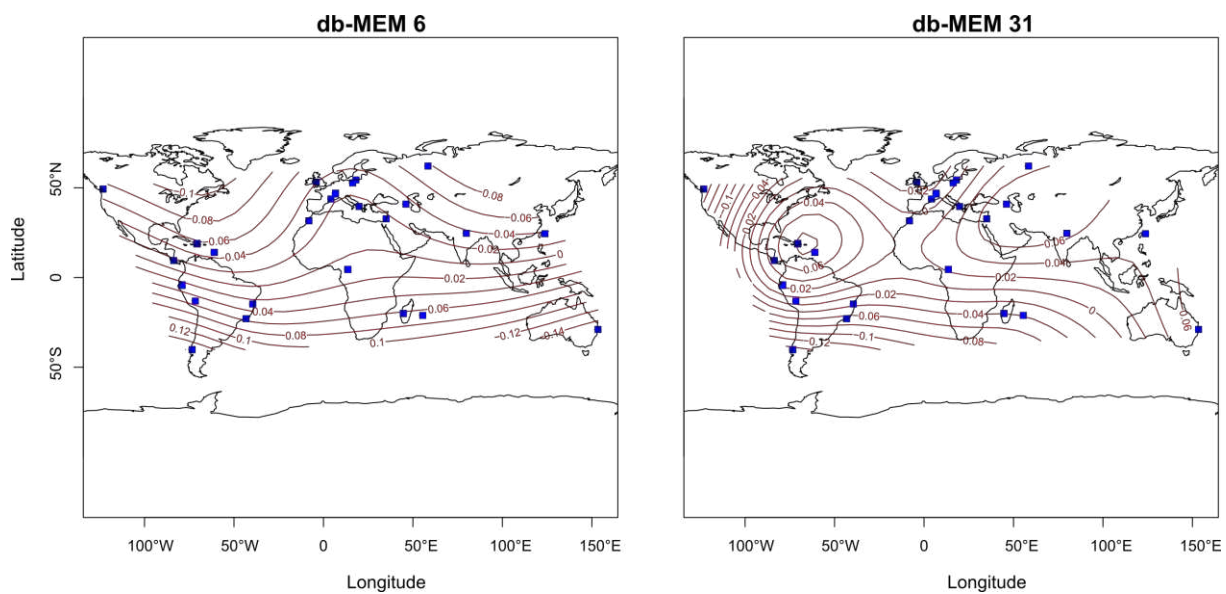
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711 Figure 4 Grey scale in print, colour online

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715 Figure 5 Grey scale in print, colour online