Mimetic host shifts in an endangered social parasite of ants

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An emerging problem in conservation is whether listed morpho-species with broad distributions, yet specialized lifestyles, consist of more than one cryptic species or functionally distinct forms that have different ecological requirements. We describe extreme regional divergence within an iconic endangered butterfly, whose socially parasitic young stages use non-visual, non-tactile cues to infiltrate and supplant the brood in ant societies. Although indistinguishable morphologically or when using current mitochondrial and nuclear sequence-, or microsatellite data, Maculinea rebeli from Spain and southeast Poland exploit different Myrmica ant species and experience 100 per cent mortality with each other’s hosts. This reflects major differences in the hydrocarbons synthesized from each region by the larvae, which so closely mimic the recognition profiles of their respective hosts that nurse ants afford each parasite a social status above that of their own kin larvae. The two host ants occupy separate niches within grassland; thus, conservation management must differ in each region. Similar cryptic differentiation maybe common, yet equally hard to detect, among the approximately 10 000 unstudied morpho-species of social parasite that are estimated to exist, many of which are Red Data Book listed.

1. Introduction

To set meaningful priorities in conservation and for practical remedies to succeed, it is vital to ascertain whether the threatened morpho-species named in Red Data lists are likely to consist of more than one cryptic species [1,2] or functionally distinct genotypes. In theory, regional (co-)adaptations may be amplified in closely coupled biological systems [3], such as obligate mutualisms [4] and host–parasite arms races [5]. In the case of insect–insect interactions, both parties may be susceptible to strong selection within small spatial scales, even between neighbouring landscapes [6,7]. Thus, unexpected subsets of host specificity, rapid evolution and cryptic speciation are an emerging feature of insect-parasitoid studies [8], and apparently exist, possibly in extreme forms, among the estimated 100 000 morpho-species of poorly studied insects that interact with ants (myrmecophiles) [9].

DNA analysis transformed biologists’ ability to detect cryptic species within the described morpho-species [10,11], and with a burgeoning array of reference sequences available (e.g. Consortium for the Barcode of Life), an increasing number of species complexes are being identified [2]. Nevertheless, commonly used, affordable techniques may be insensitive to detecting differentiation that has arisen recently or from selection on one or a few genes that affect the phenotype in major ways [12–14]. Maculinea (large blue) butterflies exemplify this problem: all
six recognized morpho-species are iconic flagship insects, long identified as global conservation priorities [15–17], that possess attributes associated with cryptic speciation [2], including socially parasitic young stages that use non-visual, non-tactile cues, including chemical and acoustical mimicry, to infiltrate and exploit ant societies [15–19]. Recent analyses of mitochondrial- (COI, COII) and nuclear sequence data (EF1α, wingless), as well as microsatellite data, suggest that each morpho-species of Maculinea that has a predatory lifestyle contains cryptic lineages, but recognized cuckoo species (species that are fed directly on regurgitations by ants; see the electronic supplementary material, figure S1e) could not be distinguished [15,20–22], despite their closer integration and local coevolution with ant societies and, typically, more extreme host specificity [9,23,24].

We studied the regional divergence that nevertheless appeared to exist within the endangered cuckoo butterfly Maculinea rebeli (Hirschke), which was itself indistinguishable from a close relative, Maculinea alcon (Denis & Schiffermüller), in recent molecular studies [20,21]. Prior to 1991, each of these congeners was classed as globally vulnerable [25], but uncertainty about their taxonomic status led to exclusion from subsequent lists. It is not disputed that Ma. rebeli and Ma. alcon are distinct ecotypes (or putative ecospecies) which inhabit different ecosystems, xerophytic and moist grassland, respectively, and exploit different plant and Myrmica species [9,22]. Both are extreme specialists whose respective larvae live for 11–23 months and acquire more than 98 per cent of their ultimate biomass (see the electronic supplementary material, figure S1) [9]. They achieve this transition by abandoning their host plant and secreting simple cocktails of hydrocarbons that resemble the chemical signatures of Myrmica grubs sufficiently well to trick foraging workers of any Myrmica species to ‘rescue’ the mimic and carry it into the underground brood chambers [9,18]. However, although each caterpillar is adopted indiscriminately by the first forager to encounter it [9], each Myrmica species whose nest it enters represents not only a different food but also a different enemy, chemical template to mimic [26] and living environment for 92–96% of the intruder’s life: unsurprisingly, caterpillars typically survive with the single, or occasionally sibling, model ant species that they mimic best [9]. Thus, within colonies of the model host species, the intruding larvae successfully compete with the ant brood for worker attention and are soon fed (and rescued) preferentially by the nurse ants (see the electronic supplementary material, figure S1e), a subterfuge that is achieved by synthesizing additional hydrocarbons shortly after adoption that more precisely mimic their host Myrmica species (but other Myrmica species less) [27]. By contrast, caterpillars carried into nests of other Myrmica species suppress their secretions and rely on the passive acquisition of their host’s gestalt odour for social integration [27]. Acquired camouflage alone, however, is insufficient to survive periods of stress or deprivation, when nurse ants become discriminatory and xenophobic [28].

We noticed that populations of Ma. rebeli in southwest Europe and Poland appear to exploit very different species of Myrmica, Myrmica schencki and Myrmica sabuleti, respectively [9,29]: ants whose chemical recognition profiles differ more than any other known pairs of Myrmica species [26] and which occupy different niches within grassland. We therefore studied the exclusivity of host specificity that has evolved in each region by measuring survival both in natural populations and in the laboratory. We then devised behavioural experiments to assess the social status achieved by Spanish and Polish larvae after infiltrating the two host ant societies, and also identified the mechanism responsible for host specificity by analysing the mimetic chemicals secreted by pre- and post-adoption larvae from each region. Finally, we described the key attribute of the niche occupied by each ecotype (or cryptic species) of this endangered butterfly, which provides the essential knowledge for their future conservation [16].

2. Material and methods
(a) Measuring host specificity in natural populations
Host specificity was measured by comparing the proportions of caterpillars that were adopted into different Myrmica nests with the proportions that survived to adulthood or pupation. Data were obtained from three populations for 5 consecutive years near Panticosa in the Spanish Pyrenees [30,31] and in one population for 4 years near Przemysł, southeast Poland [22]. The proportion of larvae adopted by different ants was estimated by baiting beneath stratified random samples of gentians and by counting the number of eggs on each plant [30,31]. Prior work had shown that there was no difference in egg or larval survival on gentians growing in different ant territories, nor in the ratio of larvae retrieved from beneath plants by different ant species: the first Myrmica worker to encounter a larva retrieved it, and where two species overlapped, there was no bias in retrieval towards one species [30,32]. The distribution of the egg population on gentians is, therefore, an accurate surrogate for the distribution of the final-instar population entering nests of each Myrmica species [30,32]. Adult estimates of Ma. rebeli were obtained by recording eclosing individuals along stratified transects across sites, and identifying the nest after confirming that it contained an empty pupal case [30,31]. Additional data were obtained by excavating all Myrmica nests near gentians along stratified transects that had supported known densities of eggs the previous year and by counting the pupae they contained. No mortality has been recorded in pupae in Myrmica cells before eclosion [30–32].

A map of regional host specificity (figure 1) was compiled from our published results [9,29–32] supplemented by additional field data. The distributions are considered to be near complete for Poland [22,29], the French and Spanish Pyrenees and southern Alps [30–32], but the northern Alps and Massif Central were less comprehensively sampled and may contain greater complexity in host use. Host use in Italy, Hungary and central Switzerland was not mapped.

(b) Laboratory experiments of host specificity
Host specificity from the two regions was measured using naive laboratory My. schencki and My. sabuleti colonies, collected from the Jura, east France, midway between the Pyrenees and Przemysł in a landscape lacking the butterfly (figure 1). Six nests of each species were excavated and divided into subcolonies, maintained on a standard diet in Brian nests [33]. After 6 to 8 weeks acclimatization, more than 100 G. cruciata flower spikes were randomly collected from the Polish and Spanish sites, and the resultant final-instar larvae were used within 12 h of leaving their foodplant to establish experiments (S2b–d). First, larval survival was measured in 22 laboratory cultures, each containing 50 workers and five ant larvae, established from the stock nests (six pairs of My. schencki, five pairs of My. sabuleti). A total of 123 Ma. rebeli larvae from Poland and 97 from Spain were introduced in
Figure 1. Host specificity of *Maculinea rebeli* in Spain and southeast Poland. (a) Field survival: outer circle, egg distribution (= larval adoption, see §2a) in different *Myrmica* ant territories (*n* = 2859 Spain, 102 Poland); inner circle, ant species where *Ma. rebeli* survived to pupae or adults (*n* = 148 Spain, 548 Poland). Map: blue, *My. schencki* recorded as sole host; pink, *My. sabuleti* primary host; red circle, source of laboratory test ants in Alps. (b) Larval survival after 17 days in paired laboratory ant colonies set from the same naive French source nests.

(c) Social status achieved in natural and unnatural host colonies

We assessed the social status achieved by each form of *Ma. rebeli* within colonies of each *Myrmica* species in a standard bioassay [23] that involved perturbing laboratory ant colonies and recording the order in which the ants’ own brood or the mimetic caterpillars were rescued. Groups of five butterfly larvae from each region were adopted into matching colonies of naive French *My. schencki* and *My. sabuleti*, using separate replicate nests to those used in §2b. Every test colony also contained five brood items each of kin ant pupae, large and small larvae, making a total of 20 immature individuals and 20 workers per replicate. Cultures were established in 413 cm$^2$ boxes containing a small moist sponge pad beneath an inverted 6 cm diameter saucer with a notched entrance, under which the ants gathered their brood and inverted 6 cm diameter saucer with a notched entrance, under which the ants gathered their brood and relocating it over another pad nearby; we then recorded the order in which the nurse ants rescued their 15 brood items or the five *Ma. rebeli* and carried them into the new nest (figure 2). The same experiment was repeated 7 days later, which represents a sufficient period for *Ma. rebeli* caterpillars to attain their maximum potential integration with a host society, yet remaining a similar size to when first adopted, i.e. the same size or smaller than the *Myrmica* pupae and large larvae [23,32]. The number of replicates for each ant–butterfly combination tested varied owing to a paucity of ant pupae and butterfly deaths (especially with unnatural hosts); *n* = 8 (figure 2a,b), *n* = 6 (figure 2c,e), *n* = 5 (figure 2d,h), *n* = 4 (figure 2f) and *n* = 3 (figure 2g). Fisher’s exact tests were used to ascertain differences in the probability of a class of item being retrieved or abandoned by worker ants after perturbations. We also made three types of non-parametric analysis of the rank order in which chosen items were retrieved, within or between treatments: Kruskal–Wallis to establish whether ants rescued items randomly or selectively; Wilcoxon to test for changes in the order of selected items after *Ma. rebeli* had lived for 7 days with the ants compared with the initial 3 h; Mann–Whitney to test for differences in the order in which ant brood or butterfly caterpillars were selected within each of the eight combinations of ants and butterflies shown in figure 2. In addition to Mann–Whitney analysis, we used a randomization procedure whereby ranks were assigned at random for each trial twice. We recorded the difference in median between the two draws and repeated the procedure 10 000 times providing a frequency distribution for differences in medians to arise without selection. We then compared the observed differences in median between the item classes and assessed their likelihood to occur at random (see the electronic supplementary material, table S1).

(d) Analysis of surface semio-chemicals on *Maculinea rebeli* larvae

To test whether observed regional differences in *Ma. rebeli*’s host specificity could be explained by variation in mimetic...
Figure 2. Status achieved by Maculinea rebeli within natural and unnatural Myrmica host societies. (a–h) The order that disturbed workers rescued ant brood or butterfly larvae 3 h and 7 days after adoption. Each replicate involved a choice between kin ant pupae (open circle), large kin ant larvae (open square), small kin ant larvae (open diamond), Ma. rebeli larvae (filled circle). Boxplots show means of median orders of rescue (symbol), 25–75% quartiles (box), and first and last individuals (tails); ‘nr’ = per cent Ma. rebeli larvae not retrieved by ants after 30 min. All treatments showed significant differences in the order in which items were retrieved (Kruskal–Wallis, $H = 10.38–25.84$, d.f. = 3, $p = 0.016$ to $< 0.001$), with fewer Ma. rebeli rescued than ant brood in (a–d,h and g) ($z = 12.83$ to $− 46.66$, $p < 0.001$). After 7 days with their natural hosts (c,h), Ma. rebeli were rescued first equal with kin pupae (Mann–Whitney $W = 38.5$, $p = 1.000$; $W = 24.0$, $p = 0.5309$, respectively), significantly ahead of kin larvae ($p = 0.024$, 0.027). See the electronic supplementary material, table S1 for full statistical tests.

To 20 μL ant workers to 50 μL and 2 μL of every sample were analysed by gas chromatography with mass spectrometric detection using a HP 5890II gas chromatograph and HP 5971A mass selective detector, and ultra-high purity helium as the carrier gas with 10 psi column head pressure. Mass spectral data were acquired in full scan mode over 40–600 m/z. Mass chromatograms were initially screened for hydrocarbons by examining the selected ion chromatogram of m/z = 57. The chromatogram was integrated at a threshold value of 12 (HP integrator) to obtain the areas under the peaks measuring the total ion count. With each sequence of samples, we also analysed alkane standards (n-C20–n-C36), and the position of each peak within that range in a sample was calculated as an equivalent chain length (ECL) [26]. Mass chromatograms were inspected to ensure that they were free of gross interferences and that peaks of interest, such as branched and straight alkanes and alkenes, were chromatographically distinct and symmetrical. We excluded peaks that were column bleed, siloxanes or phthalate plasticizers as indicated by a characteristic abundant ion at m/z 149. Peaks of interest were tentatively identified by a combination of ECL number and inspections of their full scan mass spectra and matching with the NIST-97/08 mass spectral database.

For statistical analysis, the area under each peak was expressed as the proportion of the sum of all peaks in the chromatogram [26]. Samples were compared using multivariate and non-parametric multi-dimensional scaling on the ranks of the Bray–Curtis similarities [34]. The extent of a final lack of fit...
Figure 3. Changes in cuticular hydrocarbon profiles when *Ma. rebeli* larvae are reared with or without different *Myrmica* host species. The non-parametric multi-dimensional scaling plot shows profiles of final-instar butterfly larvae from Spain (blue symbols) and Poland (red) at pre-adoption before encountering ants (diamonds), after 6 weeks (solid squares/circles) with *My. schencki* (blue arrows, boundaries and stippling) and *My. sabuleti* (red arrows, boundaries, stippling), then removal from ants for 5 days (open squares, circles, black arrows and lines), when acquired ant chemicals dissipate and those synthesized by the butterfly accumulate. Stars *My. schencki* (blue), *My. sabuleti* (red); naive test colonies (solid), Spanish (dark), Polish (pale) sites.

was assessed by a STRESS statistic [26] before pairwise differences between species and treatments were assessed using an analysis of similarities [35] in PRIMER-e v6. We used the average pairwise distance between groups, and assessed two averages with a two-sample t-test, to compare differences in the shift of similarities between groups.

(e) *Myrmica* niches on *Maculinea rebeli* sites

Baits were placed under 223 flowering *G. cruciata* plants in Spain to record the species of *Myrmica* foraging around them [31]. Vegetation structure (height) was measured using Stewart’s direct method [36] at four diagonal points 5 cm from each plant. Species’ niches were compared using two-tailed t-tests having confirmed normality of the data.

3. Results

(a) Host specificity

In three *Ma. rebeli* populations over 5 years in the Spanish Pyrenees, we found that eggs were laid indiscriminately [37] on *G. cruciata* growing in the territories of four species of *Myrmica*, yet 100 per cent of adults emerged from *My. schencki* nests the following summers, despite only 24 per cent of the larval population being adopted by that ant (figure 1a; *z* = −96.81, *p* = < 0.001). By contrast, for 4 years near Przemysl, Poland, 28 per cent of *Ma. rebeli* larvae were adopted by *My. schencki*, but no adult emerged from their nests (*z* = 9.66, *p* = < 0.001). Instead, 77 per cent of adults emerged from *My. sabuleti* nests and the remaining three species’ colonies (as tested the least maladapted individuals in the most lowly status, ranking well below small ant larvae (see figure 2a–d and the electronic supplementary material, table S1). As expected [23], workers generally selected their pupae ahead of their large larvae and retrieved both in preference to small ant larvae. However, after a week with their natural hosts, *Ma. rebeli* from Spain and Poland were chosen equal first with the kin pupae of *My. schencki* and *My. sabuleti*, respectively, and significantly ahead of the smaller ant larvae (see figure 2e–h and the electronic supplementary material, table S1). But when each was reared with its unnatural ant host, just 20 per cent of week-old butterfly larvae were rescued, and these were afforded lowly status, ranking well below small ant larvae (see figure 2i–l and the electronic supplementary material, table S1). Even this may overestimate integration, because many *Ma. rebeli* larvae were killed in unnatural host *Myrmica* nests, and we perhaps tested the least maladapted individuals in the most socially accepting colonies.

(b) Social status of caterpillars in natural and unnatural host species’ colonies

*Maculinea rebeli* larvae did not integrate with their host society in the first hours after their adoption, being the last items to be chosen by workers on the rare occasions they were rescued after colony perturbation by exposure to light (see figure 2a–d and the electronic supplementary material, table S1). As expected [23], workers generally selected their pupae ahead of their large larvae and retrieved both in preference to small ant larvae. However, after a week with their natural hosts, *Ma. rebeli* from Spain and Poland were chosen equal first with the kin pupae of *My. schencki* and *My. sabuleti*, respectively, and significantly ahead of the smaller ant larvae (see figure 2e–h and the electronic supplementary material, table S1). But when each was reared with its unnatural ant host, just 20 per cent of week-old butterfly larvae were rescued, and these were afforded lowly status, ranking well below small ant larvae (see figure 2i–l and the electronic supplementary material, table S1). Even this may overestimate integration, because many *Ma. rebeli* larvae were killed in unnatural host *Myrmica* nests, and we perhaps tested the least maladapted individuals in the most socially accepting colonies.

(c) Analysis of model and mimetic chemical profiles

Given the multi-functionality of hydrocarbons [39], perfect matches by *Ma. rebeli* secretions to the dissimilar recognition profiles of *My. schencki* or *My. sabuleti* [26] were not expected [27]. Nevertheless, *Ma. rebeli* from each region secreted a distinctive cocktail that mimicked its natural host’s signature with increasing likeness (figure 3).
larvae were absent from the simpler profiles secre-
Ma. rebeli. 3-methyl-tricosane) synthesized by isolated 7-week-old
two of these three emerging mimetic hydrocarbons (docosane,
My. sabuleti
with
pre-adoption larvae. By contrast, individuals reared
octacosane acquired from
rebeli
which were absent or just detectable on
schencki
and 3-methyl-tricosane, diagnostic hydrocarbons of
Ma. rebeli
that exploits representatives from the first two groups [9] and
Ma. rebeli from the second two. Field [40] and pre-adoption
chemical [24] evidence suggest that similar exclusive differ-
cent may have evolved between the main European
form of Ma. alcon that exploits My. scabrinodis and that of
Scandinavia and the Pyre-Bas that is adapted to My. rubra/
ruginodis. Current molecular techniques compound the confu-
sion, for no wide-scale differentiation was detected be-
tween or within Ma. rebeli and Ma. alcon [20,21], perhaps
because current forms of these extreme specialists evolved
rapidly in recent millennia [20] and/or very few genes are
involved. Unfortunately, lycaenid butterflies in general,
and Maculinea species in particular, are notoriously difficult
to pair in captivity, making large-scale cross-breeding exper-
iments on hybrids exceedingly difficult. Thus, although
some morphologists and recent genetic analyses currently
recognize one cuckoo species of Maculinea (Ma. alcon),
ecological studies suggest two cryptic species (Ma. rebeli and
Ma. alcon) [9], and our current functional/physiological
studies point towards three (possibly four) recent siblings,
drawn from the above, exploiting rubra-, scabrinodis-
and lobicornis-taxa of Myrmica.

Whatever the taxonomic status of each form, all are ill-
served by traditional conservation paradigms based on
species listing. Like ecospecies [41], each type exploits a
resource that occupies a different niche or biotope, and all
are threatened by habitat degradation or destruction. In
the case of Ma. rebeli, My. schencki requires more frequently
grazed grassland than My. sabuleti and considerably more
than My. scabrinodis. The successful restoration to the UK
of Ma. arion resulted from creating optimum habitat for
its host My. sabuleti [16]: similar management would pro-
mote Ma. rebeli in southeast Poland yet cause population
extinctions elsewhere in Poland (figure 1a) and in Spain.

Regional host shifts are not unknown in social parasites,
especially among cuckoo species [24,40,42]. However, the
more different the ecology, physiology, defence and social
organization of hosts, the less we consider it probable that
the extreme adaptations required to exploit them will be
expressed by phenotypes of a single species [43]. Indeed, all
six morpho-species of insect social parasite whose ecology,
mimicry, host use or genetics have been studied show evi-
dence of cryptic speciation (Microdon hoverflies [43],
predatory Maculinea [15,20,21]) or extreme differentiation
(cuckoo Maculinea), making it likely that the phenomenon
is common among the approximately 10,000 unstudied
morpho-species [9] of insect social parasites, many of which
are Red Data Book listed [15,25]. Other parasitic systems
may be similar, particularly where species’ interactions are
 governed by non-morphological cues such as chemical
signalling or resistance [8,10,43]. Thus, while molecular
techniques have strengthened the species paradigm by
identifying cryptic species among certain types of listed
morpho-species [8,10,11], conservationists cannot yet rely
on them [12–14] to recognize functionally distinct forms or

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<th>My. scabrinodis turf (cm)</th>
<th>Myrmica rubra turf (cm)</th>
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After living 6 weeks with laboratory ants, both types of
social parasite resembled their natural and artificial hosts
significantly more closely than did pre-adoption larvae
(t_{24} = 4.28 to −18.41, p < 0.001, see the electronic
supplementary material, table S2 for full statistics). However,
5 days of isolation, the profiles of Spanish
rebeli reared unnaturally with My. sabuleti, and Polish
rebeli with My. schencki, shifted to resemble their natural
model more closely (t_{28} = 7.38, p < 0.001 and t_{27} = 3.26;
p = 0.003, respectively). In particular, the former lost one
compound, tentatively identified as 1-methyl-tricosane (see the electronic
supplementary material, table S3), which it had evi-
dently acquired from My. sabuleti and which was absent from
My. schencki, and instead started synthesizing heptacosane
and 3-methyl-tricosane, diagnostic hydrocarbons of My.
schencki which were absent or just detectable on My. sabuleti.
Similarly, isolated Polish rebeli lost dotriacontane and
octacosane acquired from My. schencki (but undetectable on
My. sabuleti) and gained docosane, an n-alkane charac-
teristic of My. sabuleti, but not of My. schencki. It is noteworthy that
two of these three emerging mimetic hydrocarbons (docosane,
3-methyl-tricosane) synthesized by isolated 7-week-old
Ma. rebeli larvae were absent from the simpler profiles secre-
ted by pre-adoption larvae. By contrast, individuals reared
with their natural host did not change significantly (Spanish
rebeli with My. schencki, t_{28} = 0.99, p = 0.327) or became
less like it (Polish rebeli with My. sabuleti, t_{10} = 5.03,
p = < 0.001) after isolation.

(d) Niches of host ants in grassland
We found that My. schencki inhabits shorter turf than
My. sabuleti in the xerotypic grasslands that support Ma.
rebeli in the Pyrenees (table 1: My. schencki ≠ My. sabuleti
t_{28} = 3.05, p = 0.003; My. sabuleti ≠ My. scabrinodis
t_{28} = 6.37, p = 0.001; My. scabrinodis ≠ Myrmica rubra ns).
Similarly, as befits the more thermophilous ant, we observed
My. schencki predominantly in well-grazed swards on skeletal
soils in Poland.

4. Discussion
Our results reveal a major difference in the physiology of
populations of Ma. rebeli in Spain and southeast Poland,
Enabling each social parasite to infiltrate and exploit a
very different Myrmica host society—a degree of specialization
that makes each incompatible with the other’s host
species. By contrast, some taxonomists consider Ma. rebeli
itself to be a mere ecotype of Ma. alcon rather than a true
species. On current knowledge, the known hosts of these
two cuckoo Maculinea belong to three distinct groups of
Myrmica [38]: rubra (includes ruginodis), scabrinodis (includes
sabuleti) and lobicornis (includes schencki), of which Ma. alcon
exploits representatives from the first two groups [9] and

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siblings of extreme specialists which perhaps differ by a single gene or which evolve and disappear over millennia rather than epochs, and yet are among the most interesting and threatened species on the Earth.

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