Roccella ramitumidula (Roccellaceae), a new species from the tropical dry forest of Mexico

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Abstract. The new species *Roccella ramitumidula* is described from a tropical dry forest in the Pacific Coast of Mexico. The new species is characterized by fertile thalli, saxicolous habit, irregularly swollen branches and erythrin and lecanoric acid as lichen products. It differs from *R. decipiens* by its narrower and longer ascospores, irregularly swollen branches, uneven surface, and smaller branches. Sequences of the genetic markers ITS, nuLSU and RPB2 from the new species were added to a phylogenetic tree based on four genetic markers that included all the *Roccella* species known for the Americas. The biogeography and ecology of the species is discussed. We reported *R. gracilis* for the first time for the state of Jalisco, Mexico.

Keywords. New species, biogeography, Chamela-Cuixmala Biosphere Reserve, hurricane effects, lichen systematics, phylogeny.

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Roccella DC. is a fruticose genus in the Roccellaceae associated with trentepohlioid algae. Species in the genus predominantly inhabit subtropical coastal areas with Mediterranean climate but also extend into tropical and temperate areas. The genus *Roccella* with only one notable exception, *R. montagnei*, is predominantly distributed over the northern hemisphere (Tehler et al. 2010). On the American Pacific coast *Roccella* is absent south of the Atacama Desert where it is replaced by fruticose *Roccellina* and *Austroroccella* (Tehler et al. 2013). The genus *Roccella* received much attention in the last decades (e.g., Follmann 2001), but recent monographic treatments greatly increase our understanding of the genus (Tehler et al. 2004, 2009a,b, 2010). Most species of *Roccella* have limited geographical distributions, except for *R. gracilis*, *R. montagnei* and *R. tinctoria*. Currently there are 24 accepted species in the genus, all of them distinguishable by a combination of morphology, chemistry and molecular data (Tehler et al. 2010), but see Aptroot & Schumm (2011) for a different point of view, and Tehler (2012) for a response.

In the Americas, the nine species of *Roccella* present are endemic and form a monophyletic group sister to the Macaronesian species (Tehler et al. 2009b). Their main distribution is along the Pacific Ocean, from U.S.A. (California) to Peru, and with various localities in the Gulf of Mexico and the Caribbean Sea (Guzmán Guillermo et al. 2020; Tehler et al. 2009a,b). In North America, the only three species present, *R. bajasurensis*, *R. decipiens* and *R. gracilis*, form a monophyletic group.

In this study we use molecular, morphological and chemical evidence to describe a new species of *Roccella* from the Pacific Coast of Mexico that was resolved in the same monophyletic group as the other species in North America.

Methods

Study area. We collected all samples of the new species from tropical dry forests around the Chamela-Cuixmala Biosphere Reserve, in the Pacific Coast of Mexico. The reserve and surrounded areas have abundant and diverse lichen communities

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dominated by the families Arthoniaceae, Graphidaceae and Pyrenulaceae (Herrera-Campos et al. 2019; Miranda-González & McCune 2020). Detailed descriptions of the study area are found elsewhere (Maass et al. 2018; Noguera et al. 2002).

Anatomical studies. Specimens were studied using standard techniques in a Zeiss Stemi DV4 dissecting microscope and an Euromex iscope compound microscope connected to an AmScope MU1803 digital camera. Thallus photographs were taken with a Nikon D5300 digital camera. Sections and all anatomical measurements were made in tap water. Thin layer chromatography (TLC) was performed in solvent C using the standard techniques in Elix (2014) and McCune (2017). Following Tehler et al. (2009b), *Cetraria islandica* and *R. decipiens* were used as control species for the identification of secondary metabolites.

Taxon sampling. We added the new sequences generated in this study to the phylogenetic analysis of Tehler et al. (2009b) that included all the species of *Roccella* known from the Americas with an emphasis on Mexican species. The phylogenetic analyses include the following four genetic markers, although new sequences were generated for only the first three: the nuclear ribosomal internal transcribed spacer (ITS), the nuclear large subunit of the ribosomal (nuLSU), the second largest RNA polymerase subunit (RPB2), and an anonymous locus probably related to the calmodulin gene; see Tehler et al. (2009a) for further details. GenBank accession numbers of all sequences used are available as **Supplementary Table S1**.

DNA extraction, PCR, and sequencing. For specimens collected by M. A. Herrera-Campos et al., a piece of thallus of around 2 mm in diameter was detached and washed in acetone for five minutes at 70°C, followed by two more acetone washes at room temperature. Genomic DNA was isolated using the Sigma-Aldrich REDExtract-N-Amp Plant PCR Kit following the manufacturer's instructions, except only 15 μL of extraction buffer and 15 μL of dilution buffer were used per sample. For specimens collected by A. Tehler, genomic DNA was isolated from recently collected samples using the DNeasy Plant Kit (Qiagen), following the manufacturer's instructions. The genetic markers ITS, nuLSU, and RPB2 were amplified and sequenced using the following combination of primers: ITS1F/ITS2, ITS3/ITS4 (Gardes & Bruns 1993; White et al. 1990), AL2R/LR6 (Mangold et al. 2008; Vilgalys & Hester 1990), and fRPB2-7cF/fRPB2-11aR (Liu et al. 1999), respectively.

For ITS and nuLSU, each 10 μL PCR reaction consisted of 5 μL R4775 Sigma-Aldrich REDExtract-n-Amp PCR Ready Mix, 0.5 μL of each primer (10 μM), 3 μL water, and 1 μL DNA template diluted at 1:10. The PCR cycling conditions were: 95°C for 5 min, followed by 35 cycles of 94°C for 60 s, 52°C (for ITS) or 57°C (for nuLSU) for 45 s, and 72°C for 105 s, followed by 72°C for 10 min. For RPB2 we used hot-start touchdown PCR. The PCR cycling conditions included an initial denaturation at 95°C for 5 min, followed by 38 cycles of 95°C for 30 s, 58–52°C for 30 s, 72°C for 1 min and 30 s, followed by 72°C for 5 min. The annealing temperature was successively decreased where the three first temperatures (58°C, 56°C and 54°C) were run for two cycles each and the remaining 32 cycles at 52°C degrees. PCR products (2 μL each) were visualized on 1.5% TBA agarose gel stained with GelRed (Biotium). PCR products were cleaned with ExoSAP-IT (ThermoFisher).

Molecular work, including sequencing, was done at the Laboratorio de Biología Molecular, as part of the Laboratorio Nacional de Biodiversidad, in the Instituto de Biología of the Universidad Nacional Autónoma de México and at Naturhistoriska riksmuseet (Swedish Museum of Natural History).

Phylogenetic analysis. New sequences were edited in Geneious R11 (Kearse et al. 2012). All sequences from each of the genetic markers were aligned independently with MAFFT (Katoh & Standley 2013) and manually corrected. The final concatenated alignment is available as **Supplementary Table S2**. A maximum likelihood (ML) analysis of all markers partitioned by marker was performed using RAxML 8.2.11 (Stamatakis 2014), with 550 bootstrapping replicates. Furthermore, a Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist et al. 2012), with one million generations and default settings. Both analyses were done with the GTR GAMMA I model and run on Geneious R11. The final ML tree was plotted in Geneious R11 and edited in Photoshop.

Results

Phylogenetic analysis. A total of 13 new sequences were generated in this study, 12 of a

new species of *Roccella* (described below as *R. ramitumidula*) and one of *R. gracilis* (**Supplementary Table S1**). The final alignment consisted of 21 taxa, including the outgroup, and 3055 aligned characters, 698 from ITS, 919 from nuLSU, 923 from RPB2, and 515 from the anonymous locus, of which 450, 192, 235, and 369, respectively, were phylogenetically informative. The combined Maximum Likelihood and Bayesian analyses (**Fig. 1**) agreed with previously published results (Tehler et al. 2009b, 2010) and resolved all *Roccella* species from the Americas as a monophyletic group. *Roccella ramitumidula* was resolved as sister group to *R. bajasurensis* in a monophyletic group with the other North American species.

Taxonomy

Roccella ramitumidula R.Miranda, G.Epitacio, Tehler, N.Sánchez & Herrera-Camp. *sp. nov*.

Fig. 2

MycoBank MB 844376

ITS barcoding sequence accessions: ON965301–ON965306

Differing from Roccella decipiens by the narrower and longer ascospores, irregularly swollen branches, uneven surface, and smaller branches. Fertile, saxicolous, with erythrin and lecanoric acid as main lichen substances.

Type: MEXICO. Jalisco: La Huerta Mun., Negritos beach, tropical dry forest, 19°31′44.7″N, 105°04′59.7″W, elev. 7 m, on vertical rocks exposed to the sea breeze, May 2008, *M.A. Herrera-Campos et al. 2008 336-58* (holotype, MEXU!).

Description. Thallus erect to pendent, branches irregularly swollen, not completely flat but not terete either, usually up to 4 mm wide and up to 8 cm long, grey to grey-brown, surface uneven with ridges and depressions, with scattered pruina; soredia absent; medulla white to pale yellow but usually brown in the holdfast zone. Ascomata present, up to 2 mm wide, margin circular to heavily crenulate, solitary to crowded, disc brown with white pruina, initially adnate and eventually sessile; ascospores hyaline, 3-septate, fusiform, straight to curve, 16–30 × 3.5–5.5(–6) μm (mean 24.9 × 4.6 μm, n = 60). Spot test: cortex K+ faint yellowish red, C+ red; medulla K–, C–; disc C+ red.

Chemistry. Erythrin and lecanoric acid.

Etymology. The epithet refers to the irregularly swollen branches that are not flattened but not terete either.

Ecology and distribution. *Roccella ramitumidula* is, so far, only known from the coast of Jalisco, Mexico on rocks exposed to the sea breeze. At the time of collection, the species covered abundantly the exposed rocks on the type locality, but no further expeditions to the same area were made to update its current situation.

Remarks. *Roccella ramitumidula* is characterized by irregularly swollen branches that are not quite flat nor terete, a fertile and esorediate thallus, and the presence of erythrin and lecanoric acid. The new species is the sister group of *R. bajasurensis*, together forming a monophyletic clade with *R. decipiens* and *R. gracilis* (**Fig. 1**). The branches of *R. ramitumidula* are morphologically intermediate between the terete branches of *R. bajasurensis* and the flat branches of *R. decipiens* and *R. gracilis*. Besides differences in the shape of the branches, *R. ramitumidula* is morphologically similar to *R. decipiens*, but the latter usually has a smoother surface, a larger thallus, and ascospores of 16–24 × 5–6.5 μm (Tehler et al. 2009b). *Roccella gracilis* is almost always sorediate, usually corticolous, has a yellowish medulla in the holdfast area, and has a softer thallus that is easily breakable. Rarely, specimens of *R. gracilis* are fertile but those are strictly corticolous (Tehler 2011). *Roccella bajasurensis*, although genetically closer, further differs by a whitish thallus and the presence of protolichesterinic and lichesterinic acids instead of erythrin and lecanoric acid. In South America, *R. verruculosa* somewhat resembles *R. ramitumidula*, but differs by its clearly terete branches or in some specimens with clearly flat branches instead (see discussion below).

Additional specimens examined (paratypes). MEXICO. Jalisco: Cabo Corrientes Mun., Selva El Tuito, Los Corrales, 20°24′27″N, 105°40′20″W, on boulders and rocky outcrops on S side of the beach, Jan. 2016, *A. Tehler 10924* (S catalogue no. F280387). Cihuatlán Mun., Isla Navidad, 19°10′32″N, 104°41′27″W, cliffs on the south coast of the peninsula just N of the beach, on outcrops by the sea, Jan. 2016, *A. Tehler 10973* (S catalogue no. F280409). La Huerta Mun., Negritos beach, same locality as the type collection, 19°31′44.7″N,

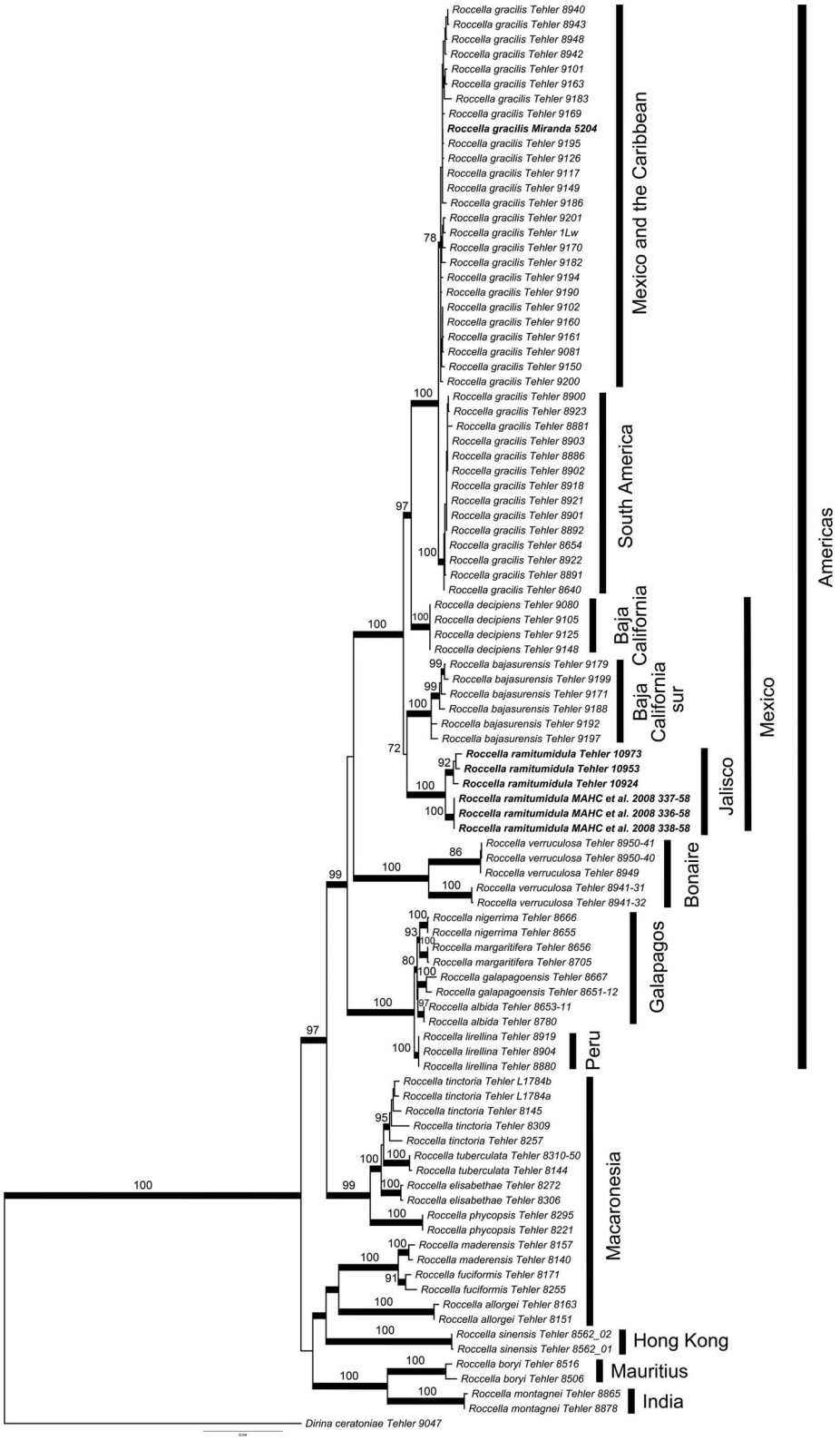

Figure 1. Phylogeny of *Roccella* species with an emphasis on the species from the Americas (after Tehler et al. 2009b), based on a Maximum Likelihood analysis of the markers ITS, nuLSU, RPB2, and an anonymous locus. Support values are shown as numbers if Maximum Likelihood bootstrap values ≥70 and as bold branches if Bayesian posterior probabilities ≥0.95. Bold names show new sequences from this study.

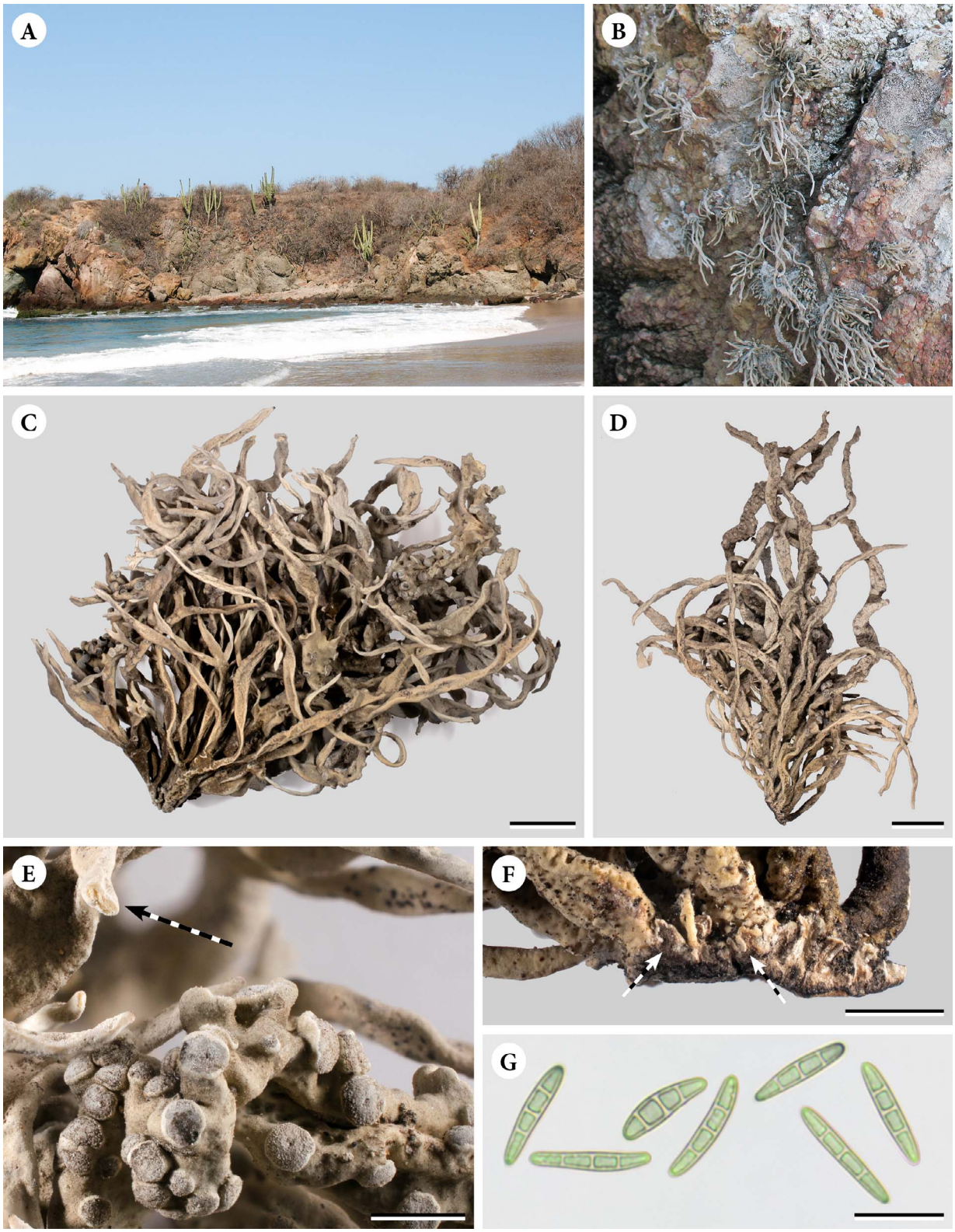

Figure 2. *Roccella ramitumidula*. **A.** Type locality during the dry season. **B.** Specimens *in situ* showing saxicolous habit. **C–D.** Thallus with irregularly swollen branches. **E.** Close up of apothecia; notice a branch with exposed pale yellowish medulla (arrow). **F.** Holdfast showing the exposed brownish medulla (arrows). **G.** Narrow ascospores. Scale: C–D 1 cm; E–F 0.25 cm; G 20 µm. Specimens: C, E *M.A. Herrera-Campos et al. 2008 336-58* (Type); D, F *M.A. Herrera-Campos et al. 2008 337-58*.

105°04′59.7″W, on vertical rocks exposed to the sea breeze, May 2008, *M.A. Herrera-Campos et al. 2008 337-58*, *338-58* (MEXU). Puerto Vallarta Mun., 3 km S of Puerto Vallarta, seaside cliff along Hwy 200, on exposed granite, elev. 20 m., Mar. 1982, *T.H. Nash III 20758* (S catalogue nos. L9702, L62126); *Ibid*.; along Hwy 200 north of Mismaloya just opposite Los Acros National Underwater Park, 20°32′34″N, 105°17′16″W, on boulders at the beach, Jan. 2016, *A. Tehler 10908* (S catalogue no. F280375); *ibid*.; Boca de Tomatlán south of Puerto Vallarta, 20°30′45″N, 105°19′36″W, along the trail to Playa las Animas, on vertical cliffs and boulders, Jan. 2016, *A. Tehler 10953* (S catalogue no. F280401).

Key to the North American species of *Roccella*

1. Thallus corticolous, mostly sorediate but rarely fertile ***R. gracilis***
1. Thallus saxicolous, mostly fertile but rarely sorediate 2
2. Thallus sorediate ..... ***R. gracilis***
2. Thallus fertile 3
3. Branches clearly terete, cortex C– (protolichesterinic and lichesterinic acids) ..... ***R. bajasurensis***
3. Branches irregularly swollen or clearly flat but not terete, cortex C+ red (erythrin and lecanoric acid) 4
4. Branches irregularly swollen, medulla in holdfast brown ..... ***R. ramitumidula***
4. Branches clearly flat, medulla in holdfast yellow 5
5. Ascospores 5–6.5 µm wide, thallus with a robust consistency ..... ***R. decipiens***
5. Ascospores 3–4 µm wide, thallus easily breakable and with a fragile consistency. Although all known fertile specimens of this species are corticolous, it is expected that saxicolous fertile specimens exist as well ..... ***R. gracilis***

Discussion

Biogeography. Our results support the idea of strongly limited geographical distribution for the *Roccella* species of the Americas, except for *R. gracilis* that has a wider range. Even for *R. gracilis*, our sequence from the Pacific Coast of Jalisco, Mexico cluster together with sequences from the Baja California Peninsula in a subgroup separated from the sequences of *R. gracilis* from South America.

The new species *R. ramitumidula* was resolved as sister to *R. bajasurensis* (**Fig. 1**). Although phylogenetically close, both species are easily separated by morphology and chemistry. Both are endemic to Mexico and have a narrow and allopatric geographical distribution; *R. bajasurensis* is only known from the south of Baja California Sur and *R. ramitumidula* is only known from the coast of Jalisco. Given that the separation of the Baja California Peninsula from mainland Mexico started only about 5 million years ago (Grismer 1994), it is probable that both species evolved following a vicariance process. This timing of species diversification is similar to the observed range for other species of lichens (e.g., Divakar et al. 2017).

Previous studies suggest that the center of origin of *Roccella* was somewhere between the Macaronesian and the Horn of Africa regions and that the first species to arrive to the Americas was the predecessor to *R. verruculosa* (Tehler et al. 2009b, 2010). From *R. verruculosa*, two groups were formed, one radiating into the *R. galapagoensis* group in Galapagos and Peru, and another one radiating into the four species around the Baja California Peninsula (Tehler et al. 2009b), including the new species described here.

Our phylogenetic analysis suggests that *Roccella verruculosa* could represent two separate species for two reasons. First, our analysis shows two different clades of *R. verruculosa*, that although monophyletic, are united by branches of similar length as the ones separating *R. bajasurensis* from *R. ramitumidula* and several times longer than the branches separating the species in the *R. galapagoensis* group. Second, according to Tehler et al. (2009b), *R. verruculosa* usually has terete branches, but some specimens develop flat branches instead. The shape of the branches is an important diagnostic character for several species in the genus. If the two different clades in the phylogenetic tree are correlated with two different morphologies, then we would suggest separating them into two species. However, as we did not review original material from that group for this study we refrain from any taxonomic changes.

Hurricane effects. Even though species of *Roccella* tend to have narrow geographical distributions, when present, they can be locally abundant. The genus is adapted to grow in coastal habitats that due to natural conditions and climate change are increasingly being subject to extreme weather conditions. This was the case for the populations of the *R. galapagoensis* group that suffered considerable damaged during the heavy and prolong rainfalls of the El Niño year in 1983 (Weber & Beck 1985). In our case, *R. gracilis* covered abundantly the canopy of the trees of the Chamela-Cuixmala Biosphere Reserve, but the hurricane Jova in 2011 destroyed most of the thalli in the area (RMG personal observations). Furthermore, the hurricane

Patricia in 2015 removed most of the canopy (Parker et al. 2018), including the majority of macrolichens in the area (Miranda-González & McCune 2020). To this day, the populations of *R. gracilis* have not yet recovered and are rarely encountered in the area. Although we did not revisit the type locality of *R. ramitumidula* after the hurricanes, we expect that the once abundant thalli suffered considerable damage as well. Fortunately, collections made in January of 2016 along the coast of Jalisco showed that the species did not disappear from the region.

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Supplementary documents online:

Supplementary Table S1. GenBank accession numbers for sequences used in the phylogenetic analyses. Bold letters indicate new sequences generated in this study. – indicates missing data. All new sequences are from specimens collected in Jalisco, Mexico, with herbarium names between parentheses.

Supplementary Table S2. Concatenated alignment of the markers ITS, nuLSU, RPB2, and an anonymous locus used for the phylogenetic analyses.