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Current knowledge and perspectives of study on benthic toxic dinoflagellates of the genus *Gambierdiscus* in the Canary Islands

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Summary
Species of the dinoflagellate genus *Gambierdiscus* are responsible of Ciguatera Fish Poisoning (CFP) which is very common in tropical areas, mainly the Pacific Ocean and Caribbean Sea. Until 2008 this disease was not reported in the NE Atlantic Ocean, when human intoxications after consumption of local fish have been reported almost yearly in the Canary Islands. Sampling on rocky shores of this archipelago revealed an unexpected diversity of the genus *Gambierdiscus*. Morphological and phylogenetic study established the presence of at least 3 species: *G. excentricus* (described in the Canaries), *G. australes*, and a third species (*G. silvae* sp nov.), close to *G. polynesiensis* and *Gambierdiscus* sp. type 4 *sensu* Xu *et al*. (2014). The evaluation of their toxicity was performed by mouse bioassay (MBA) and erythrocyte lysis assay (ELA). Additionally, identification of the CTXs congeners were conducted using liquid chromatography (LC) coupled with high resolution mass spectrometry (HRMS). Nevertheless, the link between local *Gambierdiscus* populations and ciguateric fish has not been unambiguously demonstrated. Future studies should focus on the phylogeography of *Gambierdiscus*, their distribution, abundance, and the characterization of ciguatoxins in cultures *vs* those in local fishes to understand if these are accumulated in Canaries or other areas.

Introduction
At present 12 species are recognized in the genus *Gambierdiscus*, the causative agent of ciguatera, and several of these species often co-occur in the same area (Litaker *et al*. 2010), although their diversity is difficult to recognize due to subtle morphological differences among them. In this presentation, new information on the presence of *Gambierdiscus* in the Canary Islands is given, with the first report of *G. australes* in the Atlantic Ocean and the description of *G. silvae* sp. nov., on the basis of morphological and molecular evidence.

Materials and Methods
Source of specimens and culture conditions. Seawater from macroalgal samples in tidal ponds of the Canaries archipelago (Gran Canaria, Tenerife, La Palma and La Gomera Islands) was obtained in 2004-2013. Isolated *Gambierdiscus* cells were incubated in full strength K/2 medium with a salinity of 34, at 24 ºC, 90 μmol m-2•s-1 of PAR and 14:10 L:D.

Microscopy. Cells were observed alive or fixed with formalin. For plate pattern identification staining with Fluorescent Brightner 28 (Sigma, St Louis, MO, USA) following a modified Fritz and Triemer (1985) technique, or dissected cells were used. SYBR Green (Molecular Probes, Eugene, OR, USA) was employed for nuclei staining (Figueroa and Bravo 2005). For scanning electron microscopy cultures (5 mL) were fixed with GTA 2%, rinsed with distilled water and dehydrated in EtOH followed by Hexamethyldisilazane. Samples were coated with gold and observed with a Phillips XL30 or a FEI Quanta 200 SEM (FEI Company, Hillsboro, OR, USA).

Molecular analyses. *Gambierdiscus* cultures were harvested by centrifugation. DNA extraction followed Richlen and Barber (2005). The D1–D3 and D8–D10 regions of the LSUrRNA gene were

amplified using the primers D1R/LSUB and FD8/RB (see Fraga *et al* 2011). The phylogenetic relationships were represented using the results from Bayesian inference with posterior probabibilities and bootstrap values (n=1000) from ML.

Toxins analyses. Methanolic crude extracts were purified using liquid/liquid solvent partition with dichloromethane. MBA analyses were performed by Fabiola Arévalo at the Biotoxins laboratory of the INTECMAR (Xunta de Galicia, Spain). Aliquots of the original extract, the dichloromethane and aqueous metanol soluble fractions were dried under vacuum, resuspended in aqueous Tween 60 1% and then ip injected to healthy male Swiss mice NMRI (weight 20±1g). Mice were observed over 24 hours recording signs and time of death. The Neuro-2a CBAs specific for CTXs and MTXs (detailed in Fraga *et al* 2011), were used for the determination of CTX- and MTX-like toxicity in *G. excentricus* crude extracts. Mass spectrometry analysis were done on a Thermo Scientific Dionex High-Speed LC coupled to an Exactive mass spectrometer with an Orbitrap MS analyzer and a HESI-II probe. Toxins separation followed Yogi *et al.* (2011). For toxins identification a standard solution of CTX4A, CTX4B, 52-epi-54-deoxy CTX1B, 54-deoxy CTX1B and CTX3C was kindly provided by professor Yasumoto.

Results and Discussion

Gambierdiscus diversity. Three *Gambierdiscus* species were identified in the Canaries using morphology and molecular results. *G. excentricus* was first recorded (Fraga *et al*. 2011), and described in three sampling sites (rocky shores and tidal ponds in Tenerife, La Palma and La Gomera islands) in years 2004, 2005 and 2010. *G. excentricus* was already reported as a CTX- and MTX-like compounds producer using the Neuroblastoma cell-based assays for ciguatoxins (CTX) and maitotoxin (MTX) (Fraga *et al*. 2011). In 2010 and 2013 there were isolated *G. australes* and *G. silvae* in Gran Canaria and Tenerife islands. This was the first record of *G. australes* in the Atlantic and *G. silvae*, formerly *Gambierdiscus* ribotype I, was formally characterized described (Fraga and Rodríguez, *submitted*). *G. silvae* toxins extracts injected i.p. showed symptoms typical of CTXs bioactivity. Death happened by ataxia but differences were recorded in the death times: within 4 hours with the MeOH 60% sample (containing MTX compounds), and within 7 hours with the diclorometh ane sample (containing CTX compounds). HR full MS experiments indicated presence of 2,3 dihydroxi CTX3C and CTX1B or P-CTX1 in *G. silvae*. A comprehensive study on the phylogeography and abundance of *Gambierdiscus* as well as the characterization of ciguatoxins in cultures *vs* those in local fishes is required to demonstrate a link between local *Gambierdiscus* and ciguateric fish responsible of CFP in the Canaries.

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