Genetic diversity and biological activity of *Curcuma longa* ecotypes from Rapa Nui using molecular markers

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**Abstract**

Curcuma Longa (*CL*) has been used for hundreds of years by native people from Rapa Nui for the treatment of different illness. Despite this plant was introduced from Polynesia or India, there is still scarce information about its origin. The objective of this study was to analyze the genetic variation of three *CL* ecotypes based on molecular phylogenetic and genotypification using internal transcribed spacer 2 (ITS2) and simple sequence repeats (SSR). Antioxidant and anti-inflammatory properties of rhizomes and leaves extracts of three *CL* plants were analyzed by spectrophotometric methods and cyclooxygenase 2 (COX-2) inhibition assay. Complementarily, we predicted the potential binding mode and binding energy of curcuminoids and nonsteroidal anti-inflammatory drugs (NSAIDs) into COX-2 via molecular docking. The ITS2 sequence shows two major clusters (I and II), group I consisted of *Curcuma haritha* and group II consisted of different species of Curcuma and Rapa Nui samples (MR-1, MR-2 and RK-2). Results of SSR markers show that genotype MR-2 was similar to MR-1 and RK-2 with 70.8 and 42.9% similarity, whereas genotype was similar to RK-2, MR-1 and MR-2 with 63.9, 43.2 and 42.9% similarity, respectively. MR-1 have better antioxidant and autoinflammatory activity than rest of *CL* samples due to its high concentration of polyphenols (33.68 mg/g) and curcumin (29.69 mg/g). Furthermore, docking results show that three curcuminoids of *CL* and selective NAIDs, as celecoxib, etodolac and meloxicam, share the same binding pocket into COX-2. However, three curcuminoids have a lower ΔGbinding than other COX-2 selective NAIDs as etodolac and meloxicam, except for Coxib family as valdecoxib, celecoxib and rofecoxib. Our findings suggest MR-1, MR-2 and MK-2 from Germplasm Bank (Mataveri Otai of CONAF) are closely related to *Curcuma amada* and *Curcuma montana* even though they have genetic variability.

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1. Introduction

The genus *Curcuma*, belonging to the family Zingiberaceae, includes approximately 100 species around the world and widely used in the traditional medicine in China and India (Sigrist et al., 2010). The rhizome of *Curcuma longa* (*CL*) is widely used as integral ingredient of Indian cooking, medicinal drug and to a lesser extent as cosmetic and food preservatives (Prasad and Aggarwal, 2011; Xiang et al., 2011). Among the compounds found in *CL* are essential oils, minerals, polyphenolic and terpenoid compounds (Duvoix et al., 2005; Hatcher et al., 2008). Among these polyphenolic compounds, curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the most important compound because of its therapeutic or pharmacological properties. Indeed, anti-inflammatory, antifungal and antitumoral activities are principally attributed to the content of curcumin and its analogs (Fig. 1) (Siju et al., 2010; Strimpakos and Sharma, 2008; Tomeh et al., 2019). Other species of *curcuma* are also widely used for medicine and culinary use as *C. aromatica*, *C. amada* *C. kwangsiensis*, *C. ochrorhiza*, *C. pierreana* *C. zedoaria*, *C. malabarica*, among others (Sasikumar, 2005).

*CL* is also found in Chile, specifically in Rapa Nui Island, where is considered a magical plant for the native people, being used for...
treatment of several diseases as cancer, diabetes, muscle pain and other illness (Kocaadam and Şanlier, 2017). Studies, reported between 1891 and 2005, suggest that CL is a plant introduced from Polynesia and probably native from India (Dubios et al., 2013). Nonetheless, until now its origin is unknown, and its massive use compromises the preservation of this species. Furthermore, some studies have reported that exist a difference in the content of curcumin and others active molecules among individuals of CL. This difference of chemical profile could be due to genetic variety and distribution geographic (Arya et al., 2016; Hayakawa et al., 2011). For this reason, it is necessary to use molecular markers to an appropriate characterization of CL found in Rapa Nui. DNA barcoding is a molecular diagnostic technique that allows the identification of species using a short a standardized DNA region (Arif et al., 2011). The ITS2 is part of the eukaryotic nuclear rDNA (between the 5.8S and the 28S rRNA) and allows to carry out phylogenetic analyses and classification at the species level using highly conserved priming sites to DNA amplification (Feng et al., 2016; Arif et al., 2011). In addition, genomic microsatellite markers, as the use of simple sequence repeat markers (SSR), has been utilized for a specific genotyping in a wide variety of plants (Siju et al., 2010; Sigrist et al., 2011; Tóth et al., 2000).

The objective of this study was to analyze the genetic variation of three CL ecotypes, from Mataveri Otai reservoir in Hanga Roa, based on molecular phylogenetic and genotypification using internal transcribed spacer 2 (ITS2) and simple sequence repeats (SSR). Furthermore, we evaluated the impact of the genetic variation on the content of active metabolites as curcumin, phenolic and tannins (Siju et al., 2010). Furthermore, we investigated the antioxidant properties in rhizomes and leaves to analyze the relationship between the metabolite contents and the genetic variability of three CL plants found in Rapa Nui. Additionally, we analysis the anti-inflammatory properties of extracts from CL plants through cyclooxygenase-2 (COX-2) inhibition activity assay and we predicted the potential binding mode and binding energy of curcuminoids and traditional nonsteriodals anti-inflammatory drugs (NSAIDs) into COX-2 via molecular docking. The anti-inflammatory mechanism on COX-2 may contribute in medicinal actions of CL plants - as anticarcinogenic - for being used for treatment of different types of cancer as colon, breast cancer, and other tumors that overexpress COX-2 (Xu et al., 2014). Finally, polymorphic and genetic variability information and its relationship with the biological activity of studied three CL plants could be relevant for an adequate culture and propagation of this vulnerable specie in Rapa Nui.
2. Materials and methods

2.1. Chemicals and plant material

2.1.1. Chemicals
Gallic acid, 2,2-diphenyl-1-picyrylhydrazyl radical (DPPH), AAPH, TROLOX, ethanol and n-hexane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Curcumin, Folin-Ciochette reagent, sodium carbonate and methanol (MeOH) were purchased from Merck Co.

2.1.2. Plant material
Three CL plants were obtained from the Germplasm Bank of Mataveri Otai of CONAF in Rapa Nui (geographic coordinates: 27°09’50”, 17°5-10’26”24’630”). These CL plants were named as MR-1, MR-2 and RK-2 and used in this investigation. Furthermore, a rhizome sample of CL from Peru (Continental Curcuma or CC) was used as a control to validate the results of SSR markers. The collected samples were packed into sterile polybags and its transportation was carried out by 4 °C.

2.2. Genomic diversity analysis

2.2.1. Genomic DNA extraction
Leaves and rhizomes from MR-1, MR-2, RK-2 and CC were cleaned, washed with free nuclease water. The DNA extraction of samples was performed using the NucleoSpin® Plant II kit (Machery-Nagel, Düren, Germany), according to manufacturer’s instructions. The DNA integrity was analyzed using 0.8% agarose gel, stained with ethidium bromide, the quantity and quality of the DNA was evaluate measurement the absorbance to 260 nm and the ratio 260/280 nm using EPOCH microplate reader (BioTek® Instruments).

2.2.2. Genetic identification of different CL samples
The nrRNA ITS2 region were analyzed by PCR amplification. The sequencing of PCR products (Seoul, South Korea; www.macrogen.com) were used to identify the species of CL from Rapa Nui. ITS2 sequences were retrieved from GenBank to identify homologous sequences using BLAST search. The ITS2 primers (Forward: ATGGCATACCTGTTGGAAT and Reverse: GACGCTTCTCCAGACTACAAT) were used to identify CL samples as described by Chen et al. (2010). The PCR reaction was performed in a 20 μL reaction system containing 2.5 mM MgCl₂, 0.2 mM dNTP’s, 0.25 μL of each primer, 50 ng of genomic DNA and 0.5 μL of enzyme (1U/μL, KAPA HiFi HotStart, Biosystems). PCR reactions were performed on a T100TM Thermal Cycler (BioRad, Hercules, CA, USA) using the following temperature cycling parameters: initial denaturation for 3 min at 95 °C followed by 35 cycles of denaturation at 98 °C for 40 sec, annealing temperature at 52 °C for 20 sec and extension at 72 °C for 20 sec, and a final extension step at 72 °C for 5 min. PCR product were separated using 2% agarose gel stained with ethidium bromide and the size of the amplifications was estimated using 50 bp DNA Ladder (New England, Biolabs). To construct a phylogenetic tree based on the ITS sequences of different Curcuma species, the amplified regions were aligned using ClustalW (Thompson et al., 1994). Phylogenetic relationships were analyzed using the neighbor-joining (NJ) method using the JALVIEW program (Waterhouse et al., 2009).

2.2.3. Analysis of SSR marker and interpretation of sequence of CL plants
The polymorphisms and genetic relationships of three CL plants were evaluated using a set of eight SSR markers to CL designed as Curcuma microsatellite (CuMiSat) (Siju et al., 2010) showed in the Table 1. The PCR reaction was performed as indicated above and the PCR products were analyzed using 3% agarose electrophoresis (UltraPure™ Agarose-1000) and stained with ethidium bromide. Pictures of the pattern of bands were taken and stored as digital images to be analyzed (Figure S1). Reproducible data (number of PCR fragment, sharp bands) were used to estimate the genetic similarity between CL accessions. Data were annotated as presence (1) and absence (0) of bands. A total of 37 microsatellites (PCR fragment) were selected in order to analyze the genetic variability between nine CL plants and CC. Dendrogram was generated using the UPGMA sequential clustering method through the DendoUPGMA web server (URL: genomes.urv.cat/UPGMA) (García-Valle et al., 1999), whose data were recorded in a binary matrix, as individual accessions, using the Jaccard coefficient to compare the set of variables (Chattopadhyay, 2017; Siju et al., 2010).

2.3. Determination of metabolite composition and antioxidant activity of extract from CL plants

2.3.1. Extract preparation
Total phenolic compounds as polyphenols, tannins and curcumin were determined in samples from leaves and rhizomes of MR-1, MR-2, RK-2 and CC (as control). The samples from leaves and rhizomes were cleaned, washed with deionized water, and dried at 40 °C for two weeks (see Supplementary Table S1). After drying, the rhizomes and leaves samples were ground to a powder (20 g approximately) and the lipids were subsequently extracted by Soxhlet with n-hexane as described by (Efthymiopoulos et al., 2019). Then, the extract solid from rhizome and leaves were re-extracted with 90% ethanol. Briefly, 90% ethanol extract was prepared by adding extracts solids (MR1-Leaves: 2,129 g; MR-1-Rhizome: 1,789 g; MR-2 Leaves: 1,240 g; MR-2 Rhizome: 1,355 g; RK-2 Leaves: 1,741 g; RK-2 Rhizome: 1,115 g; CC: 2,784 g), the solutions were placed in the dark to avoid oxidation of ethanolic extracts and were shaken in a shaker for 72 h at 37 °C. Then, the solutions were filtered through Whatman No. 1 filter paper. Then, these extracts were dried and concentrated using a rotary evaporator (Heidolph, Germany) under reduced pressure (100 psi) at 40 °C. Finally, the extracts were resuspended in DMSO at 1 mg/mL as stock solution and preserved at −20 °C until use.

2.3.2. Determination of total phenolic compounds
Folin-Ciocalteu reagent was used to quantify the total polyphenol content in leaves and rhizomes. To this end, ethanol extract was prepared from leaves and rhizomes as described by Ricco et al. (2015). Briefly, 100 μL of the samples of extracts from leaves and rhizomes (MR-1, MR-2, RK-2) were diluted 1:10 with water and 125 μL of Folin-Ciocalteu 1 N reagent, then were shaken vigorously. Then, 625 μL of 20% Na₂CO₃ was added and samples again were shaken for two hours. Finally, the absorbance was measured at 760 nm in a HPUV 8453 (Agilent, USA) spectrophotometer. The absorbance values were interpolated using a Gallic acid standard curve (0–10 mg/L) and the total phenolic content was expressed as mg of Gallic acid equivalents (GAE) per gram of dried extract. The experiment was performed in triplicate.

2.3.3. Determination of tannins
A modified Folin-Ciocalteu method was used for the quantification of tannins (Ricco et al., 2015). Briefly, 100 μL of extracts from leaves and rhizome (MR-1, MR-2, RK-2) and 500 μL of water were mixed with 250 μL of Folin-Ciocalteu 1 N reagent under vigorous shaken. Then, 35% Na₂CO₃ was added and the sample again was shaken for 30 min. The absorbance was measured at 725 nm and data were interpolated on a calibration curve of gallic acid. The
results are expressed as mg of GAE per g of dried extract as indicated above. These experiments were performed in triplicate.

### 2.3.4. Determination of curcumin

The amount on curcumin in leaves and rhizomes (MR-1, MR-2, RK-2) were analyzed using a curcumin standard calibration curve (1 to 25 µg/mL), according to the modified protocol by Hazra et al. (2015). These experiments were performed in triplicate.

### 2.4. Antioxidant capacity

#### 2.4.1. Antioxidant capacity by DPPH assay

The antioxidant activity of the extracts was evaluated by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Brand-Williams et al., 1995). Briefly, 1 mL of DPPH radical solution 0.1 mM in ethanol was mixed with 50 µL of the extract of leaves and rhizomes in various concentrations (50–800 µg/mL in ethanol). DPPH is reduced by antioxidant compound causing colour changes from purple to yellow. Colour change was measured [Absorbance (Abs)] at 518 nm after 20 min of reaction using an Epoch ELISA microplate reader (BioTek, VT, USA). The percentage of DPPH inhibition was calculated using the following equation:

\[
\% \text{radicals scavenging activity} = \left( \frac{\text{Abscontrol} - \text{Abssample}}{\text{Abscontrol}} \right) \times 100
\]

where Abs control is the absorbance of DPPH in the absence of a sample and Abs sample is the absorbance of DPPH in the presence of a sample or the standard (gallic acid). The radical scavenger activity was expressed in terms of concentration of antioxidants necessary to scavenge 50% of DPPH free radical (IC_{50}). The IC_{50} value for each sample was determined graphically by plotting the percentage disappearance of DPPH as a function of the sample concentration. These experiments were performed in triplicate.

#### 2.4.2. Antioxidant capacity by ORAC-FL assay

The ORAC value was measured according method described by Ou et al. (2001) with modifications from (Dávalos et al., 2004). The reaction was carried out in sodium phosphate buffer (75 mM, pH 7.4) using black-walled 96-well plates in a final volume of 200 µL 20 µL of each extract and fluorescein (120 µL; 70 nM, final concentration) solutions were placed in each well of microplate. The mixture was preincubated for 15 min at 37°C. AAPH solution (60 µL; 12 mM final concentration) and quickly added. Later the microplate was immediately read using a fluorescence reader (Synergy HT multi-detection microplate reader; Bio-Tek Instruments, Inc., Winooski, VT, USA). The fluorescence was recorded for 80 min. The area under the fluorescence decay curve (AUC) was calculated as:

\[
\text{AUC} = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}
\]

where \(f_0\) is the initial fluorescence reading at 0 min and \(f_i\) is the fluorescence reading at time \(i\). The AUC corresponding to a sample is calculated by subtracting the AUC corresponding to the blank space. Regression equations between net AUC and antioxidant concentration are calculated for all samples. ORAC-FL values are expressed as Trolox equivalents using the standard curve calculated for each assay. Results were expressed in µmol of Trolox equivalent/µmol of extract. These experiments were performed in triplicate.

#### 2.4.3. COX-2 inhibitor screening

Assessment of cyclooxygenase-2 (COX-2) enzyme inhibition was carried out by using the BioVision™ “COX-2 Inhibitor Detection Kit (Fluorometric)” kit. Briefly, the bioconversion of arachidonic acid to inflammatory prostaglandins (PGs) is not carried out as consequence of the inhibition of enzyme COX-2. Measurements were made over time, incubating MS extracts (3 µg/mL) and celecoxib (3 µg/mL) (as control of inhibition) measuring fluorescence (Ex/Em = 535/587 nm) in a multichannel reader (Thermo Scientific Skanit™ Appliskan) at 25°C for 10 min. The inhibition % was calculated from the following formula:

\[
\% \text{Relative Inhibition} = \left( \frac{\text{SlopeofEC} - \text{SlopeofS}}{\text{SlopeofEC}} \right) \times 100
\]

### 2.5. Computational details

#### 2.5.1. Molecular docking of ligand-protein interaction

We evaluate the interaction between COX-2 and curcuminoid polyphenols found in C. longa, as curcumin, demethoxycurcumin and bisdemethoxycurcumin, via molecular docking. To this end, the crystal structure of COX-2 (PDB code 3LN1) was retrieved from the Protein Data Bank (Bernstein et al., 2000), whereas the 3D structure of curcumin, demethoxycurcumin and bisdemethoxycurcumin, etodolac, rofecoxib, valdecoxib, lumiracoxib, celecoxib, etoricoxib and meloxicam were retrieved in SDF file format from the PubChem database at NCBI (Pubchem CID: 969516, 5469424, 5315472, 3308, 5090, 11960, 5090, 11960, 5090, 11960, 51166, 2662, 123619, 54677470, respectively). Etodolac, rofecoxib, valdecoxib, celecoxib, etoricoxib and meloxicam were used as reference compounds to compare their affinity to COX-2 with that of curcuminoid polyphenols because they are COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs) (Chen et al., 2008). Here we performed rigid docking taking whole receptor in order to identify the potential binding site and associated binding energy. Both ligands and protein were prepared using AutoDock Tools version 1.5.6 (ADT), as previously described (Guzmán et al., 2020). Finally, graphic analysis of molecular docking studies were performed using VMD (Humphrey et al., 1996).

### 2.6. Statistical analysis

The one-way analysis of variance followed by Tukey's post-test was used for comparisons. A \(p < 0.0001\) was considered as statistically significant difference. All the statistical analyses were calculated using the computer software GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).
Software, San Diego, CA, USA). The concentrations of total polyphenols, curcumin and tannins were expressed as percentage in the extract (mg/g).

3. Results

3.1. Phylogenetic analysis based on ITS2 sequences

In this study, the nrDNA ITS2 region was used to identify species, using phylogenetic analyses, of three *C. longa* plants collected from Germplasm Bank of Mataveri Otai localized within Rapa Nui. The size of the ITS2 sequences used in the analyses ranged from 214 to 272 bp, with an average of 243 bp. The ITS2 sequences of 15 species of *Curcuma* (i.e. *longa*, *amada*, *haritha*, *montana*, *ferruginea*, *aromatica*, *caesia*, *amarissima*, *phaeocaulis*, *yunnanensis*, *kwangsiensis*, *aeruginosa*, *aff. Prakasha*, *zedoaria* and *flaviflora*) were retrieved from GenBank (Table 2). Results indicated that all the analyzed samples of *C. longa* have a 100% of sequence identity against *Curcuma longa* voucher PH1284, and *Curcuma amada*, followed by *Curcuma haritha*, *Curcuma montana* and *Curcuma ferruginea*, with a sequence identity of 98.74, 98.34 and 96.71%, respectively. The evolutionary relationships of the taxa *Curcuma* were inferred using the Neighbor Joining clustering method (Fig. 2). The phylogenetic tree shows two major clusters (I and II), group I consisted of *Curcuma haritha* and group II consisted of different species of *Curcuma*, CC and Rapa Nui samples (MR-1, MR-2 and RK-2). The cluster II consisted of eighteen genotypes were divided into two subclusters (IIA and IIB), MR-1, MR-2, RK-2 and CC belonging to the subcluster IIB together with *Curcuma longa* voucher PH1284, *Curcuma montana* and *Curcuma amada*. Interestingly, CC is closely related to *Curcuma montana* and MR-2, whereas both MR-1 and RK-2 are closely related to *Curcuma amada*.

3.2. Analysis of genetic variability by microsatellites analysis

A total of eight SSR markers were used to evaluate of genetic variability of three samples of *C. longa* from Rapa Nui, which 36 different alleles were detected. The average size of PCR fragments were ranged from 50 to 1250 base pairs (Supplementary Figure S1). The range of allelic variants was from 2 (CMS-01) to 11 (CMS-06) with an average of 1.5 alleles per locus/amplification with a maximum

### Table 2

Identification of homologous species based in ITS2 regions of *Curcuma longa* samples.

| Species                           | CC     | MR-1  | MR-2  | RK-2  | GenBank accession number |
|----------------------------------|--------|-------|-------|-------|--------------------------|
| *Curcuma longa* voucher PH12847  | 100    | 100   | 100   | 100   | KX675001                 |
| *Curcuma amada*                  | 100    | 100   | 100   | 100   | KF304458                 |
| *Curcuma haritha*                | 98.74  | 98.74 | 98.74 | 98.74 | KJ72076                  |
| *Curcuma montana*                | 98.34  | 98.34 | 98.34 | 98.34 | KM983490                 |
| *Curcuma ferruginea*             | 96.71  | 96.71 | 96.71 | 96.71 | KJ72073                  |
| *Curcuma aromatica*              | 96.71  | 96.71 | 96.71 | 96.71 | KJ72066                  |
| *Curcuma caesia*                 | 96.71  | 96.71 | 96.71 | 96.71 | KF304479                 |
| *Curcuma amarissima*             | 96.71  | 96.71 | 96.71 | 96.71 | KF694822                 |
| *Curcuma phaeocaulis*            | 96.71  | 96.71 | 96.71 | 96.71 | KF694820                 |
| *Curcuma yunnanensis*            | 96.71  | 96.71 | 96.71 | 96.71 | KF694815                 |
| *Curcuma kwangsiensis*           | 96.71  | 96.71 | 96.71 | 96.71 | GU180361                 |
| *Curcuma aeruginosa*             | 96.69  | 96.69 | 96.69 | 96.69 | KJ72054                  |
| *Curcuma aff. prakasha*          | 96.68  | 96.68 | 96.68 | 96.68 | KJ031117                 |
| *Curcuma zedoaria*               | 96.68  | 96.68 | 96.68 | 96.68 | GU180359                 |
| *Curcuma flaviflora*             | 96.28  | 96.28 | 96.28 | 96.28 | KJ803140                 |

Fig. 2. Phylogenetic tree based on ITS2 sequences for *Curcuma* species using the Neighbor-Joining method. The numbers below the branches indicate the bootstrap value.
of three alleles, with a polymorphic loci percent between 0% and 50%. The results of presence or absence of bands from PCR amplifications were analyzed using the UPGMA clustering method (Fig. 3 and Supplementary Table ST2). Results showed two major clusters (I and II), group I consisted of CC sample and group II consisted of Rapa Nui samples (MR-1, MR-2 and RK-2). The cluster II consisted of three genotypes (MR-1, MR-2 and RK-2) were divided into two subclusters (IIA and IIB). The subcluster IIB, consisted of MR-1 and RK-2, exhibited the maximum similarity of 72.4%, as shown in similarity matrix (Supplementary Table ST2). Genotype MR-2 was similar to MR-1 and RK-2 with 70.8 and 42.9% similarity, whereas genotype CC was similar to RK-2, MR-1 and MR-2 with 63.9, 43.2 and 42.9% similarity, respectively.

### 3.3. Determination of total polyphenols, tannins and curcumin

The total content of phenolic, tannins and curcumin in the extracts from MR-1, MR-2 and RK-2 and rhizome of CC are shown in the Table 3. The results indicated that rhizomes extract of MR-1, MR-2, and RK-2 have a high content of secondary metabolites, principally polyphenols (Table 3). The Table 3 showed also that the concentration of curcumin is higher in rhizomes than leaves extracts, principally in extracts from rhizomes of MR-1 with 29.69 mg/g, being more than 6 times than the CC samples found in Peru. Compared to the other samples from Rapa Nui, MR-1 presents more than twice than those of RK-2 and almost three times more than those of MR-2.

### 3.4. Evaluation of antioxidant activity of hydroalcoholic extracts by DPPH and ORAC value

Despite that all the extracts present antioxidant activity (Table 4 and 5), leaves and rhizomes extract of MR-1 showed better antioxidant activity than the rest of samples, with an IC50 value of 0.74 μg/mL and 1.31 μg/mL, respectively. It should be noted DPPH assays showed that leaves extract of MR-1 exhibited better antioxidant activity than control sample (GA), whereas ORAC value assay showed that rhizomes extract of MR-1 exhibited better antioxidant capacity than control sample (TROLOX) with a value of 1.27 μg/mL.

### 3.5. COX-2 inhibitor screening

Assessment of the effect of CL extracts on COX-2 inhibition were carried out by using BioVision® “COX-2 Inhibitor Detection Kit (Fluorometric)”. Fig. 4 shows the effects at 1.5 μg/mL and 3 μg/mL of extract, using the celecoxib drug as a positive control. The rhizome extract of MR-1 have the higher degree of COX-2 inhibition than the rest of samples except for celecoxib, as shown in Figure 4 and 5 and Supplementary Table ST3. Furthermore, the enzyme inhibition is dependent on the concentration of the extract as well as the drug celecoxib.

Rhizome and leaves extracts of MR-1 have a greater percentage of COX-2 inhibition, followed by the rhizome extract of RK-2 (Fig. 4). Although, celecoxib has a greater inhibitory capacity than the rest of CL sample, the rhizome extract from MR-1 present only 36% less inhibition than celecoxib at the same concentration (3 μg/mL). It should be considered that rhizome extract of MR-1 has greater antioxidant capacity for the DPPH and ORAC assay. Furthermore, leaves extract of MR-1 has an IC50 value 0.74 μg/mL for DPPH and 0.73 μg/mL ORAC value. Despite ORAC value for leaves extract of MR-1 is lower than expected, it is still close to 1 and the second-best percentage of COX-2 inhibition, after the rhizome extract.

### 3.6. Molecular docking

Table 2 shows the predicted binding energies (ΔGbinding) and binding sites of curcuminoid polyphenols and NAIDs with COX-2. Bisdemethoxycurcumin has a lower ΔGbinding for COX-2 followed by curcumin and demethoxycurcumin, with ΔGbinding values −9.2, −8.8 and −8.7 Kcal/mol, respectively. Fig. 5A depicts the potential binding site and the poses of docked curcumin into COX-2. Fig. 5B shows interactions of curcumin with the COX-2 binding pocket are governed by hydrogen bonds associated with the carbonyl oxygen, which interacts with the amino of the guanidine group of Arg106 at 3.01 Å and the hydroxyl the phenolic

### Table 3

| Organ       | Ecotype | Total polyphenols [mg/g] | Tannins [mg GAE/g] | Curcumin [mg/g] |
|-------------|---------|--------------------------|-------------------|-----------------|
| Leaf        | MR-1    | 3.74 ± 0.2               | Under LOD         | 0.19 ± 0.03     |
|             | MR-2    | 3.14 ± 0.3               | 0.70 ± 0.07       | 1.04 ± 0.07     |
|             | RK-2    | 3.53 ± 0.3               | Under LOD         | 0.22 ± 0.01     |
| Rhizome     | MR-1    | 33.68 ± 0.4              | 2.57 ± 0.1        | 29.69 ± 0.32    |
|             | MR-2    | 29.73 ± 0.1              | 0.89 ± 0.1        | 10.98 ± 0.24    |
|             | RK-2    | 22.74 ± 1.6              | 2.05 ± 0.6        | 14.82 ± 0.56    |
|             | CC      | 5.52 ± 0.02              | 3.93 ± 0.5        | 4.93 ± 0.82     |

The values are the average ± SD of replicates performed for each experiment. LOD: Limit of detection.
group of Tyr341 at 3.00 Å. Additionally, the curcumin–COX-2 complex is stabilized by hydrophobic interactions with different amino acids at the binding site of COX-2, such as His75, Val335, Leu338, Ser339, Tyr341, Trp373, Ile350, Phe354, Met350, Val350, Gly352, Ala353, Ser356, Leu357.

Additionally, Table 5 shows that all studied NAIDs and curcuminoids lie in the same binding cavity into COX-2 sharing a set of amino acids as Val335, Leu338, Val509, Glu512 and Ser516.

### 4. Discussions

Study reported by Zizka (1991) suggest that probably CL was introduced to Rapa Nui from Polynesia with the American colonization. However, the origin of CL from Rapa Nui Island is still unknown. Due to lack information and the medicinal importance for native people, we carried out phylogenetic and genetic variability analysis of three plants of CL collected from reservoir from Germplasm Bank from Mataveri Otai (CONAF) in Rapa Nui, using genetic marker nrDNA-ITS2 and SSR. These molecular tools are adequate to study the conservation and ecology of this plant (Arif et al., 2011). The phylogenetic analysis, based on nrDNA-ITS2 sequences, indicated that MR-1, MR-2, RK-2, CC, Curcuma longa voucher PH1284, Curcuma amada and Curcuma montana are part of a same subgroup. Interestingly, CC is closely related to Curcuma montana and MR-2, whereas both MR-1 and RK-2 are closely related to Curcuma amada or known as mango ginger. Additionally, the phenotypic characteristic of *C. amada* is very similar to plants grown in the Rapa Nui as MR-1, for instance the color of the flowers as shown in Figure S3. Despite Curcuma is founded in South Pacific, China, Pakistan, Thailand, Malaysia, and India (Sasikumar, 2005), few reports available about its growth and distribution. On the other hand, the comparison of nrDNA ITS2 of the all studied Curcuma species, by sequences multiple alignment, revealed that there are differences in nucleotides at three different position in the DNA sequence of *Curcuma haritha*, i.e. at the alignment position 434 and 435 there were a gap, whereas at position 467 there was a “C” instead “T” (see Figure S2), which could explain that *Curcuma haritha* is distantly related to the rest of Curcuma species, including MR-1, MR-2, RK-2 and CC.

We have found two major groups, group I consisted of a sample derived from the continent (i.e. Peru) and group II consisted of Rapa Nui samples (MR-1, MR-2 and RK-2). The group II was subdivided in two subgroups, one consisted of MR-2 sample and the second one consisted RK-2 and MR-1 samples. Both groups have a similarity coefficient of 50%, whereas for both subgroups is 65% (see Fig. 3). These results could indicate that the sample MR-1, MR-2 and RK-2 come from the same lineage, being all those were collected from Germplasm Bank in Rapa Nui. However, they do not necessarily came from the same geographical location because their reservoir has received CL that have grown in different places on the Island in order to preserve and study them. It also is possible that MR-1 and MR-2 have been moved from same place to reservoir at different times, with MR-1 being closer to RK-2. Studies in CL carried out in Brazil, India and Puerto Rico using SSR, demonstrated that exist genetic variability of CL into the same country and the same region where were collected (Sigrist et al., 2011). This could explain the variation in the content of nutrients available in the soil, amount of rainfall, and temperature variations in Rapa Nui, could be favoring the production of certain secondary metabolites in the CL plants (Stevenson et al., 2015; Jungklang et al., 2017). Furthermore the genetic variability impact on the phenotype of the plant, which could explain the common trending of maintaining high genetic diversity within populations in tropical plants as reported by Hamrick and Loveless (2019).

Interestingly, our results showed a significant variation of total phenolic content in different rhizome extracts of CL. Indeed, a variety of CL rhizomes obtained in Okinawa (Japan) also showed a variation in the total phenolic content depending on the curcuma subtype (Akter et al., 2019). According to these results, the total

### Table 5

| Compound         | ΔGbinding (Kcal/mol) | binding site in COX-2                                                                 |
|------------------|----------------------|---------------------------------------------------------------------------------------|
| curcumin         | –8.8                 | His75, Arg106, Val335, Leu338, Ser339, Tyr341, Trp373, Arg349, Ile350, Phe354, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |
| demethoxycurcumin| –8.7                 | Arg106, Val335, Leu338, Ser339, Tyr341, Trp373, Trp373, Phe350, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |
| bisdemethoxycurcumin| –9.2        | His75, Arg106, Val335, Leu338, Ser339, Tyr341, Leu370, Trp373, Ile350, Phe354, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |
| meloxicam        | –7.0                 | Val102, Arg106, Val335, Leu338, Ser339, Tyr341, Leu345, Leu370, Ser339, Tyr341, Trp373, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |
| etodolac         | –8.3                 | Gin178, Val335, Leu338, Ser339, Tyr341, Trp373, Ile350, Phe354, Met350, Val350, Gly352, Ala353, Ser356 |
| lumiracoxib      | –8.9                 | Val102, Arg106, Val335, Leu338, Ser339, Tyr341, Leu345, Leu370, Ser339, Tyr341, Trp373, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |
| etoricoxib       | –9.6                 | His75, Arg106, Val335, Leu338, Ser339, Tyr341, Trp373, Phe350, Val350, Gly352, Ala353, Ser356, Leu357 |
| valdecoxib       | –10.2                | His75, Val335, Leu338, Ser339, Tyr341, Trp373, Phe350, Val350, Gly352, Ala353, Ser356, Leu357 |
| celecoxib        | –10.2                | His75, Val335, Leu338, Ser339, Tyr341, Trp373, Arg349, Phe350, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |
| rofecoxib        | –10.3                | His75, Val335, Leu338, Ser339, Tyr341, Trp373, Arg349, Phe350, Met350, Val350, Gly352, Ala353, Ser356, Leu357 |

* Blue and red colored names correspond to those amino acids involved in H-bonds and hydrophobic contacts with the corresponding COX-2, respectively.

![Fig. 4. COX-2 inhibitor screening.](image-url)

Fig. 4. COX-2 inhibitor screening.
phenolic content in the rhizome of CL from Rapa Nui is lower than those from Okinawa. However, phenolic content in these rhizomes is higher than those from Bangladesh (Tanvir et al., 2017).

Our DPPH assays and ORAC value results shows that samples from rhizomes and leaves of MR-1 exhibited better antioxidant capacity than the rest of studied samples. Although the other samples also exhibited antioxidant capacity for both tests, it is not comparable with the antioxidant capacity obtained from the standard sample (pure compound). In fact, it is important to keep in mind that other factors could play important roles in the antioxidant activities of plant materials, as structural features of the antioxidants and the complex composition of the extracts. Furthermore, polar antioxidants could remain in the aqueous phase and to be lost during the extraction process (Gálvez et al., 2005). However, our DPPH assays results are similar to the reported by Akter et al. (2019), which polar antioxidants were also found in methanolic extracts of rhizomes of C. longa, C. xanthorrhiza, C. aromatica, C. amanda and C. zedoaria.

Here we decided to carry out COX-2 inhibition assay for Curcuma Longa extracts due to that this plant has been used in traditional Rapa Nui medicine. Our COX-2 inhibition assay results showed that rhizome extracts inhibit the COX-2 activity. These findings are supported by studies reported by Mujumdar et al. (2004), which demonstrated the anti-inflammatory activity of C. amada rhizome on albino mice. Furthermore, these extracts could also reduce the synthesis of prostaglandin E2 (PGE2) by decreasing the formation of reactive oxygen species (ROS) because ROS induce the ERK activation leading to the activation of COX-2 (Hu et al., 2017). Curcumin has been widely reported as an anti-inflammatory agent due to the ability of this compound to inhibit the activity of both COX-1 and COX-2 in the arachidonic acid metabolism (Zhang et al., 1999). Complementarily, we performed docking studies in order to understand the interactions of curcuminoids of CL with COX-2 at the molecular level. According to the potential curcumin binding site on COX-2, besides those identified by Elumalai et al. (2012), i.e. Arg106 and Tyr341, we showed that three curcuminoids of CL and selective NAIDs, as drugs belonging to Coxib family, etodolac and meloxicam, lie the same binding cavity sharing a set of amino acids, including Val335, Leu338, Val509, G1u512 and Ser516. Three curcuminoids have a lower ΔGbind than other COX-2 selective NAIDs as etodolac and meloxicam, except for Coxib family as valdecoxib, celecoxib and rofecoxib.

Various plants, used in folk and traditional medicine, have been accepted as leads for therapeutic drug development in modern medicine. Studies reported by Septembre-Malaterre et al., (2016) showed that the presence of a large number of bioactive compounds, including polyphenols, tannins and flavonoids, exhibit various biological activities. These compounds are presents in many foods and hold great potential as candidate drug due to their safety and low toxicity.

5. Conclusions

Our findings suggest that three plant of CL (MR-1, MR-2 and MK-2) obtained from Germplasm Bank in Rapa Nui are closely
related to other Curcuma species as Curcuma amada and Curcuma montana even though they have genetic variability. Furthermore, rhizomes of MR-1 showed better antioxidant and anti-inflammatory capacity than the rest of samples due to its high relative concentration of polyphenols and curcumin. Therefore, these results are important to take decisions about to preservation and propagation of this ecotype. Finally, it is important to note that results are important to take decisions about to preservation and use of curcuminoids present in CL samples on cancer cell lines.

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Appendix A

Figure S1: Pattern of eight SSR markers bands to Curcuma longa samples; Representative electrophoresis agarose gel showing PCR profile from MR-1, MR2, RK2 and CC using CuMiSat primer. Lane PM indicates the molecular weight marker. Figure S2: Sequence alignment of the three Rapa Nui samples and Curcuma species listed in Table 2; Figure S3: A) Curcuma plants collected from Rapa Nui; B) Curcuma amada; C) Curcuma Longa ; D) Curcuma montana (images obtained from: Royal Botanic Gardens Kew Seed Information Database (SID), 2020). Table ST1: Extraction percentage in grams of dry weight per 100 g from Curcuma longa extracts; Table ST2: Similarity matrix of 4 Curcuma longa samples calculated by the jaccard coefficient; Table ST3: Percent of COX-2 inhibition by Curcuma longa extracts.

Appendix B Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbi.2020.10.062.

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