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New Sicydiinae phylogeny (Teleostei: Gobioidei) inferred from mitochondrial and nuclear genes: Insights on systematics and ancestral areas

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ABSTRACT

The Sicydiinae subfamily (Teleostei: Gobioidei) is the biggest contributor to the diversity of fish communities in river systems of tropical islands. These species are found in the Indo-Pacific area, the Caribbean region and West Africa. They spawn in freshwater, their planktotrophic larvae drift downstream to the sea where they develop, before returning to the rivers to grow and reproduce. Hence, they are called amphidromous. Their phylogeny has been explored using a total of 3545 sites from 5 molecular markers (mitochondrial DNA: 16S rDNA, cytochrome oxidase I, cytochrome b; nuclear DNA: rhodopsin gene and a nuclear marker specially developed for this study, the interferon regulatory factor 2 binding protein 1-IRF2PB1). Sequences were obtained for 59 Sicydiinae specimens of 9 known genera. The Bayesian and maximum likelihood analyses support the monophyly of the subfamily as well as the monophylyof all genera except *Sicydium*, which is paraphyletic. Five major clades were identified within this subfamily. One clade contained the genus *Stiphodon*. Another clade contained *Sicyopterus*, *Sicydium* and *Parasicydium* with *Sicyopterus* as sister genus of *Sicydium*. The non-monophyly of *Sicydium* subclade, because it includes the monotypic genus *Parasicydium*, challenged the validity of *Parasicydium* genus. Ancestral area reconstruction showed that the subfamily emerged in the Central West Pacific region implying that previous hypotheses proposing a dispersal route for Sicydiinae into the Atlantic Ocean are unsupported by the present analysis. Our results suggest that the hypotheses for the dispersal route of the genus *Sicydium* should be reconsidered.

1. Introduction

With 9 described genera and over 110 species, the freshwater fish of the Sicydiinae subfamily (Teleostei: Gobioidei) are the biggest contributors to the diversity of fish communities in river systems of tropical islands (Keith, 2003; Keith and Lord, 2011). However, the naturally unstable and ephemeral characteristics of these tropical systems, which have become even more so in recent years as a result of human alteration, make the Sicydiinae particularly vulnerable (Brasher et al., 2006; Walter et al., 2012). Understanding how and from where these species have emerged to occupy their actual geographic ranges may become central in a conservation framework.

To disperse through the oceans (McDowall, 2004; Resh and De Szalay, 1995) and colonise river systems on islands the Sicydiinae, along with other Gobiidae and Eleotriidae, have developed a specialised diadromous life-cycle called amphidromy, where two migrations between the sea and freshwater occur. The adults grow, feed and reproduce in streams (Keith, 2003) and hatched larvae drift downstream into the sea (Luton et al., 2005; Maeda and Tachihara, 2010) where they spend two to six months (Iida et al., 2008; Lord et al., 2010; Taillebois et al., 2012) for larval growth. After this pelagic larval phase, the post-larvae return to rivers, undergo metamorphosis (Taillebois et al., 2011), then migrate upstream to settle, grow and reproduce (Keith, 2003).

The marine larval development stage may be of major importance to explain the spatial and temporal patterns of Sicydiinae dispersal in the tropical area. The duration of the marine larval phase, as well as the strength and direction of past and present

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prevailing currents, could interact with the dispersal abilities and determine geographic distribution of the genera and species (Crandall et al., 2010; Planes, 1993). Another and non-exclusive hypothesis is that the evolutionary history of the subfamily may have played an important role in the geographic distribution patterns of the species and genera (Keith et al., 2011a). Using ancestral area reconstructions, molecular dating and geographic distributions coded into three states corresponding to the ocean basins, Keith et al. (2011a) documented the biogeography and evolutionary history of the Sicydiinae. Based on their results, the common ancestor of Sicydiinae species appeared in the Pacific Ocean between 6 and 12 Myrs ago. Members of the subfamily then dispersed throughout the Indian Ocean to reach the Atlantic basin *via* the Cape of Good Hope. In light of their results, Keith et al. (2011a) also proposed the 'age and distribution' theory, where the 'most ancient' taxa had the largest distributional range (*Sicyopterus*) or were established far from their ancestral native ranges in the Pacific (*Sicydium, Cotylopus*), whereas 'more recent' taxa had restricted distributional ranges (*Akihito, Lentipes, Sicyopus*). Although Keith et al. (2011a) provided the first insights on the subject of the emergence and biogeographic evolution of the Sicydiinae, some groups, mainly at the deeper nodes, received very low phylogenetic support preventing unequivocal conclusions about both the systematic and the biogeographic evolution of the Sicydiinae.

The Sicydiinae subfamily has traditionally been united by the presence of a sucker formed by the fusion of the pelvic fins, which adheres entirely to the belly of the fish. The pelvic suction cup is used for fastening to the substrate that allows rapid access to the upper reaches of the rivers (Keith and Lord, 2011). The nine currently accepted genera of the Sicydiinae subfamily are: *Sicydium* Valenciennes, 1837; *Sicyopterus* Gill, 1860; *Lentipes* Günther, 1861; *Sicyopus* Gill, 1863; *Cotylopus* Guichenot, 1864; *Stiphodon* Weber, 1895; *Parasicydium* Risch, 1980; *Smilosicyopus* Watson, 1999 and *Akihito* Watson, Keith and Marquet, 2007. A recent molecular phylogeny (Keith et al., 2011a) of the Sicydiinae based on samples from the Indo-Pacific Ocean and the Caribbean sea demonstrated the monophyly of the subfamily and the existence of five clades: *Cotylopus*, *Sicyopterus*/*Sicydium*, *Lentipes*/*Akihito*/*Sicyopus*(*sicyopus*), *Stiphodon* and *Sicyopus*(*smilosicyopus*). Based on morphological characters and on the split of *Sicyopus* genus into two distinct clades within their phylogeny, Keith et al. (2011a) elevated *Smilosicyopus* subgenus as a genus.

Our aim is threefold with regard to the systematic and the biogeographic evolution of Sicydiinae. First we wish to test existing phylogenetic relationships among both species and genera. Second, we aim to provide a better understanding of biogeographic patterns among Sicydiinae taxa. Third, we analyse the biogeographic patterns of speciation in a completely sampled genus (*Smilosicyopus*), with regard to species distribution. In this study, we present a new molecular phylogeny of the subfamily Sicydiinae, including all the currently recognised genera and increasing the geographical sampling compared to Keith et al. (2011a) by including the western African region. The molecular phylogeny presented herein is based on the nuclear gene, rhodopsin and the two mitochondrial genes (COI and 16S) previously used in the Sicydiinae phylogeny (Keith et al., 2011a), with the addition of one mitochondrial gene (cytochrome b) and one protein-coding nuclear gene (IFR2BP1) specially designed for this study. To examine the biogeographical hypotheses previously addressed by Keith et al. (2011a) phylogenetic analyses are supplemented with standard morphological characters classically used in the Gobiidae systematic as well as the biogeographic patterns of the species and genera. This new assessment should provide fundamental data to document the patterns of biogeographic evolution of Sicydiinae species in river systems of tropical islands. Expected outputs are: (*i*) a better phylogenetic inference of the subfamily Sicydiinae to improve our knowledge on the taxonomy of the group. We particularly aim at resolving the phylogenetic relationships between *Lentipes, Sicyopus* and *Akihito* genera and assessing the phylogenetic position of the genus *Parasicydium* and western African species of *Sicydium*; (*ii*) a discussion about the biogeographic patterns of Sicydiinae.

2. Materials and methods

2.1. Taxa sampling

The material used in this study was collected during several expeditions conducted by the Muséum national d'Histoirenaturelle, Paris (MNHN) between 2006 and 2010 (Table 1) and through several collaborations. Samples include 59 specimens collected from Reunion, Comoros and Madagascar Islands, in the Indian Ocean; New Caledonia (Grande Terre), Cook, Futuna, Papua, Palau, Japan (Ryukyu Islands), Vanuatu (Malekula, Ambae, and Pentecost Islands), French Polynesian (Moorea and Marquesas Islands) and Hawaiian (Molokai Island) archipelagos, in the Pacific Ocean; Guadeloupe in the Caribbean sea and Gulf of Guinea on the west African coast in the Atlantic Ocean. All specimens were collected by electro-fishing (Portable Dekka 3000 electric device, Dekka Ltd., Germany) or snorkelling. Either a piece of fin was clipped and the fish was released, or the fish was killed with an overdose of clove oil (10%), and a tissue sample taken. Tissues and fish were stored in 95% ethanol. Species were morphologically identified based on field guides (Keith et al., 2010, 2006, 2002; Marquet et al., 2003), taxonomic literature or by comparison with type specimens available in the collections housed in the MNHN, the RMNH (Rijksmuseum van Natuurlijke Historie, Leiden, The Netherlands), the WAM (Western Australian Museum, Perth, Australia) and the ZMA (Zoological Museum of the University of Amsterdam, the Netherlands).

One specimen of *Awaous* sp and one specimen of *Redigobius* sp (both Gobiidae, subfamily Gobionellinae) were used as closely related outgroups. Indeed, the Gobionellinae subfamily has been identified as belonging to the putative sister-group of the Sicydiinae subfamily (Birdsong et al., 1988; Nelson, 2006). Two specimens of *Rhyacichthys guilberti* (family Rhyacichthyidae) were used as a distant outgroup from Gobiidae to root the tree (Hoese and Gill, 1993; Thacker, 2003, 2009). This genus is thought to be sister group of all other Gobioidei (Hoese and Gill, 1993; Thacker, 2003) and it is distant enough to be certain that it does not form a monophyletic group with Sicydiinae.

2.2. Distribution of genera

Stiphodon and *Lentipes* can be found from the eastern Indian Ocean to Polynesia in the eastern Pacific Ocean (Keith and Marquet, 2007; Keith et al., 2007; Watson et al., 2001; Watson et al., 2002). *Sicyopterus* is distributed in the Indo-Pacific Ocean from Madagascar in the western Indian Ocean to Polynesia in the eastern Pacific (Keith et al., 2005a). *Sicydium* is found throughout western and eastern Central America, extending from Mexico to Peru along the Pacific coast, from Mexico to Venezuela along the Atlantic coast, on the islands of Greater and Lesser Antilles of the Caribbean Sea and in western Africa (mainly on volcanic islands of Gulf of Guinea) (Pezold et al., 2006; Watson, 2000). *Parasicydium* is supposed to be a monotypic genus which is known from its type locality, Bandama River in Côte d'Ivoire, from the Lokunje basin in Cameroon and the Kouilou basin in Congo (Harrison, 1993). The genus *Cotylopus* is geographically restrictedto the Mascarene and Comoros islands in the Indian Ocean (Keith et al., 2005b, 2006).

Table 1
Sampling table organised according to taxonomy and origin of specimens.

Family	Genus	*Species*	MNHN ID no	Locality
Rhyacichthyidae	*Rhyacichthys*	*guilberti*	RG15A	Vanuatu
		guilberti	RG15B	Vanuatu
Gobiidae (Gobionellinae)	*Redigobius*	sp	RediF	Vanuatu
	Awaous	*guamensis*	Awaou	New Caledonia
Gobiidae (Sicydiinae)	*Sicyopterus*	*lagocephalus*	Lago2	Vanuatu
		lagocephalus	Lago3	Vanuatu
		pugnans	Pug1A	French Polynesia
		pugnans	Pug1B	French Polynesia
		pugnans	Pug1C	French Polynesia
	Sicydium	*punctatum*	Pu12A	Guadeloupe
		punctatum	Pu13A	Guadeloupe
		plumieri	Pl001	Guadeloupe
		plumieri	Pl13B	Guadeloupe
		crenilabrum	CrCR1	West Africa
		crenilabrum	CrCR2	West Africa
		brevifile	SbCR4	West Africa
	Parasicydium	sp	PaCR3	West Africa
	Stiphodon	*rutilaureus*	Rut21	New Caledonia
		rutilaureus	Rut23	Vanuatu
		sapphirinus	Sa28B	Cook
		sapphirinus	Sa28A	Cook
		hydoreibatus	ShyF1	Futuna
		hydoreibatus	ShyF2	Futuna
		atratus	Atra1	Vanuatu
		atratus	Atra2	Vanuatu
		atratus	Satra	Vanuatu
	Smilosicyopus	*pentecost*	Spen1	Vanuatu
		pentecost	Spen2	Vanuatu
		sasali	Sasl2	French Polynesia
		sasali	Sasl1	French Polynesia
		bitaeniatus	Sbit1	French Polynesia
		bitaeniatus	Sbit2	French Polynesia
		fehlmanni	Sfeh1	Palau
		fehlmanni	Sfeh2	Palau
		fehlmanni	Sfeh3	Palau
		leprurus	Slep1	Japan
		leprurus	Slep2	Japan
		chloe	Scc03	New Caledonia
		chloe	Scc06	New Caledonia
	Cotylopus	*acutipinnis*	Acu1a	Reunion
		acutipinnis	Coty1	Reunion
		rubripinnis	Rub5A	Comoros
		rubripinnis	Rub5B	Comoros
	Lentipes	*armatus*	Larm1	Japan
		armatus	Larm2	Japan
		sp	Lesp1	Papua
		sp	Lesp2	Papua
		kaaea	Kaea1	New Caledonia
		kaaea	Kaea2	New Caledonia
		concolor	Lco83	Hawai
		concolor	Lco88	Hawai
	Sicyopus	*lord*	Lord1	Madagascar
		lord	Lord2	Madagascar
		discordipinnis	Sdis6	Papua
		discordipinnis	Sdis7	Papua
		zosterophorum	Szc01	New Caledonia
		zosterophorum	Szc02	New Caledonia
		nigriradiatus	Nigr1	Ponhpei
		nigriradiatus	Nigr2	Ponhpei
	Akihito	*vanuatu*	Avan3	Vanuatu
		vanuatu	Avan4	Vanuatu
		futuna	Afut2	Futuna
		futuna	Afut3	Futuna

Smilosicyopus is found throughout the western Pacific Ocean, extending from the Ryukyu Islands to New Caledonia for its latitudinal limits and from Philippines to Marquesas Islands for its longitudinal limits (Keith and Taillebois, in press). Our phylogeny included all the valid species of this genus. The distribution of *Sicyopus* genus was recently updated to Indian and Pacific Oceans since the discovery of *Sicyopus lord* in Madagascar (Keith et al., 2011b). It ranges from the eastern coast of Madagascar in the Indian Ocean to Fiji islands in the Pacific one. The genus *Akihito* appears to be restricted to the western Pacific Ocean in Vanuatu and Futuna Islands (Keith et al., 2007; Watson et al., 2007).

2.3. Screening for a novel gene marker

To add support to our phylogenetic analyses, a nuclear gene was specially selected for this study: the interferon regulatory factor 2

binding protein 1 (IRF2BP1). A list of shared protein-coding sequences was obtained through genome filtering in the Ensembl database release 40 using the Biomart mining tool of the Ensembl Portal (Hubbard et al., 2005). *Tetraodon nigroviridis* was used as a dataset, and only sequences having unique best hits in the genomes of *Gasterosteus aculeatus*, *Takifugu rubripes* and *Danio rerio* were retained. In the resulting list, coding sequences presenting the lowest similarity between the two tetraodontids were checked for divergence and exon length, through the Ensembl Portal, on all the available teleost genomes to maximise to the probability of identifying markers that provide information at a smaller taxonomic scale. Sequences of long exons and low similarity were downloaded from Ensembl. Within this list, the sequence coding for the 3 exons of the gene IRF2BP1 (Ref. ENSTNIG00000018992) was long (1470 base pairs). Sequences for additional teleost taxa were recovered for this marker in GenBank using the *Gasterosteus aculeatus* sequence as a blast query using the Blast tool (Altschul et al., 1997). The sequence alignment was used to identify conserved regions by visual inspection, and primers were defined in these areas. They were then checked using Oligo 4.1 Primer Analysis Software (National BioScience, Inc., Plymouth).

2.4. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fin clips using NucleoSpin^R 96 Tissues (Macherey–Nagel) and the robot Eppendorf epMotion 5075, following the manufacturer's instructions. Five molecular markers were amplified: three mitochondrial markers (partial cytochrome oxidase I – COI, partial mitochondrial 16S rDNA – 16S, and partial cytochrome b – Cytb) and two nuclear markers (partial rhodopsin retrogene – Rh, and partial interferon regulatory factor 2 binding protein 1 like – IRF2BP1). The primer sequences are detailed in Table 2. Polymerase chain reactions (PCR) were performed in 25 μL final volume, containing 2.5 μl of the corresponding buffer,5% of DMSO, 5 μg of bovine serum albumin, 300 μM of each dNTP, 1.7 pM of each of the two primers, 0.3 μM of Taq Polymerase (Qbiogen) and approximately 3 ng of template DNA. Amplification products were generated by an initial denaturation step of 2 min at 94 °C followed by 50 cycles at 94 °C for 20 s, annealing at 52 °C for COI, Cytb and Rh, 56 °C for 16S and 58 °C for IRF2BP1 for 25 s and by a final extension at 72 °C for 60 s with a terminal elongation at 72 °C for 3 min. PCR products were purified using ExonucleaseI and Phosphatase and sequenced using BigDye Terminator v3.1 kit (Applied biosystem) and the ABI 3730XL sequencer at the Genoscope (http://www.genoscope.cns.fr/) using the same primers. All gene fragments were sequenced in both directions to confirm accuracy of each sequence. Chromatograms were edited manually using Sequencher v4.8 (Gene Codes Corporation).

2.5. Tests of saturation effect

To evaluate the level of saturation of IRF2BP1 gene fragment, we plotted in Fig. 1 the average number of observed nucleotide substitutions per site (*i.e.*, the *p*-distances) as a function of the average number of inferred nucleotide substitution per site based on the Neighbour-Joining method (*i.e.*, the corrected distances) under a GTR model using PAUP 4.0b10 (Swofford, 2002). In the absence of substitution saturation, these plots should reveal linear increment of uncorrected distances in relation to NJ distance. If sequences are saturated (*i.e.*, multiple substitutions occurred at a given nucleotide position) the plots should reach a plateau beyond a certain amount of uncorrected sequence divergence. We separately evaluated the saturation at each codon position.

2.6. Dataset and partition congruence

There were six total datasets analysed for this study. Five independent analyses were performed on COI, 16S, Cytb, Rh and IRF2BP1 gene fragments for all taxa listed in Table 1. The sixth dataset consisted ina combined dataset of the five gene fragments and is referred to as CD. We first conducted separate Maximum Likelihood (ML) and Bayesian Analyses (BA) for each dataset and surveyed corresponding nodes for support and congruence. Nodes that differed between methods but possessed low support values (under 0.5) were treated as congruent.

2.7. Phylogenetic analysis

Sequences were aligned with the program MUSCLE using default parameters (Edgar, 2004). Hyper-variable regions of 16S gene were excluded from further analyses due to ambiguities in the alignments.

The best-fit substitution model for each subset of our partitioned data set was selected using PartitionFinder (Lanfear et al., 2012). COI,Cytb, Rh and IRF2BP1 were partitioned by codon position. 16S gene was considered as one partition. The model selection and partitioning scheme comparison was performed using the Bayesian Information Criterion (BIC; Posada and Buckley, 2004). Each partition had its own set of independent branch lengths and all models were analysed for each one. The best-fitting substitution model according to the BIC, for each of the subset, was implemented in the following phylogenetic analyses.

Phylogenetic analyses were conducted using two different probabilistic approaches. A heuristic ML search was conducted using RaxML HPC2 (Stamatakis, 2006) on Teragrid v.7.2.7, implemented in the CyberInfrastructure for Phylogenetic Research (CIPRES) portal v.3.1. (http://www.phylo.org/portal2). One hundred independent searches, each starting from distinct random trees, were conducted. Robustness of the nodes were assessed

Table 2
Forward and reverse primers used for the PCR. Details on fragment size and hybridisation temperature are given for the five genes analysed in this study.

	Gene	Frag. Size	Name	Dir.	Primers	T° of hyb. (°C)	Sources
Mitoch.	COI	≈670 bp	TelF1	F	5′TCGACTAATCAYAAAGAYATYGGCAC3′	52	Dettai et al. (2011)
			TelR1	R	5′ACTTCTGGGTGNCCAAARAATCARAA3′		
	Cytb	≈844 bp	F216	F	5′TCCGAAAYATACATGCYAATGG3′	52	Briolay et al. (1998)
			R15537	R	5′CGTTCTGRGCTGAGCTAC3′		
	16S	≈570 bp	L2510	F	5′CGCCTGTTTACCAAAAACAT3′	56	Palumbi (1996)
			H3084	R	5′AGATAGAAACTGACCTGGAT3′		
Nuclear	Rhodo.	≈760 bp	Rh193	F	5′CNTATGAATAYCCTCAGTACTACC3′	52	Chen et al. (2003)
			Rh1030r	R	5′TGCTTGTTCATGCAGATGTAGA3′		
	IRF2BP1	≈710 bp	F34	F	5′CARTGGTGCTACCTSTGCGA3′	58	This study
			R751	R	5′CGTGGTCYTTCCKGAAGCG3′		

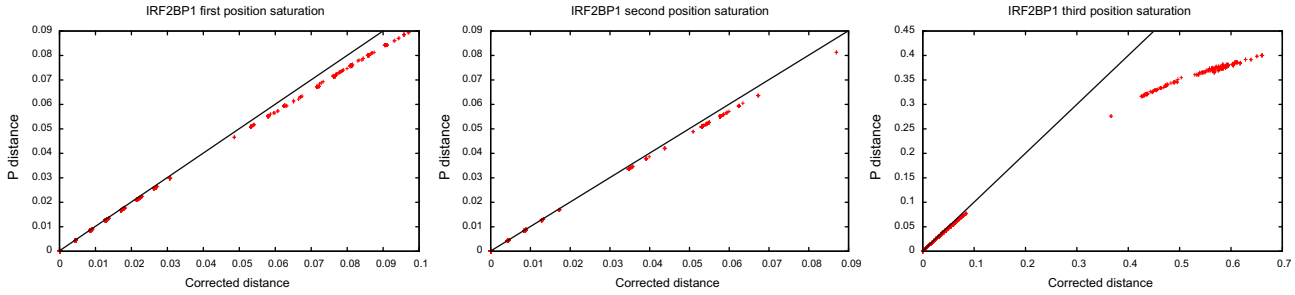


Fig. 1. Saturation plots of each codon position character set for IRF2BP1 gene. The straight line ($y = x$) represents the situation for which there is no homoplasy (*i.e.*, the number of inferred substitutions equals the number of observed differences).

using nonparametric bootstrapping (Felsenstein, 1985) with 1000 bootstrap replicates. Bayesian analyses (BA) were performed running two parallel analyses in MrBayes(Huelsenbeck and Ronquist, 2001), consisting each of four Markov chains of 50,000,000 generations with a sampling frequency of 1 tree every 1000 generations. The number of swaps was set to 5, and the chain temperature at 0.02. Convergence and mixing of the chain of each analysis was evaluated using Tracer v1.4.1 (Rambaud and Drummond, 2007) to check that ESS values were all superior to 200. A consensus tree was then calculated after omitting the first 25% trees as burn-in. For the treatment of CD using ML and BA, the data were separated into nine unlinked partitions as suggested by PartitionFinder: 16S, Rh, IRF2BP1 and the three-codon positions of COI and Cytb genes.

2.8. Biogeographic range evolution

An ancestral biogeographic range reconstruction was inferred using the likelihood-based program package Lagrange Configurator (beta) version 20120508 (Ree et al., 2005; Ree and Smith, 2008). This tool was used to configure and download our Python script analysis. The Lagrange dispersal-extinction-cladogenesis (DEC) model was explored using a ML best tree topology discarding duplicate species and outgroups compared to previous data. Likelihood searches were performed using the software RaxML HPC2 (Stamatakis, 2006) on Teragrid v.7.2.7, implemented in the Cyber-Infrastructure for Phylogenetic Research (CIPRES) portal v.3.1. (http://www.phylo.org/portal2) with the single model of evolution underlying the complete concatenated matrix and a partitioned analysis allowing for estimating of parameters within the subsets given by PartitionFinder. All species included were assigned to one or more of 11 biogeographic regions corresponding to geographic provinces on which the Sicydiinae are extant: (1) Carribean sea (A); (2) West Africa (B); (3) Comoros, Madagascar and Mascareignes islands (C); (4) the Indonesian shelf and the Ryukyus islands (D); (5) Papua New Guinea and Queensland region (E); (6) Micronesia (F); (7) Solomon, Vanuatu islands and New Caledonia (G); (8) Fiji (H); (9) Futuna, Samoa, Tonga and Cook islands (I); (10) Hawai'i archipelago (J) and (11) Marquesas and Polynesian islands (K). The biogeographic areas are represented on a map in Fig. 3. Ranges were constrained (adjacent matrix) according to current proximity of the regions and the maximum number of ancestral areas was set to 8 allowing ocean scale widespread ancestors.

3. Results

3.1. Genetic diversity and sequence divergence

Out of the 59 Sicydiinae samples used to reconstruct the molecular phylogeny of the subfamily, 59 were sequenced for the COI, Cytband 55 for 16S and IRF2BP1. For Rh, we used 7 sequences of our samples that were available in GenBank database (KF016035–KF016040 and HQ639169) and sequenced 52 other individuals. For COI, 670 bp were sequenced (53 different haplotypes, displaying 257 polymorphic sites, 232 parsimony informative sites and a high haplotypic diversity (0.996) were found, GenBank accession numbers including outgroups KF668814–KF668876). A fragment of 844 bp was obtained for Cytb (57 different haplotypes were found, displaying 375 polymorphic sites, 337 parsimony informative sites and a high haplotypic diversity (0.999) were found, GenBank accession numbers including outgroups KF668995–KF669057). After removal of hyper-variable regions, 16S sequences included 555 bp (52 different haplotypes displaying 137 polymorphic sites, 98 parsimony informative sites and a high haplotypic diversity (0.996) were found, GenBank accession numbers including outgroups KF668877–KF668935). A fragment of 764 bp was obtained for Rh (31 different haplotypes displaying 174 polymorphic sites and 135 parsimony informative and a high haplotypic diversity (0.976) were found, GenBank accession numbers including outgroups KF669058–KF669113). For IRF2BP1 gene, a fragment of 713 bpwas obtained (38 different haplotypes were found, displaying 224 polymorphic sites and 166 parsimony informative sites and a haplotypic diversity of 0.987, GenBank accession numbers including outgroups KF668936–KF668994).

The interspecific pairwise differences in gene sequences between pairs of species within the Sicydiinae ranged between 0.6% and 23.07% for COI, 0.71% and 22.36% for Cytb, 0.18% and 7.79% for 16S, 0% and 2.83% for Rh and 0.14% and 3.32% for IRF2BP1.

3.2. Properties and substitution model of genes

The saturation plots were drawn for each of the three-codon positions for IRF2BP1 gene and involved all possible species pairs within the Sicydiinae. No saturation was observed for any codon position (Fig. 1). The five genes displayed striking differences in base composition. The nuclear gene Rh appeared poor in A (17.1%) and IRF2BP1 appeared poor in A+T (38.1%). The two mitochondrial markers (COI and Cytb) had an excess of respectively C+T (58.6%) and A+C (63.3%) while they have a notably small proportion of G (13.8% and 9.9%). The third mitochondrial gene 16S exhibited an excess of A+C (56.4%) and an equal proportion of G and T. For the concatenated data set, a total of 6 partitions were identified using PartitionFinder (Table 3).

3.3. Phylogenetic analysis

For each of the five single genes, the consensus tree showed that the Sicydiinae are monophyletic. Since no incongruence was revealed from the single gene analyses, we analysed the CD comprising the data of the 5 genes resulting in a 3545 bp fragment, and only the results obtained for the CD are presented (Fig. 2). Topologies derived from ML analyses of the CD were congruent with the

Table 3
Substitution models for nucleotide data partitions selected using the BIC in PartitionFinder.

Partition	Model
COI and Cytb1st-codon + 16S	SYM+I+G
COI 2nd and 3rd-codon, Cytb and Rh 2nd-codon	GTR+I+G
Cytband Rh 3rd-codon	GTR+G
Rh 1st-codon, IRF2BP1 1st and 2nd-codon	HKY+I+G
IRF2BP1 3rd-codon	K80+G

topology derived from BA analyses. From these combined analyses, the Sicydiinae were found monophyletic (Posterior Probabilities PP = 1, Bootstraps $B = 1$; Fig. 3). Within Sicydiinae, five clades hereafter referred to as Clades D to H, are delineated. All of the five clades are highly supported (D: PP = 1; $B = 1$; E: PP = 1; $B = 0.99$; F: PP = 1; $B = 1$; G: PP = 1; $B = 1$; H: PP = 1; $B = 0.89$) but relationships among them are most of the time poorly resolved according to either bootstrap and/or posterior probabilities (Fig. 3). The relationships between clades E and F–G–H and between clades F and G were not supported (PP = 0.66; $B = 0.31$ and PP = 0.64; $B = 0.36$). However, the relationship between clades H2–H3 and H1 was strongly supported (PP = 1; $B = 0.89$) as well as the relationship between clades E1 and E2 (PP = 1; $B = 0.99$).

The clade D includes 4 species from the genus *Stiphodon*. The clade E is split into two subclades: E1 includes the species of the genus *Sicyopterus*; E2 includes the species of the genera *Sicydium* and *Parasicydium*. The clade F includes the 2 species from the genus *Cotylopus*. The clade G includes the 6 species from the genus *Smilosicyopus* and the species *Sicyopus nigriradiatus*. The clade H includes 3 subclades: H1 includes *Lentipes* species; H2 includes *Akihito* species; and H3 includes *Sicyopus* species.

3.4. Biogeographic range evolution

Results for ancestral reconstruction are presented in Fig. 3b and Table 4. Lagrange estimates the inheritance of a range after a

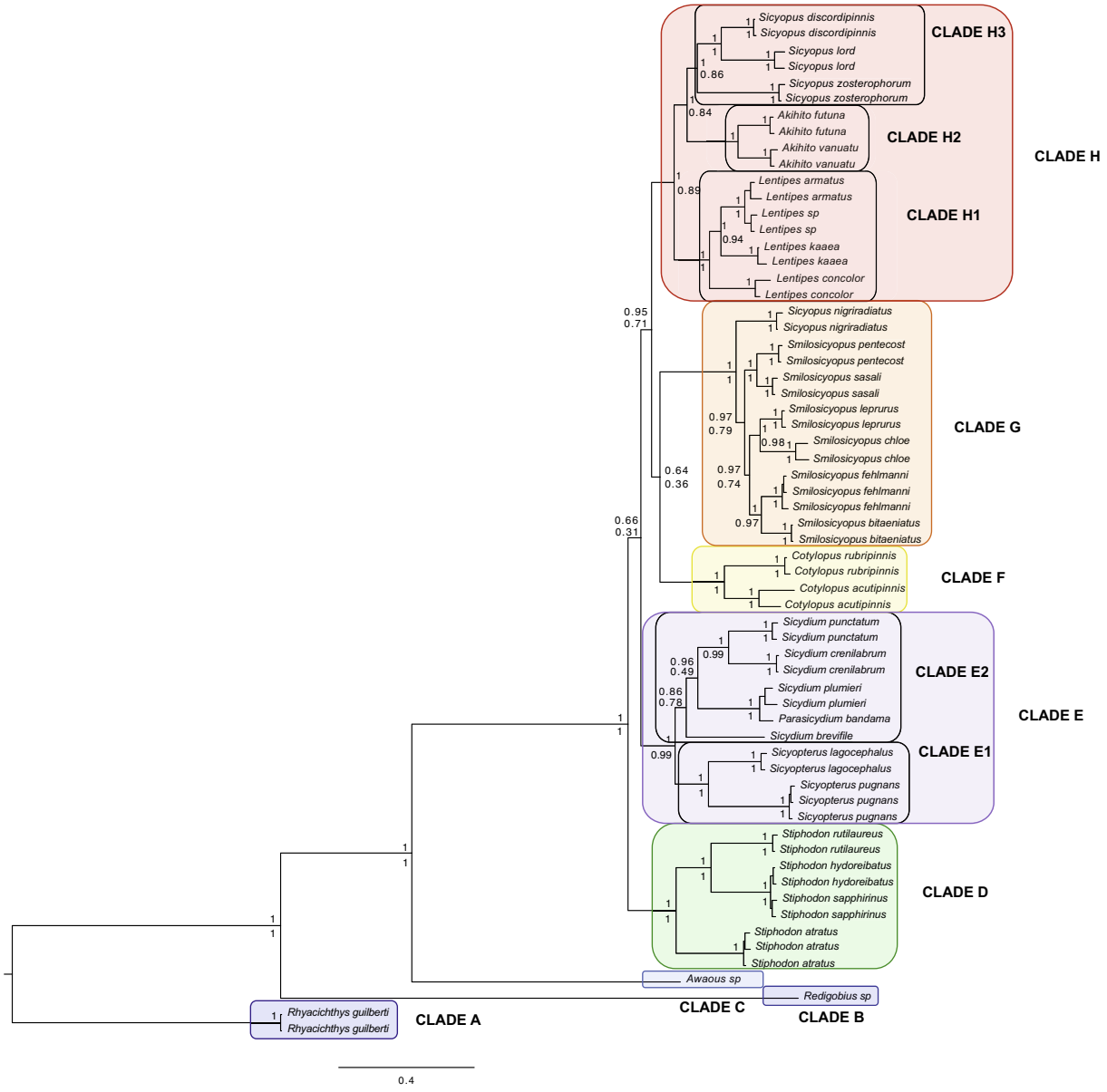
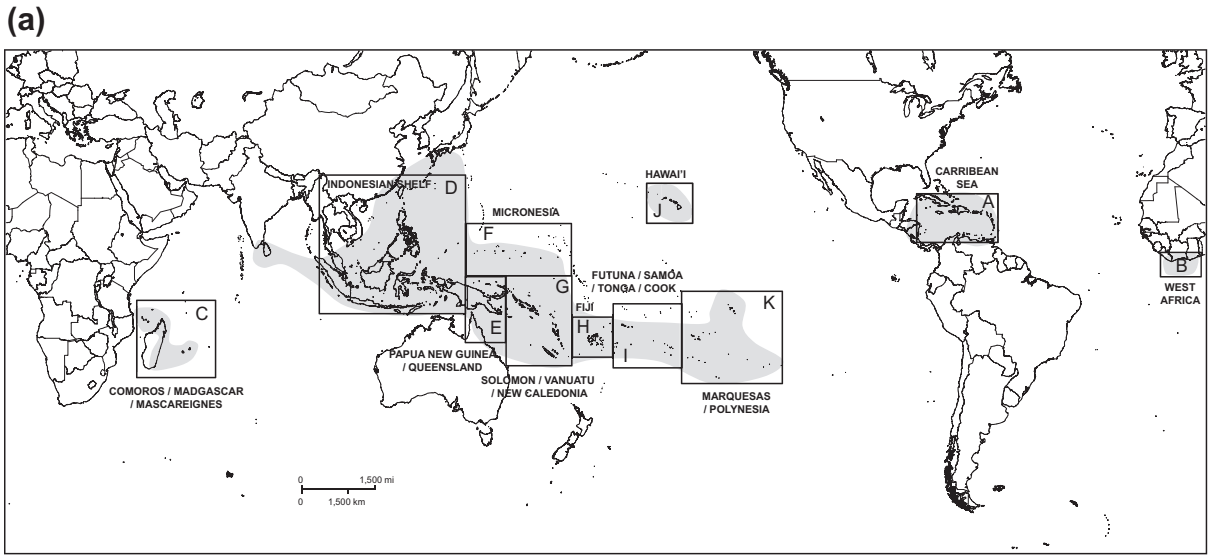


Fig. 2. Phylogram inferred from Bayesian analysis of the combined molecular markers (COI, Cytb, 16S, Rh and IRF2BP1). Posterior probabilities and Bootstrap values are indicated above and below, respectively, the corresponding nodes.



(b)

A = Carribean sea
B = West Africa
C = Comoros, Madagascar and Mascareignes islands
D = Indonesian shelf
E = Papua New Guinea and Queensland region
F = Micronesia
G = Solomon, Vanuatu and New Caledonia
H = Fiji
I = Futuna, Samoa, Tonga and Cook islands
J = Hawai'i archipelago
K = Marquesas and Polynesia

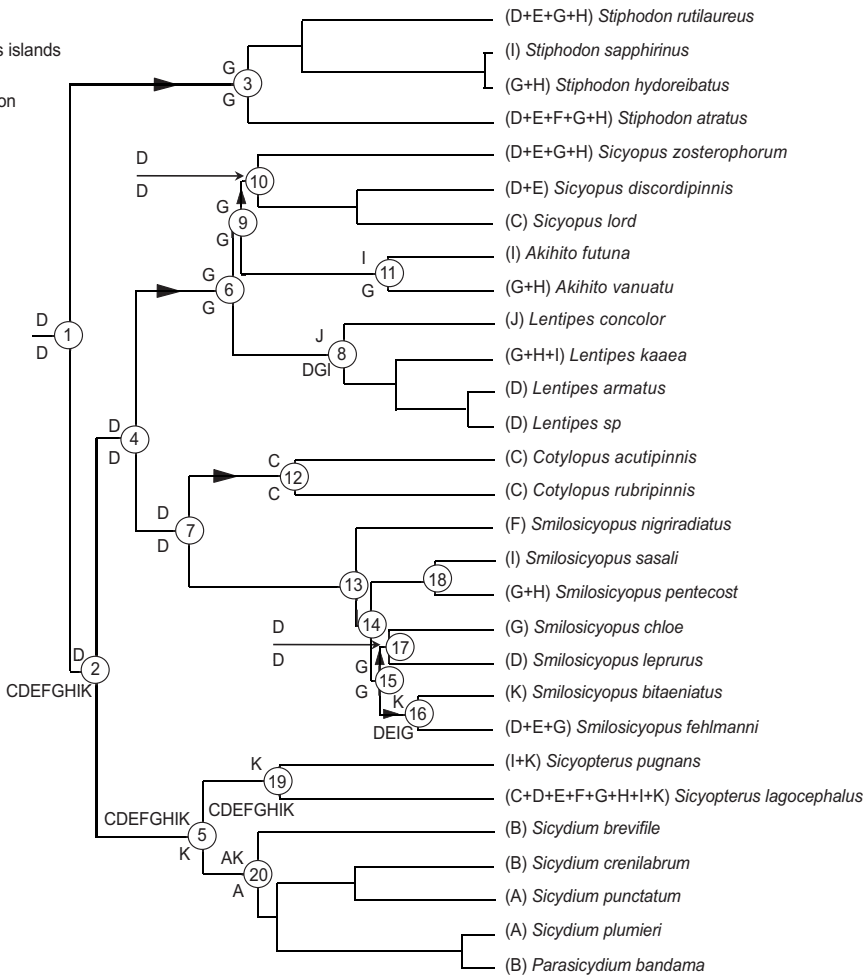


Fig. 3. Geographic distribution of the subfamily Sicydiinae (grey shading) showing endemic areas A–K used for the biogeographic analysis (a). Ancestral area reconstruction of Sicydiinae (b) using the DEC method as implemented in the software Lagrange (Ree and Smith, 2008). Arrows indicate dispersal events between designated regions. At each node, each ancestral area is made either of the combination of two ranges inherited from the two descendant lineages (each placed above and below the corresponding node/branch). At each node, the most likely inferred ancestral area is drawn.

Table 4
Inferred ancestral ranges for branches (separated by vertical bar) descending from each numbered node from Fig. 3. Area abbreviations are explained in the text (see Section 2.8). For example, the first row refers to the split at node 1 into its daughter lineages node 2 and node 3; these are both reconstructed as Indonesian Shelf (first among two likely reconstructions). The "–" are nodes that are not resolved for DEC analysis. The ln L and relative probabilities are listed for each likely reconstruction. Only alternative scenarios that fall within two log-likelihood units of the optimal reconstruction and have a relative probability ⩾0.1 are provided. The most likely ancestral areas at each node that are drawn in Fig. 3 are shown in bold.

Node No.	Infer. Anc. Area	ln L	Rel. Prob.
1	**[D|D]**	**−126.4**	**0.2723**
	[G|G]	−126.5	0.2429
2	**[D|CDEFGHIK]**	**−126**	**0.4136**
	[G|CDEFGHIK]	−126.1	0.3594
3	**[G|G]**	**−26.8**	**0.1809**
4	**[D|D]**	**−126.3**	**0.3011**
	[G|G]	−126.8	0.1774
5	**[CDEFGHIK|K]**	**−125.1**	**0.9976**
6	**[G|G]**	**−126.6**	**0.2308**
	[D|D]	−126.8	0.1832
7	**[D|D]**	**−126.4**	**0.2703**
8	**[J|DGI]**	**−126.6**	**0.2203**
	[J|DGHI]	−127.1	0.1352
9	**[G|G]**	**−126.4**	**0.27**
	[D|D]	−126.7	0.2001
10	**[D|D]**	**−126.1**	**0.3453**
11	**[I|G]**	**−126.6**	**0.211**
	[I|GH]	−126.8	0.1793
	[G|G]	−127.2	0.1208
12	**[C|C]**	**−125.2**	**0.8532**
13	–	–	–
14	–	–	–
15	**[G|G]**	**−127.3**	**0.1134**
16	**[K|DEGI]**	**−126.6**	**0.2308**
17	**[D|D]**	**−125.9**	**0.4252**
	[G|D]	−126.3	0.3048
	[G|G]	−127	0.1426
18	–	–	–
19	**[K|CDEFGHIK]**	**−125.7**	**0.5663**
	[I|CDEFGHIK]	−126.1	0.372
20	**[AK|A]**	**−126.4**	**0.2663**
	[A|A]	−126.7	0.1953
	[A|AK]	−127.1	0.1382
	[B|ABK]	−127.3	0.1106

phylogenetic split, and therefore, the ancestral range of Sicydiinae cannot be directly estimated. However, Lagrange reconstructs the range at nodes 1 and 2 to the Indonesian shelf (area D), supporting the idea of a Pacific ancestral range of the subfamily. At node 2, the range splits into a restricted range (node 4, area D) and a widespread one (node 5, area CDEFGHIK). From node 1 to node 3 a dispersal event occurred from the Indonesian shelf area to the Solomon–Vanuatu–New Caledonia area, from where *Stiphodon* diversified. At node 5, the range splits into a widespread branch leading to *Sicyopterus* species and a restricted one in the Polynesian area leading to current *Sicydium* and *Parasicydium* species. The common ancestor to all other genera ranges in the Indonesian shelf (node 4) and splits into two branches: one stays in the Indonesian shelf (node 7) and the other branch leads to node 6, which has a Solomon–Vanuatu–New Caledonia ancestral range. The genera *Sicyopus–Akihito–Lentipes* have a common ancestral area corresponding to the region Solomon–Vanuatu–New Caledonia (node 9). From node 7, a split leads to the genus *Cotylopus* on one branch and on which a dispersal event occurs from the Indonesian shelf to the Indian Ocean. The other branch leads to the genus *Smilosicyopus* but only few ancestral reconstructions were resolved for this clade.

4. Discussion

A robust phylogenetic context has been used to both clarify the systematics of the Sicydiinae and document the patterns of biogeographic evolution of the group. The molecular phylogeny of the Sicydiinae presented here strengthens the previous large-scale phylogeny of the group (Keith et al., 2011a). Here we present a higher number of specimens and species including all of the 9 accepted genera, an extended sampling to the East Atlantic Ocean and we use two additional genes including a new nuclear marker specially designed for this study. The IRF2BP1 gene has a higher divergence level than the other nuclear gene Rhodopsin, thus offering a promising power for phylogenetic inferences. Even if the addition of IRF2BP1 did not allow strengthening deeper nodes, this new 5-gene phylogeny, now including the genus *Parasicydium* and all species from *Smilosicyopus* genus, has implication for the whole subfamily's systematics.

4.1. Sicydiinae taxonomy and morphology

The phylogenetic trees in our analyses support the monophyly of the subfamily Sicydiinae as shown by Keith et al. (2011a) and the existence of five major clades identified here as Clade D to H (Fig. 2).

4.1.1. Stiphodon clade (Clade D)

Our phylogenetic analyses confirm the monophyly of the genus *Stiphodon* (Keith et al., 2011a) and suggest that this clade may be the sister group of all other Sicydiinae species analysed in this study. The sister group relationship of clade D to all others is not well supported (PP = 0.66; *B* = 0.31) and clade D could also be sister to clade FGH or clade E. However, this position of clade D was found in all the phylogenetic analyses he have run considering any combined or not data set and using maximum likelihood or Bayesian analyses. *Stiphodon* is the only genus among the Sicydiinae characterised by the presence of three anal pterygiophores prior to the first haemal spine (Birdsong et al., 1988; Keith et al., 2011a) instead of two. The new phylogenetic position of *Stiphodon* highlighted here updates the evolution of the Sicydiinae, placing *Stiphodon* as the sister clade to all other clades.

4.1.2. Sicydium–Parasicydium–Sicyopterus clade (Clade E)

Our phylogeny strongly supports the monophyly of the genus *Sicyopterus* (Berrebi et al., 2006; Keith et al., 2005a) and its sister relationship with *Sicydium* that was predicted on both molecular and morphological characters (Keith et al., 2011a). Indeed, these two genera shared two synapomorphies: a short, blunt ascending process of the premaxilla and a unique oculoscapular canal pore pattern (Parenti and Maciolek, 1993; Pezold, 1993). Moreover, the high supports obtained for the clade including *Sicyopterus* and *Sicydium* (Clade E) confirm their sister relationship. The genus *Sicydium* is not monophyletic because the group that includes all the *Sicydium* species and their common ancestor also includes the monotypic genus *Parasicydium*. This latest genus shares the two synapomorphies with *Sicyopterus* and *Sicydium* cited above. The clade formed by *Sicydium* and *Parasicydium* appears to be the sister group of *Sicyopterus*. The addition of African *Sicydium* and *Parasicydium* species in our phylogeny brings new insights into the diversification of the clade E. These new results challenge the validity of the genus *Parasicydium*. Morphological and meristic analyses with a revision of these two genera are needed to confirm these genetic results. Further work is needed to clarify the phylogenetic relationships within this clade.

4.1.3. Cotylopus clade (Clade F)

Our molecular analyses suggest that the genus *Cotylopus*, which includes only two species, is monophyletic. In the study by Keith et al. (2011a), the genus *Cotylopus* was found to be the sister group of all the Sicydiinae species. Here, even if its relationship with other clades is poorly supported, the genus *Cotylopus* appears more

closely related to the clades G and H including the genera *Smilosicyopus, Sicyopus, Lentipes* and *Akihito*. Indeed, the three clades F, G and H form a well-supported clade (PP = 0.95; *B* = 0.71). Morphological characters furthermore support the hypothesis of a close relationship between *Cotylopus* and *Lentipes*. In particular, *Cotylopus* shares with *Lentipes* numerous morphological characteristics and especially osteological ones such as the tongue fused to the floor of the mouth, the pelvic disk adherent to the belly between all five rays and a narrow ascending process on the premaxilla at the dorsal tip (Watson, 1995; Watson et al., 2002).

4.1.4. Smilosicyopus and Sicyopus clades (Clade G and part of Clade H)

In 1999, Watson proposed a subdivision within *Sicyopus* species based mainly on dental characteristics found in both jaws. Three sub-genera were proposed: *sicyopus*- with conical teeth sharply recurved in both jaws and without canines, and with *Sicyopus (sicyopus) zosterophorum* as type species; *smilosicyopus*- with conical teeth slightly recurved in both jaws, lateral teeth and in-between at least one canine tooth well developed in males, with *Sicyopus (smilosicyopus) leprurus* as type species; and the monospecific subgenus *juxtastiphodon*- with conical teeth in both jaws crowded closely together and without canine, and with *Sicyopus (juxtastiphodon) nigriradiatus* as type species. In 2011, Keith et al. showed that *Sicyopus* was paraphyletic. They thus elevated *smilosicyopus* to genus level, but the case of *juxtastiphodon* was not resolved.

Our study that included all the known species of *Smilosicyopus* confirms the split of *Sicyopus* found by Keith et al. (2011a) into two monophyletic genera. The two genera are highly supported by our phylogenetic analyses: *Sicyopus* (Clade H3, comprising in our study *S. discordipinnis*, *S. lord* and *S. zosterophorum*) and *Smilosicyopus* (Clade G, comprising in our study all the described species of the genus *Smilosicyopus* plus *Sicyopus* (*juxtastiphodon) nigriradiatus*). The presence of this latter speciesin the clade G is congruent with the morphology showing conical teeth slightly recurved in both jaws, lateral teeth and in-between at least one canine tooth that is well developed in males. The high support of the clade G and the morphological similarities observed between *Sicyopus* (*juxtastiphodon*) *nigriradiatus*and other species from the genus *Smilosicyopus* suggest that *Sicyopus nigriradiatus* should be reassigned to *Smilosicyopus* genus.

4.1.5. Lentipes–Sicyopus–Akihito clade (Clade H)

Our phylogeny strongly supports the sister relationships between the genera *Lentipes* (Clade H1), *Akihito* (CladeH2) and *Sicyopus* (CladeH3) and the monophyly of each of these genera (Keith et al., 2011a). Here we furthermore resolve the relative position of the three genera with well-supported nodes. These results are congruent with the morphological characters as *Akihito* shares with *Sicyopus* the free tongue and the pelvic disk adherent to the belly between fifth rays only. These two genera share with *Lentipes* the dorsal tip of ascending process on premaxilla narrower than the process below (Keith et al., 2011a).

4.2. Evolutionary history and ancestral range of Sicydiinae

Although the phylogenetic position of *Stiphodon* is not highly supported, the maximum likelihood tree resulted from ML analyses as well as the majority consensus tree from Bayesian analyses placed the clade *Stiphodon* as the sister group of all other Sicydiinae species. *Stiphodon* species are distributed from the eastern Indian Ocean to Polynesia. This new result differs from the results of Keith et al. (2011a) since their phylogeny placed the genus *Cotylopus* – geographically restrictedto the Mascarene and Comoros islands in the Indian Ocean – as the sister group of all other genera. Using a new phylogenetic context and a likelihood-based method of ancestral biogeographic range reconstruction, our work challenges the conclusions of Keith et al. (2011a) about the ancestral area of the subfamily.

Because Sicydiinae may have emerged quite recently (6–12 Myrs ago, Keith et al., 2011a), we can expect that the current distribution of species reflects their biogeographic evolution. Our results of ancestral area reconstruction suggest that Sicydiinae might have emerged within the Indonesian shelf and that it may have been a key area in the diversification of some clades such as *Sicyopus* and *Cotylopus*/*Smilosicyopus* groups. Our results are consistent with previous studies that suggested the West Pacific Ocean as a centre of origin for the Sicydiinae subfamily (Keith et al., 2011a; Parenti, 1991). In the diadromousAnguillid species, it has been suggested that these species also emerged in the Pacific tropics (Aoyama, 2009; Minegishi et al., 2005). However, our results suggest that Melanesia, and especially the region including Solomon–Vanuatu–New Caledonia, might also have been a centre of diversification of some genera and species groups (*Lentipes*, *Akihito*, *Stiphodon* and some *Smilosicyopus*species).

In congruence with Keith et al. (2011a), we found that the sister group relationship between *Sicyopterus* – distributed in the Indo-Pacific, from Madagascar to Polynesia- and *Sicydium*-restricted to each side of Central America – is highly supported. Based on their molecular work, Keith et al. (2011a) suggested that the colonisation of the Atlantic Ocean by the common ancestor of *Sicydium*/*Parasicydium* might have taken place from the Pacific Ocean by dispersal through the Indian Ocean and around South Africa (Keith et al., 2011a). Conversely and considering the extended sampling of *Sicydium* and *Parasicydium* species from Caribbean and West coast of Africa, our results suggest that their common ancestor may have emerged in the Polynesian region and dispersed into the Caribbean Sea through the Panama Isthmus. In the Panama route hypothesis, the ancestors of the clade *Sicydium–Parasicydium* would have reached the western and north part of Central America from the centre of the Pacific, crossed the Isthmus of Panama before its completion (3–4 Myrs ago) and reached the Caribbean Sea. This hypothesis would also explain the presence of *Sicydium* on the western margin of Central America. The little known past equatorial currents cannot help us to contradict or confirm the Panama route theory (Iturralde-Vinent and MacPhee, 1999). Moreover, the panama route scenario is considered to explain the presence of Anguillid species in the Atlantic Ocean (Lin et al., 2001; Minegishi et al., 2005). Our phylogenetic results add information and suggest that *Sicydium* probably independently colonised the western coast of Africa and the Caribbean Sea several times. Indeed, in our phylogeny, African and American *Sicydium* do no group geographically. *Sicydium punctatum*, distributed in the Caribbean Sea is sister group of *Sicydium crenilabrum* which is found in West Africa. The second species from the Caribbean area, *Sicydium plumieri* groups with the African species *Parasicydium bandama*, and *Sicydium brevifile*, the sister group of all *Sicydium* of our phylogeny, is from West Africa. A phylogeny including all described species of *Sicydium* and *Parasicydium bandama* species would help to test this hypothesis.

4.3. Endemic species are in outskirts of Smilosicyopus genus range

Our data including all the known species of *Smilosicyopus* revealed several pairs of closely related species within this genus(*S. Sasali + S. pentecost*, *S. bitaeniatus + S. fehlmanni,* and *S. chloe + S. leprurus*). Moreover, we detected a recurrent geographic pattern of genetic structure among these species pairs. First, for the three species pairs, the two sister species have no overlap in geographic distribution (Keith and Taillebois, in press). Second, for each of these three pairs, one species has a widespread distribution and the second one a restricted distribution (endemic)

located at the outer edge of the distribution range of the genus. Indeed, the genus *Smilosicyopus* is found throughout the western Pacific Ocean, extending from southern Japan to New Caledonia for its latitudinal limits and from Indonesia to Marquesas Islands for its longitudinal limits. All the restricted-range endemic species of the group are distributed in the northern and eastern borders of the distribution range of the genus: *S. sasali* (endemic from Futuna, on the eastern Pacific limit of the genus distribution range), *S. nigriradiatus* (endemic from Ponhpei, on the northern limits of the genus distribution range) and *S. bitaeniatus* (endemic from Marquesas Islands, on the eastern Pacific limit of the genus distribution range). The recurrent biogeographic and phylogenetic patterns based on a robust phylogeny and presenting disjoint distributions may indicate a common mechanism of cladogenesis. Two allopatric species can be explained as the result of either vicariance of a widespread ancestor or peripatric speciation (Heads, 2009). Our results suggest that speciation patterns may result from peripatric processes in which one of the new species is formed in a small and isolated peripheral population (Bird et al., 2012). Similar patterns have been observed in the family Kuhliidae (Teleostei) that includes diadromous, marine and estuarine species (Feutry et al., 2013a). For the amphidromous and marine fauna, the isolation of peripheral islands in the Pacific or the Indian Ocean has been shown to contribute significantly to genetic subdivision within and among species. For example, significant population structure has been reported in numerous taxa including diadromous prawns (Castelin et al., 2013), gastropods (Crandall et al., 2010) and fish (Feutry et al., 2013b; Lord et al., 2012), or marine reef fish (Gaither et al., 2011; Gaither et al., 2010; Planes and Fauvelot, 2002; Winters et al., 2010).

For the *Smilosicyopus*, which have a marine dispersive phase in their life cycle (Taillebois et al., 2012), the peripatric mode of speciation could be difficult to achieve because oceanic currents potentially allow larvae to connect distant habitats separated over hundreds and even thousands of kilometres (Keith, 2003). Furthermore, given the great number of tropical islands in the western Pacific Ocean, one can expect that these islands have constituted effective stepping-stones among archipelagos and may prevent any divergence process. In Neritiidae, connectivity between high-island archipelago populations is higher in marine species than in amphidromous ones when connection between these populations requires an atoll stepping-stone (Crandall et al., 2012). For such amphidromous species, the geographic separation of the populations and allopatric divergences might have occurred on the outskirts of the species range thanks to biogeographical events, such as Pleistocene sea level changes or oceanic barriers driven by past changes in climate and water mass circulation (see the review by Hoeksema, 2007). However, the stepping-stone model for connectivity requires to be tested at the intra-specific level in *Smilosicyopus* with a high density of taxonomic sampling from intermediate islands.

Our study revealed other pairs of recently diverged sister-species within the Sicydiinae. In the genus *Cotylopus*, the sister-species co-occur in the same geographic area and have restricted and overlapping species ranges. In the genus *Sicydium* two pairs of closely related species present no overlapping distributions and are distributed on either sides of the Atlantic Ocean. These results suggest a large diversity of speciation patterns in the subfamily and much remains to be elucidated with regards to the speciation mechanism in the group. Our study does not exclude that other processes, such as habitat selection or ecological switching, that may act conjointly with geographic speciation (Coyne and Orr, 1998, 2004) and which suggest that speciation with gene flow might be possible within Sicydiinae as in the genus *Cotylopus*, for example, where sister-species appear sympatric (Papadopulos et al., 2011). Sicydiinae may thus constitute a well-suited model to investigate speciation patterns.

5. Conclusion

Although we used extended taxonomic and geographic sampling in our 5-gene phylogeny, certain nodes were still not resolved. Our results thus indicate that further phylogenetic investigations are needed to obtain complete resolution of the Sicydiinae phylogeny and confidently document their evolutionary history. The presence of short branch lengths between the five clades may indicate that Sicydiinae genera have diversified within a short amount of time, and this may pose a challenge to the complete resolution of the Sicydiinae phylogeny. These results suggest that analyses of genes with higher evolutionary rates compared to this study should be considered in future phylogenies of Sicydiinae.

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