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Pyrrhosoma and its relatives: a phylogenetic study (Odonata: Zygoptera)

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The placement and relationships of the red-and-black zygopteran *Pyrrhosoma*, currently considered to be part of the Teinobasinae, has long been uncertain. DNA fragments (COI and ITS) reveal that *Pyrrhosoma* s.s. is restricted to the West Palaearctic, with two morphologically distinct name-bearing clades (*nymphula*, *elisabethae*), and with a morphologically indistinct third clade in the Middle Atlas, Morocco, that might be close to the common ancestor of all three. *Chromagrion*, the closest relative of *Pyrrhosoma*, is found in North America, not in South Asia. Two isolated Chinese taxa (*tinctipenne* and *latiloba*) are morphologically similar to *Pyrrhosoma*, but their molecular distance is so large that a new genus, *Huosoma*, is required to accommodate them. Past climate change is suggested as the driver of the biogeography and evolution of this group of zygopterans. The origin of the Moroccan isolate and of *elisabethae* might predate the glaciations, and be of Pliocene age. The much wider disjunction between the American and South Asian groups and the western group suggests an older, perhaps Miocene age.

Keywords: Odonata; phylogeny; *Pyrrhosoma*; *Huosoma*; *Chromagrion*; COI; ITS; disjunction; biogeography

Introduction

The small zygopteran genus *Pyrrhosoma* is remarkable in several respects. It is conspicuously red-and-black coloured, a pattern that is rather infrequent but not extremely rare among "coenagrionids" and not always homologous among the genera where it occurs. The few temperate-zone species are vernal and among the first damselflies to be on the wing after winter. At higher altitudes (1000–3500 m), *Pyrrhosoma* extends or shifts its adult life to summer, however. Structurally, *Pyrrhosoma* belongs to the coenagrionids with an angular frons, a group that perhaps should be singled out in a separate (sub)family, the mainly tropical Teinobasinae *sensu* De Marmels, 2007 (see Bybee, Ogden, Branham, & Whiting, 2008; Carle, Kjer, & May, 2008; Dumont, Vierstraete, & Vanfleteren, 2010 for molecular support for this idea). Within this large clade, it stands out by the males having a spur that springs from the ventral base of the superior appendage or cercus (see e.g. Askew, 2004), a character it shares with the genus *Chromagrion* (and *Teinobasis*), but not with *Ceriagrion* (De Marmels, 2007).

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The range of *Pyrrhosoma* consists of two disjunct parts. The western branch extends from a nucleus in the Atlas Mountains in North Africa in the West, across Western Europe eastwards as far as Moscow, narrowly reaching the southern Urals in Bashkhiristan (see Skortsov, 2010, for a map). Further south, it occurs in the Black Sea countries, including Turkish Thracia, and it fringes the south coast of the Black Sea in north-western Anatolia (Hacet, 2009), although it is rare there (Olthoff & Ikemeyer, 2012). Possibly, it even reaches Western Iran (Ghahari, Tabarai, Sakenin, Ostovan, & Imani, 2009) but these latter data need independent confirmation. A curious endemic taxon, *P. elisabethae* Schmidt, 1948, inhabits southern Albania, Corfu Island and the Peloponesos (see Kalkman & Lopau, 2006 for a distribution map). Its range is thus surrounded on all sides by that of *P. nymphula*. There have been claims that a cline with *P. nymphula* exists and for a subspecies rather than a species status for this animal (Buchholz, 1954), but Lieftinck (1966) and Dumont, Mertens, and Miho (1993) did not confirm this, and recent handbooks list it as a full species (Dijkstra & Lewington, 2006). In Morocco, a group of *Pyrrhosoma* "*nymphula*" populations lives at around 1600 m asl around Ifrane in the Middle Atlas Mountains, disconnected from the species' range in continental Europe (Jacquemin & Boudot, 1999). The Asian or "eastern branch" is even more widely separated from the western one and lives on the south and south-east flanks of the Himalaya mountain chain, at altitudes up to 3300 m. Its westernmost populations occur in Bhutan (A. Mitra, unpublished data), possibly representing an as yet undescribed species. This branch, with two species, *P. tinctipenne* and *P. latiloba* (but see below for their transfer to a new genus), described so far, is mainly found at altitudes up to 3300 m in the provinces of Sichuan and Yunnan, China (Asahina, 1973; Mclachlan, 1896; Needham, 1930; Yu, Yang, & Bu, 2008).

There is no *Pyrrhosoma* in North America, yet De Marmels (2007) claims that it is most closely related to the monotypic genus *Chromagrion.* The two genera are quite different in body coloration but share the basal spur on the male cercus (Westfall & May, 1986).

Here, we use molecular information, *in casu* the ITS and COI DNA fragments to explore phylogenetic relationships within the two branches of *Pyrrhosoma*, and with putative related groups such as *Chromagrion* and *Ceriagrion*. In addition we compare *Pyrrhosoma* with other red-and-black genera of coenagrionids, to test the long-held idea that this colour pattern arose several times independently.

Material and methods

In all, 41 specimens were successfully analysed, with duplicates from one or adjacent populations in *Pyrrhosoma nymphula* Morocco (2 specimens), *Chromagrion conditum* (4 specimens) and *Pyrrhosoma latiloba* (7 specimens). A list of species examined, with geographic origin and collector, is shown in Table 1. Field-collected specimens were preserved in 80% ethanol until transferred to the laboratory for further work (see below), except in cases where only dried specimens were available. In some cases (Finland and South Albania) we succeeded in obtaining good quality DNA from them but managed to amplify only one of the two gene segments studied here.

Preparation of DNA

Genomic DNA was extracted using a modified CTAB protocol (Kocher et al., 1989). Tissue was crushed using a bead-beater and afterwards incubated for a minimum of 3 h at 60°C in 500 µl CTAB buffer with 6 µl proteinase K (100 µg ml^{-1}). After an overnight incubation at 37°C the remaining protein was precipitated by adding 0.5 volumes of 7.5 M ammonium acetate and centrifugation for 10 minutes 14,000 rpm at room temperature. Supernatant was transferred to a new tube, and DNA was precipitated with an equal volume of isopropanol and rehydrated in

Table 1. Species list.

Taxon	Origin	Collector
Oxyagrion terminale	Lake Dom Helvecio, Brazil	H. Dumont
Ceriagrion glabrum	Limbe, Cameroon	H. Dumont
Ceriagrion cerinorubellum	Asan Lake, N India	H. Dumont
Ceriagrion cerinorubellum	Kuala Lumpur, Malaysia	V. Alekseev
Ceriagrion fallax	Kanglung, Bhutan	H. Dumont
Ceriagrion olivaceum	Chiang Mai, Doi Inthanon, Thailand	V. Alekseev
Ceriagrion coromandelianum	Asan Lake, India	H. Dumont
Chromagrion conditum	USA	M. McPeek, E. Bright
Pyrrhosoma nymphula	Near Ifrane, Morocco	S. Ferreira
Pyrrhosoma nymphula	Escarei, Portugal	A. Cordero
Pyrrhosoma nymphula	Afonsin, Portugal	A. Cordero
Pyrrhosoma nymphula	Marvão, Portugal	S. Ferreira
Pyrrhosoma nymphula	Bertiandos, Portugal	S. Ferreira
Pyrrhosoma nymphula	Malkoçlar, Devletliagaç, Turkey	N. Hacet
Pyrrhosoma nymphula	Kirklareli, Thracia, Turkey	N. Hacet
Pyrrhosoma nymphula	Affligem, Belgium	H. Dumont
Pyrrhosoma nymphula	Finland	M. Hamalainen
Pyrrhosoma nymphula	Macedonia	S. Ferreira
Pyrrhosoma elisabethae	South Albania	H. Dumont
Pyrrhosoma elisabethae	Corfu Island	C. Brochard
Pyrrhosoma elisabethae	Kalavrita, Greece	J. Vermeir
Pyrrhosoma tinctipenne	Kangding, Sichuan, China	X. Yu
Pyrrhosoma latiloba	Shangrila, Yunnan, China	Z. Guan
Rhodischnura nursei	Jaipur, India	H. Dumont
Ischnura fluviatilis	Rio Doce, Brazil	A. Rietzler
Xanthocnemis zealandica	Canterbury, New Zealand	M. Marinov

25 μl of water. Small aliquots of extracted nucleic acids were used as template for polymerase chain reaction amplification (PCR). The ITS region (ITS1 + 5.8S + ITS2, in all *c*.620 bp) was amplified using the F2F (5′-RGY AAA AGT CGT AAC AAG GT-3′) and V2R (5′-TTT CAC TCG CCG TTA CTA AGG GAA TC-3′) primers. Cycle conditions were 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min. The COI "barcoding" region was amplified (*c*.640 bp) using the primers 1490F (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and 2198R (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′). Cycle conditions were 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min.

Sequence analysis

Sequencing was performed using an Applied Biosystems ABI 3130XL Genetic Analyser (Hitachi, Tokyo, Japan). Excess primer and dNTP were removed with exonuclease I (Fermentas EN0581, Thermo Fisher Scientific Inc., Sint-Leon-Rot, Germany, 10U/10 μl PCR) and calf intestine alkaline phosphatase (Fermentas EF0342, 1U/10 μl PCR) for 15 min at 37°C, followed by 15 min at 85°C to inactivate the enzymes. This material was then used for cycle sequencing without further purification, using the ABI Prism BigDye V 3.1 Terminator Cycle Sequencing kit. The sequencing conditions were 30 s at 96°C, 15 s at 50°C and 1 min at 60°C for 27 cycles. Primers used for sequencing were the same as for the PCR. Cycle sequence products were precipitated by adding 25 μl of 95% ethanol and 1 μl 3 M sodium acetate, pH 4.6 to each cycle sequencing reaction (10 μl). The samples were placed at room temperature for 15 minutes and centrifuged at 14,000 rpm for 15 minutes. After precipitation, an additional wash of the pellet was performed with 125 μl of 70% ethanol followed by centrifugation at 14,000 rpm for 5 minutes. The pellet was dried in a Speedvac concentrator (Savant, Instruments inc., Farmingdale, NY, USA), re-dissolved in formamide and run on 50 cm capillaries with POP7 polymer.

Alignments were made with the software package Mafft Version 6 (http://mafft.cbrc.jp/alignment/server/), using standard settings (Katoh & Toh, 2008), and later optimized using DAMBE (see below). Sequences of all gene–taxon combinations are shown in Supplementary Tables S1 and S2 (Supplementary content is available via the article webpage).

The model of DNA evolution that best fitted the data was determined for each fragment separately with Modeltest version 2 (Darriba, Taboada, Doallo, & Posada, 2012). Based on the Akaike Information Criterion (AIC), the general time-reversible substitution model with a discrete c correction for among site variation, and corrected for invariable sites (GTR + G + I model) was chosen for maximum likelihood analysis in MrBayes (Version 3.1.2; Altekar, Dwarkadas, Huelsenbeck, & Ronquist, 2004; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003).

MrBayes trees were calculated for 10,000,000 generations, and resulted in an average standard deviation of split frequencies of 0.002342 for ITS and 0.002775 for COI. Convergence is documented using Tracer (Rambaut & Drummond, 2007) and AWTY (Wilgenbusch, Warren, & Swofford, 2004) in Supplementary Figures S3 and S4.

We tested for substitution saturation, using DAMBE (version 5.3.15) (Xia & Xie, 2001). Substitution saturation was measured on fully resolved sites; any unresolved sites were deleted. For COI, we retained 547 positions in the alignment, 180 of which were informative, under the model of evolution GTR + I + G. We found a critical substitution saturation (Iss.c) of 0.71 and an Iss of 0.34, significantly lower ($p < 0.0001$, two-tailed test). For ITS, 460 positions were left in the alignment, 184 of which were informative, and the model now was TIM3 + G. For a symmetrical tree, we found Iss = 0.21 and Iss.c. = 0.70 ($p < 0.000$). There was thus insignificant saturation and a clear phylogenetic signal. The resulting trees, moreover, only differed marginally from the ones estimated without a saturation correction (not shown).

A haplotype map for COI in the *Pyrrhosoma nymphula* group and in *P. latiloba* was estimated as well, using the TCS program with standard settings (Clement, Posada, & Crandall, 2000). K2P pairwise genetic distances were obtained for the *Pyrrhosoma* s.l.–*Chromagrion* clade using Mega4 under standard settings.

Results

For each gene fragment, we derived a maximum likelihood and a Bayesian tree (Figures 1–4). The ITS trees (Figures 3, 4) all show the same overall topology, with one clade composed of the "western branch" of the *Pyrrhosoma* clade, and *Chromagrion conditum* as its sister, itself sister to the "eastern" *Pyrrhosoma* clade (but note that no ITS gene sequence was obtained for *P. elisabethae* from Albania). The hierarchy within *P. nymphula* populations from different geographic origins is polytomous and not well resolved, and *P. elisabethae* is sometimes enclosed within *P. nymphula*. The *P. nymphula* from Morocco is enclosed within *nymphula*. *P. tinctipenne* is well differentiated from *P. latiloba.* The sister clade to the two branches of *Pyrrhosoma* plus *Chromagion* is *Ceriagrion*. In the outgroup, the New Zealand endemic *Xanthocnemis* is in polytomy with a small clade composed of *Ischnura* and the red *Oxyagrion* and all of these are confirmed as non-Teinobasinae. All clades are well supported.

The COI based estimate is somewhat different (Figures 1, 2): in the western *Pyrrhosoma* clade, the Moroccan population either forms a clade with *elisabethae* or forms a subclade in its own right. *Chromagrion* is recovered as sister to the western *Pyrrhosoma.* The eastern "*Pyrrhosoma*" spp. are sister to the clade "western *Pyrrhosoma* plus *Chromagrion*". The monophylum *Ceriagrion*, as before, is sister to all the above.

A haplotype map for COI (Figure 5) shows the reason for the special position of the Moroccan taxon: it is several mutations away from all other *nymphula*and from *elisabethae*. Both *nymphula*

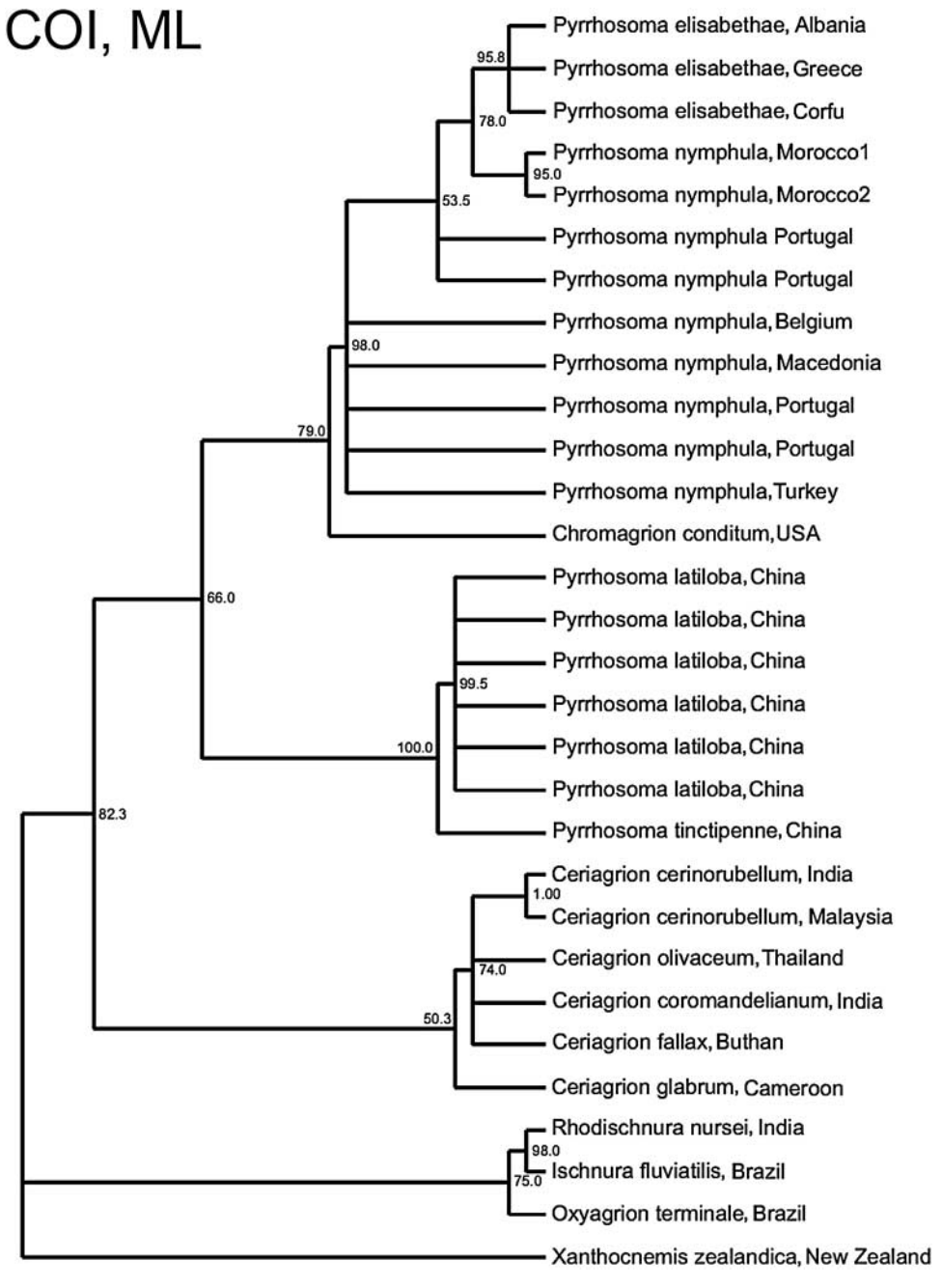

Figure 1. COI, barcoding gene fragment, maximum likelihood estimate of phylogenetic relationship of *Pyrrhosoma* and its closest relatives. Percentage bootstrap values on nodes. Outgroup composed of non-teinobasine coenagrionids.

"Europe" and *P. elisabethae* may easily be derived from it, and we think it has conserved some primitive features.

The K2P values (Table 2) show percentage genetic distances of 10% and more (up to 20%) between teinobasid genera, including for *Pyrrhosoma* and *Huosoma* (see below, for a formal

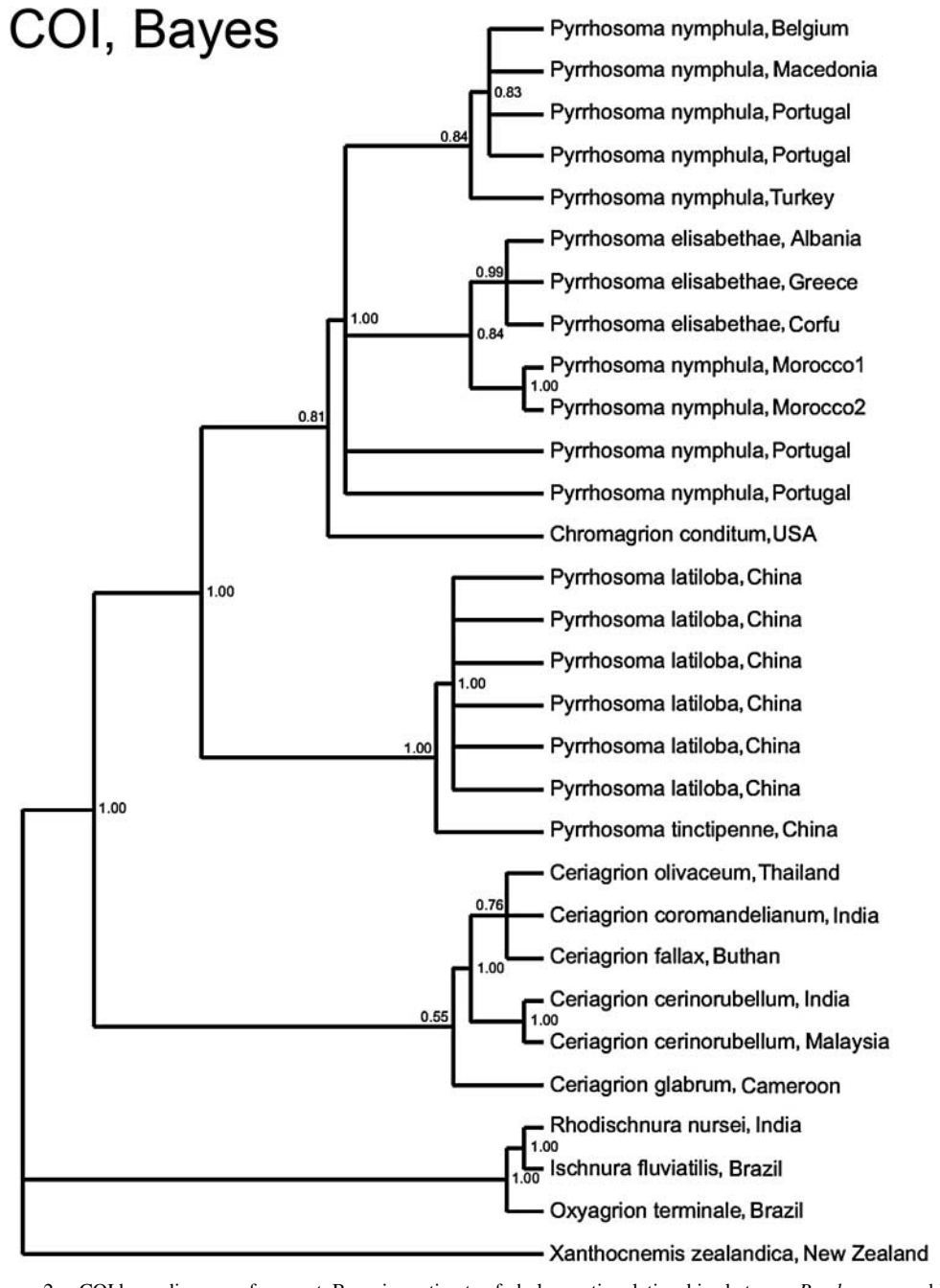

Figure 2. COI barcoding gene fragment, Bayesian estimate of phylogenetic relationships between *Pyrrhosoma* and its relatives. Posterior probability values on nodes.

definition of this genus). Here, both gene fragments tell a similar story, but some species and genera are recovered with considerable differences in genetic distance for the two fragments. For example, the distance between *P. nymphula* and *elisabethae* was recovered as up to 3% for COI (conventionally considered as a species level distance), but only 1% for ITS.

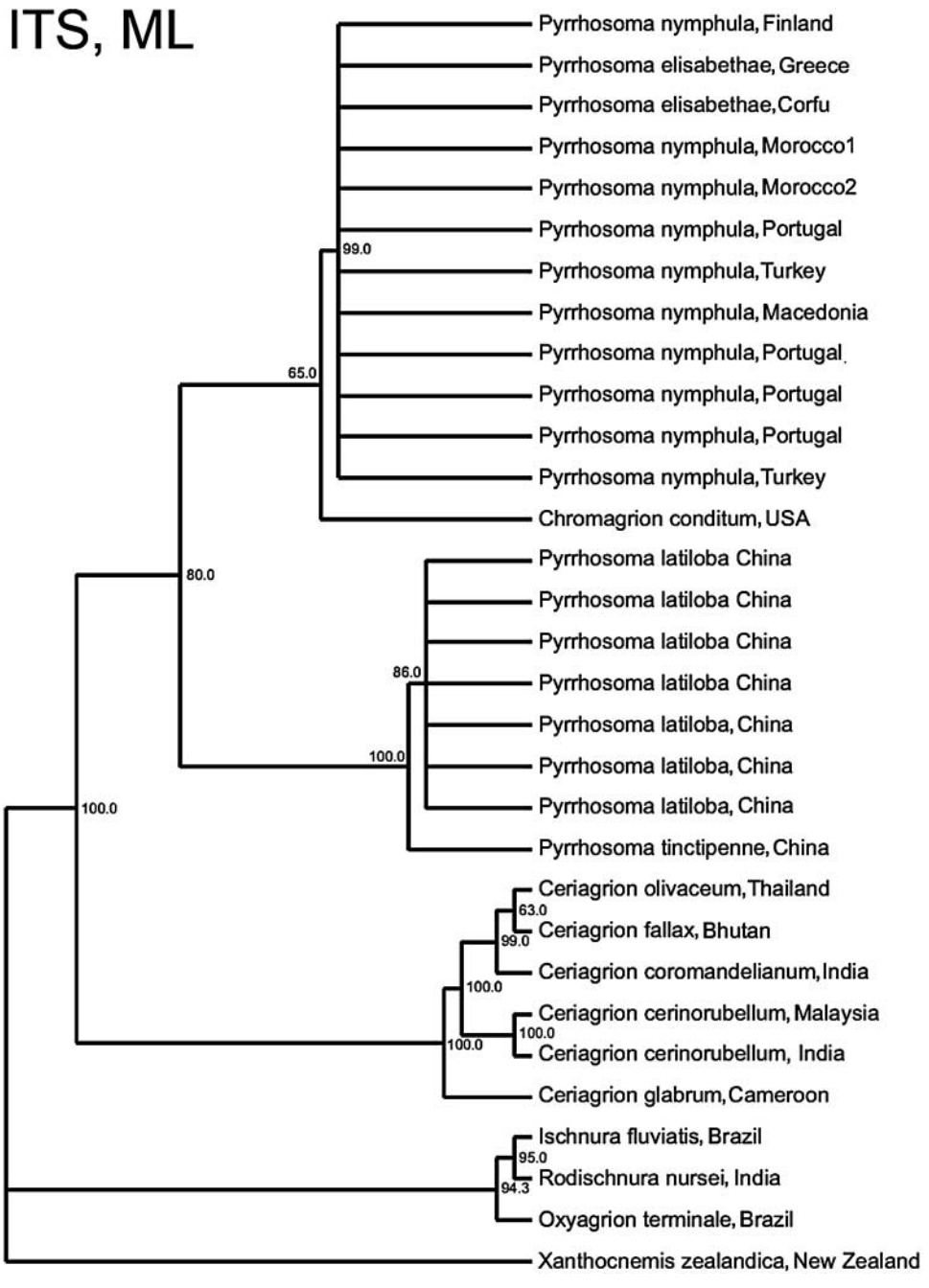

Figure 3. ITS1-5.8.S-ITS2 ("ITS"), maximum likelihood estimate of the phylogenetic relationship between *Pyrrhosoma* and its relatives, percentage bootstrap values on nodes. Outgroup composed of non-teinobasine coenagrionids.

Discussion

Our results confirm that western *Pyrrhosoma* are composed of more than one taxon, and the validity of *P. elisabethae* is confirmed, although some doubt remains as to its full specific status. More unexpected was the discovery that the Moroccan isolate is also a genetic entity of

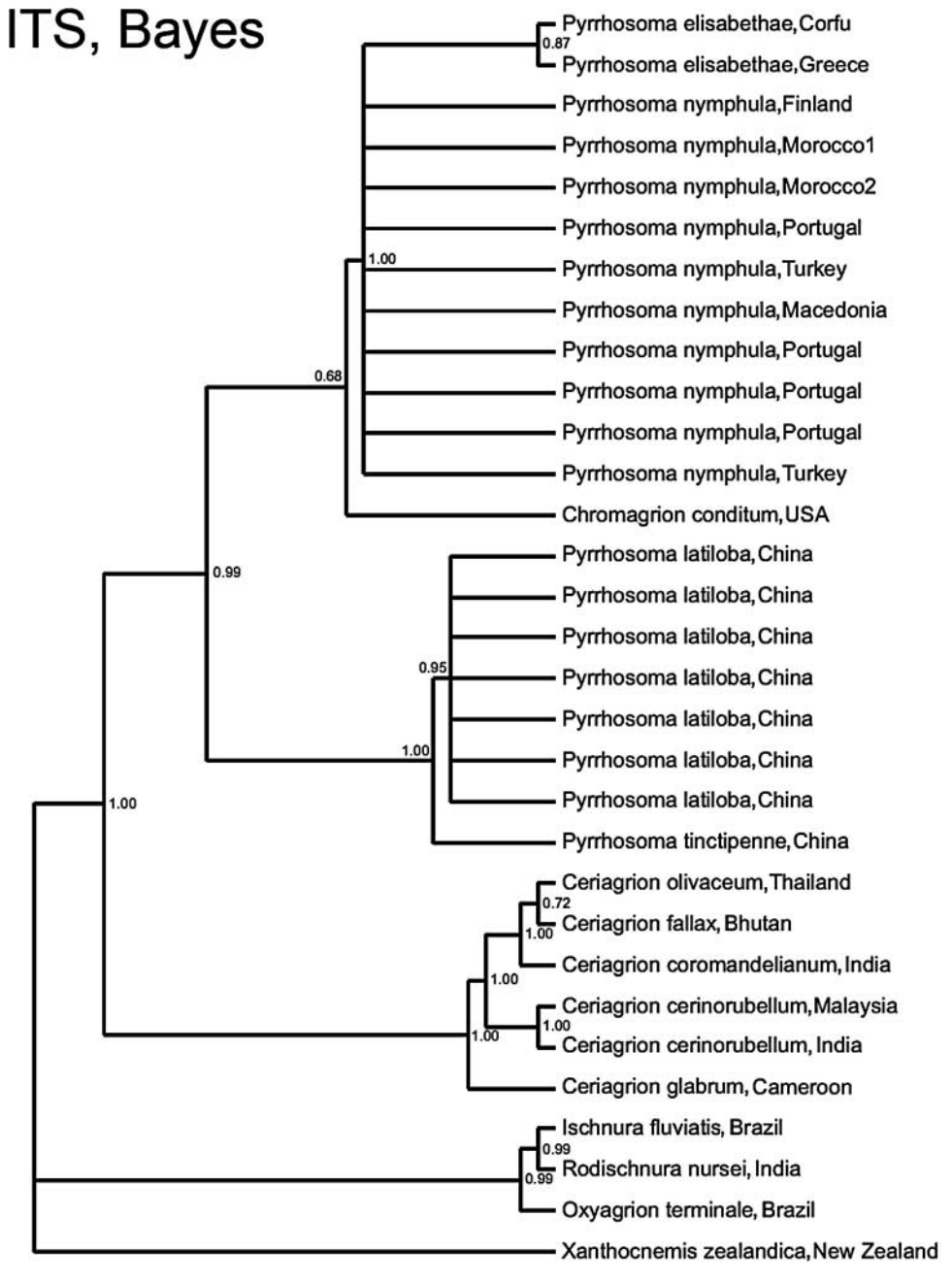

Figure 4. "ITS", Bayesian estimate (Mr Bayes) of the phylogenetic relationship between *Pyrrhosoma* and its neighbours, posterior probabilities on nodes.

old age. Although it is not separable from typical *nymphula* by traditionally used morphological characters, it appears to share a sister relationship with the ancestor of both *nymphula* and *elisabethae*. We here abstain from formally naming it, but it is clear that its status, including that of putative populations in the Rif mountains of Northern Morocco (Jacquemin & Boudot, 1999) and perhaps even in the south of Spain need to be re-evaluated by novel morphological methods.

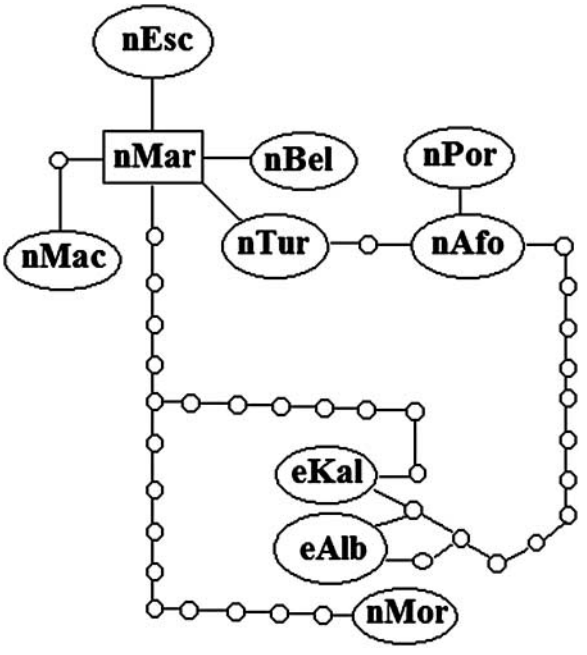

Figure 5. COI-based haplotype map within populations of the *Pyrrhosoma* species complex. Three groups were singled out: *elisabethae*, a Moroccan population, and "mainland" European *nymphula* (abbreviations: n, *nymphula*; e, *elisabethae*; Afo, Afonsin, Portugal; Alb, Albania; Bel, Belgium; Esc, Escarel, Portugal; Mac, Macedonia; Mar, Marvao, Portugal; Mor, Morocco; Por, Bertiandos, Portugal; Tur, Thracia, Turkey-in-Europe.

Table 2. Percentage pairwise genetic distances (K2P).

	COI	ITS
Chromagrion–Huosoma	16.0–16.2	—
Chromagrion–Huosoma latiloba	11.6	—
Chromagrion–Pyrrhosoma	10.7–11.4	6.4–6.6
Pyrrhosoma nymphula–elisabethae	2.0–3.0	0.4–0.8
Pyrrhosoma nymphula–Huosoma	14.8–15.7	9.8–10.0
P. nymphula Morocco–other	2.2–3.0	1.9–3.0

Both European taxa seem to have been deeply influenced by glaciations, with *elisabethae* possibly an early glacial relict, sequestered in western Greece to southern Albania. A second zygopteran that broadly shares the same geographic range (and probably the same age as well) is *Platycnemis pennipes nitidula* (Dumont, 1977). The Moroccan isolate co-occurs with West Mediterranean or Maghrebian endemics such as *Calopteryx exul*, *Platycnemis subdilatata*, *Enallagma deserti*, *Cordulegaster princeps*, and *Gomphus lucasi.* For *C. exul*, a divergence time estimate was derived by Dumont, Vanfleteren, De Jonckheere, and Weekers (2005). It predates the glacial epoch, and is likely of Pliocene age.

We confirm De Marmels' (2007) prediction that *Chromagrion* is the "North American *Pyrrhosoma*", but were surprised by the position of the East Asian "*Pyrrhosoma*". We expected a confirmation of their specific status, but our results reveal that this clade apparently warrants full generic rank. We therefore transfer the two eastern species to a new genus. Aside from the molecular support, we diagnose this genus as follows (both sexes).

Huosoma, gen. nov.

Color red and black, but red color less deep than in *Pyrrhosoma*, with slight orange tinges. Body generally less robust than true *Pyrrhosoma*. Males with both pairs of appendages reddish brown, without a trace of black. Superior appendage seen from above not straight but curved inwards. The basal spur of the superior appendages turned inwardly (not upwards, in the axis of the appendage, as in true *Pyrrhosoma*). The origin of the name is from the Chinese noun *huo*, fire, combined with the neuter Greek noun soma, body, and thus the genus name has the same meaning as *Pyrrhosoma*. The type species is *Pyrrhosoma tinctipenne* McLachlan, and the genus also includes *P. latiloba*.

We hypothesize that the widely disjunct *Huosoma* and *Pyrrhosoma* became isolated at a time when free faunal exchange between Eurasia and North America was coming to an end, as climatic impediments to such exchange were evolving and winters in Siberia and Central Asia became too long, too cold, and too arid. We therefore speculate that the last common ancestor of the entire *Pyrrhosoma–Huosoma–Chromagrion* clade may well be situated in the (late?) Miocene.

We also conclude that the red and black colour pattern of *Pyrrhosoma* and *Huosoma* carries only a faint phylogenetic signal: genera like *Oxyagrion* and *Xanthocnemis* are absolutely unrelated to them, and acquired a similar habitus independently. *Ceriagrion*, finally, is perhaps the best illustration of the relative value of colours: it includes live red-and-black species, beside orange, brownish, yellow, and even blue species.

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