This study characterizes the phylogenetic relatedness of non-SARS human coronaviruses (HCoVs) in southern Taiwan by sequencing the nucleocapsid (N), spike (S), and RNA-dependent RNA polymerase (RdRp) genes directly from ten HCoV PCR-positive respiratory samples collected during 2012–2013. In the N, S1, and RdRp phylogeny, HCoV-OC43 in one and three samples was clustered with genotypes F and G, respectively, and HCoV-OC43 in sample YC101/TWN/2013 represented a recombination event between genotypes F and G. Amino acid substitutions in the S1 protein of HCoV-OC43 were also identified. In the N phylogeny, HCoV-HKU1 in one and two samples clustered with genotypes A and B, respectively, and HCoV-229E in two samples was clustered with genogroup 6. The genotypes and genogroup detected here were in line with the prevalent phylogenetic lineages reported outside of Taiwan during the contemporary period. In summary, three species of non-SARS HCoVs with different genotypes cocirculated in the community, with genetic evolution observed in HCoV-OC43.
reported rate of 3.6% in adults and 7.4% in children [3,4]. In addition, our prior work examining nasopharyngeal or throat swabs from 267 adults with RTIs attending outpatient and emergency departments in a medical center and a regional hospital in southern Taiwan between October 2012 and June 2013 revealed HCoVs in 13 (4.9%) patients, which included HCoV-OC43, -HKU1, and -229E in seven, four, and two patients, respectively, by using PCR/electrospray ionization mass spectrometry (PCR-ESI/MS) [5].

Despite an increase in the molecular detection of non-SARS HCoVs in Taiwan, the phylogenetic characteristics of local non-SARS HCoV strains remain undescribed because isolation of non-SARS HCoVs in cell culture or sequencing viral nucleic acids directly from clinical specimens are not routinely performed for diagnostic purposes at hospital’s virology laboratories. Therefore, this study aimed to investigate the phylogenetic relatedness of non-SARS HCoVs in Taiwan by comparing Taiwanese and non-Taiwanese HCoVs.

Materials and methods

The nucleocapsid (N), spike (S), and RNA-dependent RNA polymerase (RdRp) genes of HCoVs in the abovementioned 13 HCoV-positive respiratory samples detected by PCR-ESI/MS (PLEX-ID®, Abbott Laboratories, Illinois, U.S.) in our earlier study were amplified and sequenced by using previously described PCR primers and conditions for HCoV-OC43, -HKU1, and -229E, respectively [6–8]. The corresponding positions and lengths of the N, S, and RdRp genes used for analysis are provided in Table S1.

The phylogenetic trees based on the N, S, and RdRp genes of HCoVs from this study and representative sequences retrieved from GenBank and literatures were constructed using neighbor-joining method and Kimura’s two-parameter model in MEGA X (http://www.megasoftware.net/) and evaluated with 1000 bootstrap pseudoreplicates [2,7,9–13]. The assignment of genotypes or genogroups followed those described in earlier studies [2,7,9–13]. Profiles of amino acid substitutions in the S1 domain of the S protein of HCoV-OC43 were also analyzed.

The study was approved by the Institutional Review Board (B-ER-101-031) of the study hospital with informed consent from all patients.

Results

The N, S, or RdRp sequences could be obtained from ten (77%) out of 13 HCoV PCR-positive respiratory samples and were submitted to the DDBJ/EMBL/GenBank databases under the accession numbers LC543620 to LC543641 (Table S2). All ten patients presented with upper RTIs and did not travel abroad, except one patient who tested positive for HCoV-229E (sample ID221/TWN/2013) developed upper RTI one day after returning to Taiwan from Macau, suggesting an imported case.

The N, S (S1 receptor binding domain), and RdRp genes were successfully amplified and sequenced in five of seven HCoV-OC43-positive samples. HCoV-OC43 in four samples had congruent positions in the phylogenetic trees of the N, S, and RdRp genes: sample YC031/TWN/2013 belonged to genotype F and samples YC006/TWN/2013, YC021/TWN/2013, and YC029/TWN/2013 belonged to genotype G [Fig. 1]. However, for

Fig. 1 Phylogenetic analysis based on the nucleocapsid, S1 domain of spike, and RNA-dependent RNA polymerase (RdRp) genes of human coronavirus OC43 from Taiwanese patients enrolled in this study (marked with symbols) and representative sequences retrieved from GenBank (accession number) and literatures [2,9–12]. Symbols ●, ○, ▲, ◆, and ※ referred to sequences from sample numbers YC101/TWN/2013, YC029/TWN/2013, YC021/TWN/2013, YC006/TWN/2013, and YC031/TWN/2013, respectively.
HCoV-OC43 in sample YC101/TWN/2013, the S and RdRp genes were clustered with genotype F, whereas the N gene was clustered with genotype G, implying a recombination event between genotypes F and G.

Through alignment to S1 (678 nucleotides assessed) of the reference genotype D strain HK04-02, six and five shared amino acid substitutions were identified in genotype F (T25P, R26K, K90L, L152S, Y176H, K184N) and genotype G (P22T, T25P, K90L, L152S, and K184N), respectively (Table S3). Of two samples with S1 domain designated as genotype F, YC101/TWN/2013 shared similar substitutions in S1 with a Malaysian strain MY-U868-2012, though the former had an additional P38L substitution, and YC031/TWN/2013 shared identical substitutions with a French strain MDS6 [2,10]. Of samples designated as genotype G, additional substitution P38S in YC006/TWN/2013 and Y72F in YC029/TWN/2013 were identified.

The N and S (S2 membrane fusion domain) genes were successfully sequenced in three and one out of four HCoV-HKU1-positive samples, respectively. Phylogenetic analysis of the N gene revealed that HCoV-HKU1 in samples YC008/TWN/2013, YC008/TWN/2013, and ID193/TWN/2013 were clustered with genotype B, and phylogenetic analysis of both the N and S genes showed that HCoV in sample ID193/TWN/2013 was clustered with genotype A [Fig. 2A].

The N and S (S2 membrane fusion domain) genes were successfully sequenced in two and one out of two HCoV-229E-positive samples, respectively. Phylogenetic analysis of the N gene revealed that HCoV-229E in samples ER207/TWN/2013 and ID221/TWN/2013 were clustered with genogroup 6, and the former had a congruent position in the phylogenetic tree of the S2 gene (genogroup 6) [Fig. 2B].

Summary of sequence identities of N, S and RdRP within (intra-) and among (inter-) genotypes or genogroups of three HCoV species is presented in Table S4, in which all the sequences shown in Figs. 1 and 2 were included for analysis. Of HCoV-OC43, S1 domain had the highest genetic diversity (92.0%–100%), whereas RdRp remained highly conserved (99.4%–100%), followed by N (97.1%–100%).

**Discussion**

HCoV-OC43 was the major HCoV species detected in respiratory tract infections in our previous study in 2012–2013, which was in line with observations elsewhere [14,15]. To date,
eleven genotypes (A to K) have been identified [2,9]. Genotypes A and B emerged around the 1950s and 1990s, respectively, while genotypes C, D and E were detected around the 2000s with the latter two originating from natural recombination [2,6,14]. Genotypes F and G were first described in a molecular surveillance study during 2012–2013 in Malaysia, in which analysis inferred that genotypes F and G might probably diverged concurrently around the late 2000s to early 2010s from a genotype D-like common ancestor through natural recombination and have co-circulated in China, Japan, Thailand, and Europe [2]. The presence of genotypes F and G in Taiwan in 2012–2013 is consistent with the Malaysian report. Our study also revealed a recombination event occurred between genotypes F and G, which has not been reported yet. Later, novel genotypes H (from recombination between genotypes B, D-like and E strains), I (no recombination event observed), J (from recombination between genotypes H and I), and K (derived from genotype I) were sequentially recovered after 2014 in China [9,16,17].

Besides recombination, this study also identified non-synonymous (amino acid) substitutions in the S1 protein of HCoV-OC43 and revealed a greater genetic diversity in S1 than in N and RdRp in HCoV-OC43. S1 protein is a major antigenic surface protein exposed to human humoral immunity. Whether and how substitutions in S1 identified here affect viral transmissibility or pathogenicity warrants further investigation. However, studies demonstrated that in HCoV-OC43, S1 accumulated adaptive substitutions faster than S2 and RdRp [18]. Such antigenic evolution (or antigenic drift) of S1 plays an important role in response to continuous selective pressure exerted by host immunity and genotype persistence in human populations [18]. On the contrary, RdRp exhibits a lower rate of nonsynonymous substitutions and hence remains highly conserved over time given its essential role in viral replication and lack of antibody exposure [18]. Taken together, these observations underline the importance of recombination and nucleotide substitution in driving the evolution of HCoV-OC43 and the need for continuous molecular surveillance to monitor emergent variants.

HCoV-HKU1 has evolved into three distinctive genotypes (A to C) with genotype A dated to late 1990 and both genotypes B and C traced back to the early 2000s [15]. HCoV-HKU1 detected in Malaysian patients in 2012–2013 belonged to genotype A (27.3%) or B (72.7%), while HCoV-HKU1 strains detected in Thai patients all belonged to genotype B [15,19]. Later, HCoV-HKU1 strains detected among children in South China and Hong Kong belonged to either genotype A (46.7%) or genotype B (53.3%) in 2014–2015 [20]. The HCoV-HKU1 genotypes identified herein (A and B) were consistent with the prevalent genotypes reported in nearby countries during the contemporary period.

To date, six distinct HCoV-229E genogroups have been revealed based on the phylogeny of S gene, with the former five genogroups 1, 2, 3, 4, and 5 comprising strains detected from 1979–1982, 1982–1984, 1989–1995, 2001–2005, and 2005–2011, respectively [13]. The latest group, genogroup 6, was first described by Lau et al., in 2011 and comprised strains detected in 2011–2020, including those from China (2011), Germany (2015), Haiti (2016), the United States (2015–2019), and patients with upper or lower RTIs in Hong Kong (2011–2020) [13]. The detection of HCoV-229E genogroup 6 in our two patients, one of whom had recently returned from Macau, suggesting that genogroup 6 extended its geographical distribution to cover Macau and Taiwan.

In conclusion, this study demonstrates for the first time the phylogenetic characteristics of non-SARS HCoVs in Taiwan and co-circulation of three non-SARS HCoV species belonging to different genotypes in a city during one influenza season. Although Taiwan is geographically separated from the main Eurasian continent, HCoVs in Taiwan shared similar prevalent phylogenetic lineages with those outside of Taiwan and continued to evolve. A periodic surveillance program would be warranted to monitor emergent HCoV variants and to assess their impacts on viral diagnostics and disease severity.

Conflicts of interest
The authors have no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2022.08.001.

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